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Nutritional Habits and Interventions in Childhood

Edited by
Silvia Scaglioni, Alessandra Mazzocchi and Valentina De Cosmi

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About the Editors

Silvia Scaglioni

Silvia Scaglioni is a medical doctor who has a specialty in Pediatrics and in Endocrinology. The main lines of her research activities concern pediatric nutrition and endocrinology. In particular, she has dedicated herself to the following topics. In the area of nutrition, the assessment of eating habits and body composition; early risk factors for obesity development; nutritional approach to children and adolescent obesity; the development of programs for assessment of eating habits; eating disorders in children (selective feeding, refusal of food); the diagnosis and treatment of child malnutrition; cognitive and behavioural therapy for children and adolescents suffering from overweight and obesity; food education for the prevention of carious disease; factors influencing children's eating behaviours; and early taste experiences and later food choices. In the field of endocrinology of children and adolescents, her topic areas of interest are the management and therapy of children and adolescents suffering from growth failure, precocious and delayed puberty, hypogonadism, and thyroid disorders.

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Alessandra Mazzocchi is a Biologist who graduated in Human Nutrition with a PhD in Experimental and Clinical Nutrition. She is a fixed-term Research Fellow A at the Department of Clinical Sciences and Community Health, University of Milano, Italy. Since 2018, she has been a nutritional consultant at the Pediatric Clinical Nutrition Outpatient Clinic, IRCCS Ca' Granda Foundation Ospedale Maggiore Policlinico, Milano, Italy. Her original research projects are related to the nutritional status (energy expenditure, fatty acid profile, nutritional habits, vitamin D status) in pediatric patients hospitalized for both acute and chronic diseases. Her scientific reviews field are nutritional aspects in pediatric age; the role of docosahexaenoic acid in the development and maintenance of correct brain functions and protective effect against cognitive processes and the neuropathology's onset; nutrition in the prevention and management of food allergies (study of the diet in the first years of life and the impact on health and correct growth of the different types of formulas used as an alternative to breast milk); the role of diet and nutrients in the prevention of non-communicable diseases; Mediterranean diet; and sustainable diet.

Valentina De Cosmi

Valentina De Cosmi is a Biologist who graduated in Human Nutrition with PhD in Epidemiology. She is a post-doc researcher in the field of pediatric nutrition and nutrition epidemiology at the Department of Clinical Sciences and Community Health, University of Milano, Milano, Italy. Her topic areas of interest are the evaluation of resting energy expenditure of healthy and critically ill children; the evaluation of nutritional habits and body composition; the analysis of fatty acids profile; the Mediterranean diet and the adherence to the Mediterranean diet in children; the dietary patterns and their sustainability for the planet; the role of diet and nutrients in preventing non-communicable diseases; nutrition in the prevention and management of food allergies; the epidemiological determinants of infertility.

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Editorial

Nutritional Habits and Interventions in Childhood

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The present Special Issue of *Nutrients* aims to host scientific articles contributing to enriching the knowledge in the field of nutritional habits and intervention in childhood. The role of the diet in the achievement and maintenance of a healthy status is well recognized. This is especially important in the pediatric age since children need an adequate intake of energy and nutrients for growth and development with respect to their full potential. This collection includes a large group of articles dedicated to the nutrition of the healthy child and its preventive and therapeutic role in numerous diseases (sarcopenia, idiopathic nephrotic syndrome, obesity, anaemia, dyslipidemia, and cardiovascular disease).

Most papers emphasize the importance of establishing proper eating and lifestyle habits to prevent chronic noncommunicable diseases in the first 1000 days and assess different individual and population strategies. Healthy and sustainable food models have been identified: the Mediterranean diet, the new Nordic diet, and the oriental diet, which are the result of ancient traditions but are currently difficult to spread. The main difficulties in identifying effective strategies to improve eating and healthy-living habits are linked to the specificity of the developmental age, the possibility of accessing the various initiatives and, therefore, the socio-economic level, the influence of media and peers, and local traditions. In this editorial, we will provide a look at the topics discussed in each article, providing the novel contributions of the authors on the main theme.

Optimizing nutrition in infancy and establishing healthy lifestyles from preschool years help prevent all forms of malnutrition and diet-related non-communicable diseases in future life. Obesity treatments and preventions in childhood are topics that always offer reasons for study and reflection. In this volume, various therapeutic approaches and suggestions for the treatment and prevention of obesity in the pediatric age are presented and discussed. Alaina P. Vidmar et al. [1] conducted a pilot study on the feasibility and safety of the time-limited eating (TLE) combined with continuous glucose monitoring in 50 adolescents. TLE strategy limits the eating window and may be a feasible approach for treating adolescents with obesity. Calcaterra V. et al. [2], following promising interventions in adults, propose a review of the role of the Ketogenic Diet (KD) as a possible therapeutic tool to counteract metabolic alterations and systemic low-grade inflammation in children and obese adolescents and explain the hypothesized mechanism of action of KD. It is well known that obesity intervention programs should prioritize the low socio-economic families and those with overweight or obese parents. Roberto Franceschi et al. [3] present the "Smuovi La Salute" ("Shake Your Health") project. This project was targeted to prevent and treat overweight and obesity in low socioeconomic status and minority groups. An app and a cookbook promoting transcultural nutrition and a healthy lifestyle were developed, and no-cost physical activities were organized. Healthy lifestyle teaching was implemented in 30 primary school classrooms. Students' knowledge of good nutrition significantly improved, and they started to eat more fruits and vegetables. No modifications to the physical activity levels occurred. However, at the individual level, the comprehensive and integrated management of obese patients remains mostly ineffective.

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In the context of the prevention of obesity, Kwon et al. [4] described the association between daily milk consumption and the risk of obesity in children aged 30–36 months in Korea. Their data confirmed that participants who consumed an amount higher than the recommendations (more than 500 mL milk per day) were at an increased risk of obesity at the age of 42–72 months, underlining the importance in providing nutritional education to the parents or caregivers. Another study that explored the consumption of milk is the one by Cristine Couto de Almeida et al. [5], which aimed to assess the protein quality and essential amino acid content of both starting and follow-up formulas from different manufacturers in Brazil. The authors found that only some brands exhibited total protein content in accordance with product labels. Protein composition and essential amino acid ratios were variable.

Stival s article [6] is dedicated to both the epidemiological aspect of childhood obesity and overweight in a Nord Italian region and some conditions associated with obesity. In the population examined (2916 adolescents aged 11–15 years), a direct relationship between obesity and increased psychological distress or being victims of bullying has been demonstrated, especially in those with low levels of physical activity. The authors underline the importance of psychological wellbeing since being overweight and having poor physical activity were both related to several shortcomings (i.e., feeling nervous and irritable) and being a victim of bullying. These observations suggest the need for physical activity to always be included in the prevention and treatment of overweight not only for its positive effects on the metabolism but also because participation in sports and physical activity reduces mental health problems developing in adolescence.

Nutritional interventions tailored to specific pathologies are needed to prevent nutritional deficiencies and maintain an adequate nutritional status too. In particular, children and adolescents with chronic or inflammatory diseases are more vulnerable and are at major risks of developing malnutrition. In the presence of cardiovascular risk factors, all guidelines propose dietary behavioural intervention as a first step in treating overweight/obesity. The classification and treatment of an inflammatory condition and metabolic derangement present in numerous pathologies are addressed in some articles in this volume. Giussani et al. designed a study on a cohort of 276 children and adolescents (4–18 years) based on the modification of dietary habits and the general lifestyle, demonstrating an improvement in the weight status and, in most cases, the metabolic alterations (e.g., alterations in the lipid profile, insulin resistance, and hyperuricemia) [7]. In the article by De Cosmi et al., the authors performed a randomized, double-blind, controlled study to evaluate the effects of vitamin D and docosahexaenoic acid (DHA) co-supplementation for six months on vitamin D status, body composition, and metabolic markers of obese children with a vitamin D deficiency. During the supplementation period, all subjects received nutritional advice. At the end of the study, all subjects had fat mass significantly reduced, even if still in a condition of obesity. Children receiving both vitamin D and DHA presented a higher increase in DHA levels that could be relevant to prevent inflammatory-associated complications of obesity, but co-supplementation was no more effective than vitamin D plus placebo [8].

Two articles [9,10] dedicated to cardiovascular diseases and familial hypercholesterolemia underline the role of the preventive and therapeutic intervention of nutrition and discuss dietary recommendations for children [9,10]. In the case of pathology, such as familial hypercholesterolemia, current guidelines support the dietary and lifestyle approach as the primary strategy of intervention in children and adolescents, but additional interventions with nutraceuticals having cholesterol-lowering effects, both as functional foods and as supplements, have been proposed. Banderali et al. in their updated literature review concluded that the use of functional foods as supplements is an interesting strategy for paediatric patients; however, it may have some risks as trials on nutraceuticals have been frequently carried out on a limited study population and the availability of nutraceuticals as supplements without medical prescription could result in uncontrolled use such as auto-prescription, therapy discontinuation, and/or excessive dose, with a consequent reduced therapeutic effect and/or increased adverse events [10]. Capra et al. underline

the importance of prudent diet, lifestyle modifications, and the pursuit of psychological wellbeing to prevent the development of dangerous serum lipid levels, excessive adiposity, and elevated blood pressure through intervention in childhood [9].

Rutigliano et al. evaluated the capacity to identify the presence of cardiovascular and metabolic risk of the new guidelines on the diagnosis of hypertension in paediatrics. The authors retrospectively analysed data from 489 overweight and obese children and adolescents [11]. They classified hypertension according to the 2017 American Academy of Pediatrics classification and according to the 2004 classification. The newest ones offer the opportunity to better identify overweight and obese children at risk for organ damage, permitting the design of an earlier prevention strategy. Helgadottir et al. analyzed the macronutrient intake and blood lipid profile at 6 years of age by comparing results from two earlier population-based cohorts of healthy children. They found that a lower intake of saturated and trans-fatty acids, replaced by unsaturated fatty acids, may have contributed to an improved lipid profile in that population. This research work was aimed at the preparation of new national dietary surveys and interventions in childhood [12].

The following articles rely upon nutrition intervention or monitoring and their role in specific disease conditions. Two reviews evaluate the effectiveness of nutritional interventions in improving the quality of the diet of the child population. Qiu et al. analyzed the possible role of the consumption of protein-rich breakfast as a strategy for weight management by declining short-energy intake and suppressing appetite [12]. Most eligible studies were of low quality; therefore, the results ought to be interpreted cautiously. Naroa Andueza et al. systematic reviewed 12 studies and reported that interventions that modify the school environment or provide different meals or snacks may be effective in improving children's dietary patterns, both in the short and long term [13].

The Developmental Origin of Health and Disease theory, also known as the "Barker hypothesis", is mentioned in some articles [9,14]. According to Barker's hypothesis, an individual is programmed toward nutritional thrift during gestation and early postnatal life so that she/he can survive environmental insults caused by poor nutrition. The review by Inzaghi et al. summarized the nutritional roles in the regulation of growth from fetal life to adolescence, attempting to better understand the interplay between nutrients and the endocrine system [14]. The aim was the development of strategies for optimizing nutritional status and allowing the recovery of a normal growth pattern. In this article, the authors recall the fundamental importance of measuring the growth parameters that allow the documentation of regular growth, which is recognized as an excellent and reliable indicator of the child's general good health. The knowledge of the endogenous and exogenous factors (genetic, endocrine, environmental, nutritional, and socioeconomic) that specifically influence the different growth periods makes it possible to target diagnoses in the case of slowdowns or accelerations with respect to the growth rate.

Achieving an adequate intake of nutrients is an important goal for all ages of life and, particularly, for the pediatric age since it is crucial for cognitive development. Giordani et al. addresses the issue of the Adherence to Dietary Recommendations. The authors used Nutrient Adequacy Ratio (NAR) and Mean Adequacy Ratio (MAR) approaches to evaluate adequacy to Italian dietary reference values at nutrient- and overall-diet levels in 381 seven-year-old children who were previously enrolled within a cohort study aimed at evaluating the effects of mercury on infant neurodevelopment. The study showed a distribution of macronutrient intakes, in the percentage of energy, and it was unbalanced in favour of protein and fats and inadequate with respect to defects for vitamin D, zinc, and folates. Finally, the suboptimal adequacy of the overall diet in the study population emerged [15]. Roberts et al. found significant advances in cognitive outcomes in undernourished preschool-age children who received micronutrient supplementation and improvements in cognitive abilities in nourished children who increase fish consumption [16].

Nutritional status is strictly linked to nutritional habits and, therefore, to nutritional preferences. The cross-sectional study presented by Mumena et al. assessed the role of parents in shaping the dietary behaviours of children. In particular, the impact of maternal

knowledge and attitude related to free sugar (FS) was evaluated. Saudi children numbering 424 and aged 6–12 years with their mothers were included, and the results showed that, despite the limited awareness about FS and their influence on health, mothers were making efforts to limit their children's consumption of this nutrient [17]. Guzek et al. explored the associations between food preferences and appetitive traits in adolescents within the Polish Adolescents' COVID-19 Experience study population using two validated questionnaires. The results support the association of food preferences with both food approach traits and food avoidance traits [18].

The review from Caffarelli et al. is part of articles that clarify and reinforce EFSA's messages regarding the timing of egg introduction during complementary feeding. A delayed introduction has no preventive benefit and may negatively influence the growth and psychological wellbeing of children and their families. The authors suggest that HE or HE-containing products should be a regular part of the diet from around 6 months of age, and they should not be introduced earlier than 4 months of age [19].

Vandenplas et al. tested, in a real-life situation, the usefulness of the Cow's Milk-related Symptom Score (CoMiSS®) for the diagnosis and management of infants presenting with symptoms attributable to cow's milk allergy, suggesting, therefore, that it is an effective tool in aiding the awareness of disease in primary health care [20].

Turolo et al. performed a cross-sectional study to evaluate the positive potential role of the Mediterranean dietary pattern in patients with idiopathic nephrotic syndrome (INS) thanks to their anti-inflammatory properties related to the high levels of omega-3 fatty acids. The authors investigated the adherence to the Mediterranean diet and fatty acid profile and the results in 54 children with Idiopathic Nephrotic Syndrome (INS) and found a negative correlation between proteinuria and the anti-inflammatory omega-3 series [21].

In a cross-sectional study by Sunardi et al., in a sample of 180 children aged 6–36 months living in a poor urban area of Jakarta, results from a nutritional survey detected two dietary determinants as risk factors for anaemia, that there was no cow's milk formula consumption, and an inadequate zinc intake [22].

The aspect of validated screening tools in paediatrics is intriguing too, in addition to the training of health professionals for their utilization, for preventing both nutritional and psychopathological consequences. The paper by Bertrand et al. reported data about the prevalence of Feeding and Eating Disorders in the general pediatric population, diagnosing the presence of these conditions in at least 12.7% of the children and adolescents [23]. Screening became more than important in the contest of oncologic patients because, in children hospitalized for cancer, malnutrition constitutes a very common complication. Romano et al. examined nutritional and sarcopenia statuses and their clinical impact on pediatric patients' prognosis affected by bone and soft tissue sarcomas [24].

The message that can arise from all the articles is represented by the fundamental role of the paediatrician as being responsible for health from infancy to adolescence. In particular, the fundamental usefulness of the periodic assessment of nutritional habits and growth parameters, nutritional status, and cognitive development, as part of pediatric check-ups, is examined. Such surveys would allow the early identification of any nutritional errors, eating disorders, and growth alterations and would allow prompt treatment.

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Article

Time-Limited Eating and Continuous Glucose Monitoring in Adolescents with Obesity: A Pilot Study

Alaina P. Vidmar [1,2,*], Monica Naguib [1,2], Jennifer K. Raymond [1,2], Sarah Jeanne Salvy [3], Elizabeth Hegedus [1,2], Choo Phei Wee [4] and Michael I. Goran [1,2]

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Abstract: Due to its simplicity, time-limited eating (TLE) may represent a more feasible approach for treating adolescents with obesity compared to other caloric restriction regimens. This pilot study examines the feasibility and safety of TLE combined with continuous glucose monitoring (CGM) in adolescents. Fifty adolescents with BMI ≥95th percentile were recruited to complete a 12-week study. All received standard nutritional counseling, wore a CGM daily, and were randomized to: (1) Prolonged eating window: 12 h eating/12 h fasting + blinded CGM; (2) TLE (8 h eating/16 h fasting, 5 days per week) + blinded CGM; (3) TLE + real-time CGM feedback. Recruitment, retention, and adherence were recorded as indicators of feasibility. Weight loss, dietary intake, physical activity, eating behaviors, and quality of life over the course of the intervention were explored as secondary outcomes. Forty-five participants completed the study (16.4 ± 1.3 years, 64% female, 49% Hispanic, 75% public insurance). There was high adherence to prescribed eating windows (TLE 5.2 d/wk [SD 1.1]; control 6.1 d/wk [SD 1.4]) and daily CGM wear (5.85 d/wk [SD 4.8]). Most of the adolescents (90%) assigned to TLE reported that limiting their eating window and wearing a CGM was feasible without negative impact on daily functioning or adverse events. There were no between-group difference in terms of weight loss, energy intake, quality of life, physical activity, or eating behaviors. TLE combined with CGM appears feasible and safe among adolescents with obesity. Further investigation in larger samples, with a longer intervention duration and follow-up assessments are needed.

Keywords: intermittent fasting; continuous glucose monitor; obesity; pediatrics; adolescents

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1. Introduction

In the United States, one in five adolescents has obesity, and 30–50% of those go on to develop early onset type 2 diabetes, which is associated with a high risk of complications [1–4]. With increasing prevalence, an aggressive disease phenotype with risk for both short- and long-term health complications, and increasing cost for care, pediatric obesity in adolescents is expected to result in extensive financial costs, significant life-limiting complications, and negative impacts on quality of life [5–8]. Conventional pediatric obesity treatment addresses nutritional, physical activity, and behavioral topics with the goal of achieving clinically meaningful weight loss, defined as a weight loss of 5% or more of baseline weight [4,5,9–14]. Adherence to comprehensive lifestyle intervention recommendations is challenging for adolescents, in part because these approaches

require monitoring and engaging in multiple behavioral targets (e.g., caloric intake and/or macronutrients, physical activity, impulse control). There is increased interest in finding effective and sustainable alternatives to improve weight loss and overall health and well-being in adolescence.

Multiple trials, conducted globally, in adult populations have examined the efficacy of various fasting regimens, including alternate day fasting, fasting mimicking diet, and time-restricted eating (TRE) [15–31]. Time-restricted eating involves shortening the eating window to a pre-specified number of hours per day (6 to 10 h) and fasting for the remaining hours of the day, without altering diet quality and quantity [30,31]. TRE has been shown to be well-tolerated and safe in adult populations, while promoting β cell responsiveness and reduction in fat mass [20,21,24,25,32–35]. However, the feasibility and effectiveness of TRE in adolescents has been questioned due to concerns of poor adherence, fear of iatrogenic adverse events (such as increased disordered eating behaviors [33–36]), and consequences on development. Because of its simplicity, TRE may result in greater intervention adherence than comprehensive and costly approaches, while preserving autonomy and dietary preferences [35].

This pilot study was undertaken to examine the feasibility and safety of TRE or time-limited eating (TLE, as it will be referred to moving forward) combined with a continuous glucose monitor (CGM) relative to eating during an extended eating window among adolescents with obesity. We were primarily interested in the feasibility of recruiting and retaining adolescents in the study, and examining adherence to the intervention and assessment procedures, while monitoring possible iatrogenic effects of TLE on eating attitudes and practices. CGM was used to capture glycemic excursions, monitor adherence to TLE and control intervention protocols, and monitor for hypoglycemia. Weight loss, dietary intake, percent time in range, quality of life, physical activity, and eating behaviors and attitudes were collected as secondary outcomes. We hypothesized that TLE would be feasible, safe, and not negatively impact any of the secondary outcomes during the 12-week trial.

2. Materials and Methods

2.1. Study Design

This 12-week pilot randomized controlled trial examined the feasibility, safety, and preliminary efficacy of TLE (8-h eating/16-h fasting, intervention) compared to the control (12-h eating/12-h fasting). The trial was implemented remotely between March 2020 and June 2021 [36]. The protocol was reported by Vidmar et al.; however, due to the timing of implementation, there were several protocol changes made before implementation. Briefly, adolescents (ages 14–18) with obesity (BMI $\geq$ 95th percentile) were recruited from clinical programs at Children's Hospital Los Angeles (CHLA). All participants and their families received a one-time, two-hour nutritional counseling session promoting low added sugar and carbohydrate intake delivered by a healthy educator. Participants chose and paid for their own food for the entire intervention. Research visits were conducted at 0, 4, 8, and 12 weeks and lasted 120 min (5 total visits, including initial consent visit). All participants wore a continuous glucose monitor (CGM) daily for the study duration. After a one-week run-in period, all participants were randomized (block size = 3 and 6 and balanced by sex and age) to: (1) Control: 12 h eating/12 h fasting + blinded CGM; (2) TLE (8 h eating/16 h fasting 5 days per week) + blinded CGM; and (3) TLE + real-time CGM feedback.

Due to COVID-19 restrictions, this pilot study was conducted virtually. Study material (body scales and CGM supplies) were shipped to the participants' homes, and all study interactions with participants, including the informed consent process and enrollment into the study, occurred via a secure HIPPA-compliant videoconference platform. Experienced staff guided the participants to conduct anthropometric measurements throughout the study period. Participants completed validated patient-reported outcome surveys at each visit via Research Electronic Data Capture (REDcap). Weekly contacts with participants were conducted over the phone by the study team, lasting approximately 15 min per session.

The purpose of these calls was to review participants' experience with the prescribed eating window, provide support and guidance, and monitor for adverse events. Participants were also asked to report any adverse events or changes in their health or physical function since the last contact. See the full study protocol for details on study team training and fidelity monitoring [37].

All study procedures were approved by the CHLA Institutional Review Board (CHLA-000193, date of approval—20 December 2019). The study was reported according to the Consolidated Standards of Reporting Trials (CONSORT) statement and is registered with ClinicalTrials.gov (NCT03954223). Written informed consent was obtained from the adolescents and one parent or guardian. The study was conducted in accordance with the Declaration of Helsinki and all participants provided written informed consent prior to participation. Participants received compensation in the form of gift cards to complete study assessments.

2.2. Participants

Inclusion criteria were: (1) age 14–18 years; (2) BMI $\geq$ 95th percentile; (2) participant and/or parent/guardian or family member had a personal smart phone that was CGM compatible and/or was willing to come to the study center for manual data upload monthly for the study duration; and (3) participant was willing and able to adhere to the assessments, visit schedules, and eating/fasting periods Adolescents were ineligible for the study if they: (1) had a documented diagnosis of Prader Willi Syndrome, type 2 diabetes, brain tumor, hypothalamic obesity, binge eating disorder, serious developmental or intellectual disability, or previously diagnosed eating disorder; (2) were unable or unwilling to complete study assessments (e.g., inability to wear CGM, inability to be in the imaging modality without sedation); and/or (3) were enrolled in a weight loss intervention or previously underwent bariatric surgery; or (4) were taking weight-altering medication (e.g., antipsychotics, sedatives, hypnotics, off-label obesity medication, insulin).

2.3. Intervention Components

Components Common to All Study Arms. All participants received two hours of nutrition counseling focusing on reduction of carbohydrate and added sugar intake prior to randomization. The education session provided dietary recommendations for intake of added sugars (<5% of daily energy intake) and carbohydrate (<100 g per day). No specific caloric restriction was recommended, and participants were not required to keep logs of food intake. Recommendations were made in terms of avoiding sugar-sweetened beverages, juices, and food high in added sugars. In addition, physical activity consistent with physical activity guidelines for adolescents was encouraged but not formally prescribed.

Time Limited Eating. Participants were instructed to consume all their food in an eight-hour time window (i.e., from 11 AM to 7 PM) with a 16-h fasting period. Participants selected their eating window based on feasibility and daily routine. At baseline, participants' eating windows were recorded based on dietary recall. At the consent visit, baseline eating windows were re-assessed, and participants were required to select their eating/fasting windows. Noncaloric, non-artificially sweetened beverages (water, tea, coffee) were allowed during the fasting period. All participants were asked to record the time they started and finished eating daily, and to report their eating windows with the study staff weekly.

Control. Participants assigned to the control arm were instructed to consume food over a 12-h or more eating window. No energy restriction was required. All participants were asked to record the time they started and finished eating daily and to report their eating windows to the study staff weekly. As described above, the nutrition and physical activity recommendations were received by all participants regardless of treatment group.

Continuous Glucose Monitor. All participants wore Dexcom G6 continuous glucose monitors (Dexcom, San Diego, CA, USA) continuously for 13 weeks (week −1 to week 12 of the study period). All participants were blinded to CGM data for seven days for

baseline data collection (1 week), and then randomized to one of three intervention arms: Control + blinded CGM; (2) TLE + blinded CGM; and (3) TLE + real-time CGM feedback. Participants in the control and TLE + blinded CGM groups were blinded to the CGM data in that they did not have access to the smartphone app or web browser platform that housed the glycemic data, throughout the study period and therefore did not have real-time access to their glycemic profiles. Participants in the TLE + real-time CGM feedback group were coached to use a personal smart phone with Bluetooth capabilities to access real-time blood glucose levels throughout the 12-week intervention period. Participants were provided with a transmitter and enough sensors to replace the sensor every 10 days. The participants and guardians were educated on how to use the CGM and received 1:1 coaching on how to change the sensor, which was completed either independently or under study team guidance. No glucometer calibration was required. At each weekly phone meeting, study staff monitored any adverse events and challenges related to CGM wear, including participant discomfort, skin adherence, and other issues.

2.4. Measurements

At baseline, adolescents and their parents were asked to complete a demographic questionnaire (age, gender, race/ethnicity, household composition, education, household income), baseline eating window (assessed with dietary recall and semi-structured interview), and medical history. The primary endpoint of the study was feasibility. Feasibility was determined by assessing the number of days adolescents complied with their prescribed eating window, number of days they wore their CGM, number of weekly phone calls and scheduled research visits they attended, Satisfaction Questionnaire, and exit interview. Secondary goals for the study were to compare clinical outcomes (weight loss, dietary intake and quality, physical activity, eating behaviors and practices, and quality of life) for adolescents in the TLE versus control groups throughout the study period. Participants were also asked to complete a series of self-reported survey measures at baseline, mid-study, and three months. Measures included the Nutrient Data System Recall (NDSR) 24 Hour Dietary Recall, Pediatric Quality of Life Scale (PedsQL), Patient Reported Outcomes Measurement Information System (PROMIS®) Physical Activity Scale, and Binge Eating Disorder Screener (BEDS) [35,38–43]. Exploratory goals of the study were to compare glycemic profiles (percent time in range, average glucose) between TLE and control throughout the study period.

2.4.1. Primary Outcome—Feasibility

Compliance with the recommended eating windows was collected from adolescents during the weekly phone calls with the study team. Adolescents were asked to record the time they started and finished eating daily, the number of days they adhered to their prescribed eating schedule, and barriers to adherence. Adolescents were instructed to wear their CGM daily for the duration of the study and to report deviation from the protocol during the phone calls. In addition, study staff reviewed the Dexcom Clarity platform to verify the number of CGM wear days per week. The number of calls completed over the course of the study was recorded. Assessment of satisfaction with the eating window included a 5-point scale from 1 = 'strongly agree' to 5 = 'strongly disagree for the following domains: (1) perceived effects of eating window on daily functioning, (2) would recommend to friends, (3) perceived hunger, and (4) how the assigned eating window impacted their family. During weekly phone calls with the study staff, adolescents were asked open-ended questions about their experience with either TLE or control, likelihood of continuing their current eating window after the study was over, and any barriers to adherence. A one-time exit interview was completed at week 12.

2.4.2. Secondary Outcome

Anthropometrics. All participants received a wireless Bluetooth scale upon consent. Participants' height and weight were collected by the participant and parent/guardian at

home with the research coordinator monitoring the measurement collection via a HIPAA compliant virtual platform. Height was measured using a portable wall height indicator tape ruler, accurate to 0.5 cm (Posh Rulers, Quick Medical, Issaquah, WA, USA). Weight was measured on a self-calibrating Etekcity Digital Body Weight Scale, accurate to 0.2 kg (Etekcity, San Diego, CA, USA). Adolescents wore minimal clothing during the height and weight measurements. BMI was calculated as kilograms per meter squared and BMI z-score (zBMI) and excess percent of the 95th percentile (%BMI_{p95}) was determined utilizing the CDC growth charts.

Dietary Intake [38,41–43]: Twenty-four-hour dietary recalls using the Nutrient Data System Recall (NDSR) 24 Hour Dietary were conducted in duplicates (one weekend day and one weekday in control and one TLE day and one non-TLE day for those in the TLE groups) at three timepoints throughout the study. The procedures used in the 1985–1986 United States Department of Agriculture Continuing Survey of Food Intakes of Individuals (USDA-CSFII) were followed, and all recalls were collected in a personal interview via the virtual platform using a standardized protocol based on the "multiple pass" method, which was developed and tested by the USDA for use in the 1994–1996 CSFII in an effort to limit the extent of under-reporting.

Pediatric Quality of Life Scale (PedsQL): Quality of life was measured utilizing the Pediatric Quality of Life Scale (PedsQL), which is a 10-item questionnaire designed to assess quality of life parameters for youth under 19 years of age [36,44–47]. The PedsQL is a brief, standardized, generic assessment instrument that systematically assesses patients' and parents' perceptions of health-related quality of life (HRQOL) in pediatric patients [44,46,48].

Physical Activity: Physical activity was assessed using the Patient Reported Outcomes Measurement Information System (PROMIS®) [39]. The PROMIS® instruments were developed using rigorous qualitative and quantitative methods and standardized to a reference population. The PROMIS® measures for children have been found to demonstrate feasibility, internal consistency, construct validity, and responsiveness to change in a clinical setting. The physical activity survey is a self-administered 7-day recall instrument developed to assess the general levels of physical activity of children and adolescents.

Binge Eating Disorder Screener (BEDS) [49–52]. Given that adolescents with obesity are at high risk of binge eating disorder (BED) symptoms, our goal was to screen participants at baseline to ensure appropriate referrals are made in a timely manner. In addition, we monitored for BED symptomatology as a safety metric throughout the study period. Binge Eating Disorder Screener (BEDS-7) is a brief, valid, patient-reported screening tool for use in primary care and general psychiatry settings to identify individuals most likely to have BED and to facilitate further evaluation or referral to specialists. It has been validated in youth aged 12–21 years.

2.4.3. Continuous Glucose Monitoring

CGM data were downloaded weekly by the study team. CGM data were evaluated continuously over the study period. This data was utilized to compute the following measures: mean, maximum, and minimum glucose levels; standard deviation of glucose; mean amplitude of glycemic excursion; and overall percent of total time spent in euglycemic range (percent time in range = 70–180 mg/dL). All CGM data were housed in Clarity (Dexcom and Dexcom CLARITY are registered trademarks of Dexcom, Inc., San Diego, CA, USA) and the study team had weekly access to assess all glycemic excursions that occurred during the self-reported fasting periods [50–53]. For those in the TLE + real-time CGM feedback group, every time they viewed their CGM data in the app, the event was captured, and time stamped in the Clarity system.

2.5. Statistical Analysis

The study was a pilot trial, thus we opted for a convenient sample size of 50 participants to estimate parameters for a larger, fully powered trial. NDSR, PedsQL, PROMIS®

PA, and BEDS questionnaires were summarized according to prior literature. Analyses were based on the intention to treat (ITT) population using the last observation carried forward. The ITT population was defined as at least two visits (baseline and 1 month). The study was designed as a three-group intervention analysis; however, given that very few adolescents in the TLE+ real-time CGM feedback group looked at their real-time data, we completed a post hoc analysis combining both TLE groups compared to control for all analyses performed.

Baseline characteristics (age, sex, race, BMI status, household income) were summarized descriptively across arms for the ITT population using mean and standard deviation (SD) or median and interquartile range as appropriate for the distribution of continuous variables. Categorical variables are described as a frequency and percentage. Continuous variables that are skewed were analyzed in log scale. The differences in demographics, anthropometrics baseline, and eating window distributions among intervention groups were examined using analysis of variance (ANOVA) and Fisher's Exact test. Adherence was operationalized as the number of days adolescents complied with their prescribed eating window, number of days they wore their CGM, and number of weekly phone calls and scheduled research visits they attended, and satisfaction scores were summarized using mean, standard deviation (SD), minimum, and maximum score between TLE and control.

To assess the TLE effect on the secondary outcomes, mixed-effects models were used to evaluate the change in clinical outcomes from week 0 to week 12 between intervention groups. The TLE effect on mean change of BMI z-score and $\%BMI_{p95}$ between week 0 and week 12 was assessed by using ANOVA. Then, a mixed-effects generalized linear model based on a Gaussian or Gamma distribution as appropriate was used to further assess the TLE effect on change in weight outcome from week 0 to week 12. The mixed-effects generalized linear model based on a Gaussian or Gamma distribution was used as appropriate for the distribution of continuous outcome variables. Whereas a mixed-effects logistic regression model was used for binary clinical outcome variables. In addition, a mixed-effects Tobit regression model was used to evaluate the TLE effect on the change in quality-of-life assessment, where the scores are reported in percentages, with no data below 0 or above 100. Then, the non-additive effects of TLE were also examined; specifically, whether the change on clinical outcomes during the study period varied by intervention groups by including the interaction term in the mixed-effect models. In addition, sensitivity analysis was performed to examine whether the weight change observed in the data was influenced by one adolescent who achieved a weight loss of greater than 15% from baseline weight. All results are described in beta estimate, β, percent change, and odds ratio with its associated 95% confidence interval and *p*-value. The statistical significance level was set at 0.05 with two-sided throughout the analyses. All statistical computations were done in Stata/SE 17.0 (StataCorp, College Station, TX, USA).

3. Results

3.1. Primary Outcome—Feasibility

3.1.1. Characteristics of Participants Recruited

Descriptive statistics are provided in Table 1 describing participants' demographic characteristics and anthropometrics at week 0. In total, 511 adolescents with obesity were screened (Figure 1). Eligible adolescents were identified through various recruitment methods including clinic referral, hospital-wide advertising, community advertising, social media, and direct contact from the research team either by phone, email, or self-referral. Of the 183 adolescents screened, 99 did not meet the eligibility criteria. Of the remaining 84, 34 declined to participate. Thus, 50 adolescents were enrolled in the study, which achieved a recruitment rate of 60% of those who were contacted about the study. Five participants withdrew, two of whom developed type 2 diabetes and required initiation of pharmacotherapies, two withdrew because of unexpected changes to their school and work schedules, and one became pregnant. Forty-five adolescents completed the study

(Figure 1). Consistent with the demographics of patients served by CHLA, most participants were Hispanic (60%), publicly insured (74%), and had an annual household income <$50,000 (70%) (Table 1). There was no significant difference in demographics or baseline characteristics between study completers and non-completers (all *p*-values > 0.5).

Table 1. Demographic characteristics and baseline anthropometrics.

	Total (*n* = 50)	**Control (*n* = 15)**	**TLE + Blinded CGM (*n* = 19)**	**TLE + Real-Time CGM Feedback (*n* = 16)**	***p***
Age (in year) [1]	16.43 ± 1.17	16.38 ± 1.25	16.16 ± 1.16	16.80 ± 1.09	0.3 [a]
Sex [2]					0.8 [b]
Male	14 (28.0)	3 (20.0)	6 (31.5)	5 (31.2)	
Female	36 (72.0)	12 (80.0)	13 (68.4)	11 (68.7)	
Race [2]					0.05 [b]
White	5 (10.0)	3 (20.0)	1 (5.2)	1 (6.0)	
Black	3 (6.0)	1 (6.6)	2 (10.5)	0 (0)	
Asian	4 (8.0)	3 (20.0)	1 (5.2)	0 (0)	
Hispanic	27 (54.0)	7 (46.7)	13 (68.4)	7 (43.8)	
Am. Indian	2 (4.0)	0 (0)	1 (5.2)	1 (6.2)	
Mixed race	6 (12.0)	1 (6.6)	0 (0)	5 (31.2)	
Ethnicity [2]					0.1 [b]
Non-Hispanic	15 (30.0)	8 (53.3)	4 (21.1)	3 (18.7)	
Hispanic	32 (64.0)	7 (46.6)	14 (73.6)	11 (68.7)	
Weight (kg) [3]	101.4 (87.9, 123.8)	104.3 (74.8, 123.1)	99.5 (84.6, 123.2)	110.5 (92.2, 128.3)	0.9 [c]
%BMI_{p95} [3]	125.9 (111, 158)	141.1 (114.4, 167.0)	122.6 (110.0, 158.5)	123.9 (109.8, 159.1)	0.9 [c]
BMI z-score [1]	2.30 ± 0.5	2.34 ± 0.5	2.28 ± 0.4	2.30 ± 0.5	0.9 [a]

[a] Analysis of variance; [b] Fisher's Exact test; [c] Analysis of variance in log scale; [1] Mean ± standard deviation; [2] Frequency (percentage); [3] Median (interquartile range).

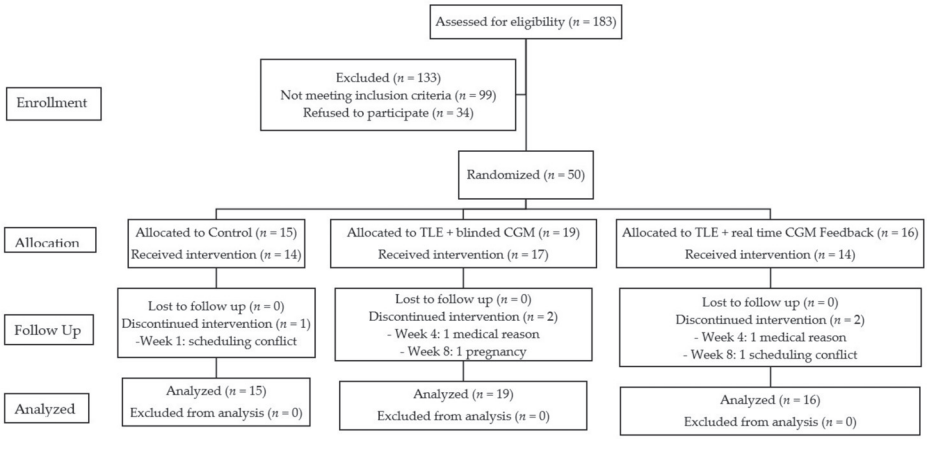


Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flow diagram of participant inclusions.

3.1.2. Adherence

The baseline average eating period for this cohort was 9.7 h per day (SD 3.3, range: 1–21 h) with no significant difference between an eating window <10 h and ≥10 h among the three intervention groups (χ^2 = 2.4, p = 0.3). Ninety percent of adolescents in the TLE groups selected to start their eating window between 10 AM and 12 PM (11 AM-7 PM—16/31, 52%, 12 PM and 8 PM—9/31, 29%), and 10 AM and 6 PM 3/31, 10%). The remaining three adolescents selected 1 PM-9 PM, 2 PM-10 PM, and 3 PM-11 PM. Eighty percent of adolescents in the control group selected to start their eating window between 10 AM and 1 PM and to end their eating window between 9 PM and 11 PM. Over the course of the 12-week study period, there was a significance difference in eating windows between TLE (TLE + blinded CGM and real-time CGM feedback: 7.0 h, SD 2.3) and control

(9.8 h ± 2, SD 3.1, $p < 0.001$). Overall, adolescents were highly adherent to the prescribed eating periods (mean number of days in which TLE was completed per week: 5.2 d/week (SD 1.1) and mean number of days in which control was completed per week: 6.1 d/week (SD 1.2), Table 2). To better characterize adolescents' eating windows, we compared self-reported fasting periods with CGM data to classify what glycemic excursions occurred during fasting. During fasting periods, only four adolescents had excursions ≥60 mg/dL, two of them being later diagnosed with T2D. The remaining participants had excursions from 0–99 mg/dL with a mean excursion of 65 mg/dL during fasting periods.

Table 2. Mean number of days (SD) in which the assigned eating window was completed across intervention arms using intention to treat with carry forward of the last weeks data.

Week	Control (n = 15)	TLE + Blinded CGM (n = 19)	TLE + Real-Time CMG Feedback (n = 16)
1	5.1 (1.9)	4.03 (1.9)	4.5 (1.9)
2	5.6 (1.3)	5.3 (1.3)	5.3 (1.2)
3	5.6 (1.3)	5.4 (1.3)	6.7 (1.3)
4	6.1 (1.6)	5.1 (1.6)	5.3 (1.4)
5	5.9 (1.6)	5.1 (1.6)	5.4 (1.7)
6	4.9 (1.3)	5.4 (1.3)	5.9 (1.1)
7	6.7 (1.3)	5.3 (1.3)	6.0 (1.0)
8	5.4 (1.1)	5.4 (1.1)	4.9 (1.1)
9	5.9 (1.0)	5.1 (1.0)	4.8 (1.5)
10	6.1 (1.2)	5.3 (1.2)	5.3 (1.4)
11	5.5 (1.5)	5.3 (1.5)	5.1 (1.0)
12	4.9 (1.0)	5.3 (1.0)	4.9 (1.1)

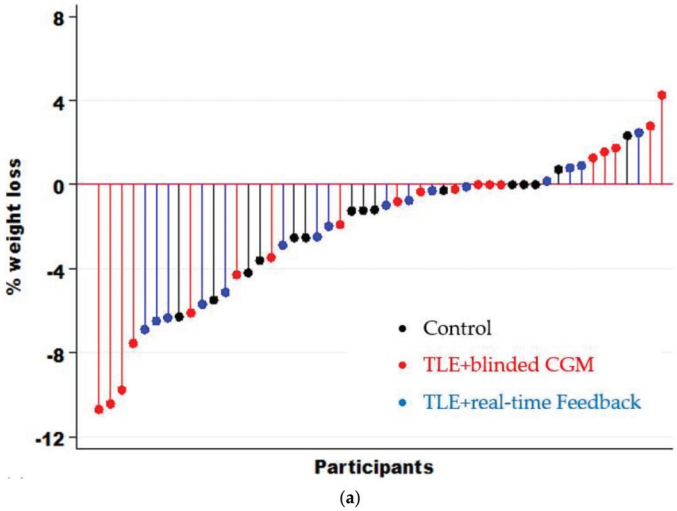
Based on adolescents' responses to the satisfaction survey and exit interviews, TLE was viewed favorably. Overall, 90% of adolescents reported that the study was worthwhile and 95% reported that they would recommend it to others. Only 15% of adolescents reported barriers to implementing their assigned eating window into their daily schedule including conflict with work or sleep schedule, social commitments, and explaining eating patterns to family. Adolescents denied any negative compensatory behaviors (i.e., excessive exercising, binge episodes, or excessive dietary restraint). Adolescents in the TLE groups reported that eating within an 8-h daily period would be feasible for most adolescents and that they would recommend it to their peers. All participants reported they would be willing to continue to eat during their assigned eating window after the study was completed. When asked how helpful TLE was, on a scale from 1 (not helpful) to 5 (very helpful), the mean score was 4. Similarly, when asked how enjoyable the study was on a scale of 1 (not enjoyable) to 5 (very enjoyable), the mean score was 4, with no difference between groups. In addition, adolescents reported favorable experiences with wearing a CGM daily for 12 weeks. Adolescents wore their CGM for a mean of 5.85 (4.08 SD, median 7 days) days per week over the study period with no difference between groups (p = 0.9). No significant barriers to wearing the CGM daily were identified. One-third of participants (15/50) reported at least one minor barrier to daily CGM (i.e., skin irritation, mild bleeding at insertion site, etc.).

3.2. Secondary Outcomes

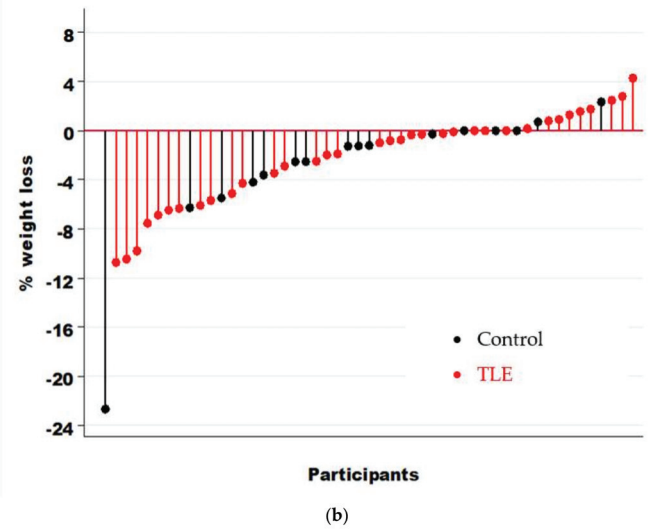
3.2.1. Weight Loss

There was great heterogeneity in weight loss across participants. Overall, 68% of adolescents lost weight during the intervention period (Figure 2). Post-intervention, 26% of the TLE + blinded CGM group lost ≥5% of their baseline weight vs. 31% in the TLE + real-time CGM feedback and 13% in the control group (no between-group difference p = 0.5). Consistent with intention-to-treat analysis, across the study period, there was a significant decrease in median weight loss (kg), $\%BMI_{p95}$, and zBMI across all three groups, with no significant difference in weight loss between groups from the mixed-effect

generalized linear models (Tables 3 and 4, and Figure 3). Sensitivity analysis was conducted to exclude one participant in the control group that lost >15% of their total body weight and the results remained the same. In addition, given that very few adolescents in the TLE+ real-time CGM feedback group looked at their real-time data, we completed a post hoc analysis combining both TLE groups compared to the control and the results remained the same (all $p > 0.05$).



(**a**)



(**b**)

Figure 2. Percent weight loss at week 12 compared to baseline by individual participants across: (**a**) all three intervention arms and (**b**) TLE groups (TLE + blinded CGM and TLE + real-time CGM) compared to control.

Table 3. Weight change between baseline and week 12 across intervention arms.

Weight Change	Control (n = 15)	TLE + Blinded CGM (n = 19)	TLE + Real-Time CGM Feedback (n = 16)	p	Effect Size
BMI z-score change	−0.05 ± 0.09	−0.09 ± 0.14	−0.11 ± 0.19	0.6	0.04
$\%BMI_{p95}$ change	−3.27 ± 3.34	−3.76 ± 5.76	−4.85 ± 5.08	0.7	0.04

Table 4. Gamma mixed-effects generalized linear model on $\%BMI_{p95}$ and BMI z-score.

$\%BMI_{p95}$	% Change	95% CI	p
Week			
0	Ref	–	–
4	−2.0	(−2.7, −1.3)	<0.0001
8	−2.9	(−3.8, −1.9)	<0.0001
12	−3.3	(−4.4, −2.1)	<0.0001
Intervention group			
Control	Ref	–	–
TLE + blinded CGM	−3.4	(−17.6, 13.3)	0.7
TLE + real-time CGM feedback	−4.3	(−17.9, 11.7)	0.6
BMI z-score	β	95% CI	p
Week			
0	Ref	–	–
4	−0.05	(−0.1, −0.03)	<0.0001
8	−0.08	(−0.1, −0.04)	<0.0001
12	−0.09	(−0.1, −0.05)	<0.0001
Intervention group			
Control	Ref	–	–
TLE + blinded CGM	−0.08	(−0.4, 0.2)	0.6
TLE + real-time CGM feedback	−0.06	(−0.4, 0.3)	0.7

3.2.2. Dietary Intake and Quality

Overall, all adolescents showed a 25% reduction (~375 calories/day) in their total daily caloric intake on both weekdays and weekend days, and TLE and non-TLE days for those in the TLE groups (all $p < 0.05$). There was no difference in caloric reduction between groups (all $p > 0.05$). There was a small but significant reduction in the percent of calories consumed from added sugars (−2%, decrease of ~14.5 g/day) and carbohydrates (−5%, decrease of ~50 g/day) at week 12 compared to baseline across all participants, with no significant difference between groups.

3.2.3. Physical Activity

All participants reported an increase in the number of days per week they participated in physical activity across the study period (mean +1 day/week (Range: −1 to 3 days/week). The mean t-score on the PROMIS® Physical Activity questionnaire increased over the study period by 3.12 points ($p = 0.01$, minimally important clinical difference on PROMIS® Physical Activity is 2–3 points) across all participants, with no significant difference between intervention arms ($p = 0.7$).

3.2.4. Eating Behaviors and Attitudes

At baseline, 25% of adolescents reported some excessive eating behaviors, with 18% of them reporting distress from excessive eating. Participants who endorsed excessive eating at baseline were referred to their primary care provider for evaluation, and referral to a psychiatrist. Of those 25% identified at baseline, upon further evaluation by a psychiatrist, none met criteria for an eating disorder based on the DSM V criteria. The mixed-effect logistic regression model showed there was no significant change in excessive eating behaviors, binge eating episodes, or distress related to eating over the study course within

and between groups (control vs. TLE—OR 3.1, 95% CI 0.64, 17.89, $p = 0.1$). During the exit interviews, none of the adolescents reported unhealthy eating behaviors, such as excessive exercise, dietary restraint, or eating disorder symptomatology, upon completion of the study.

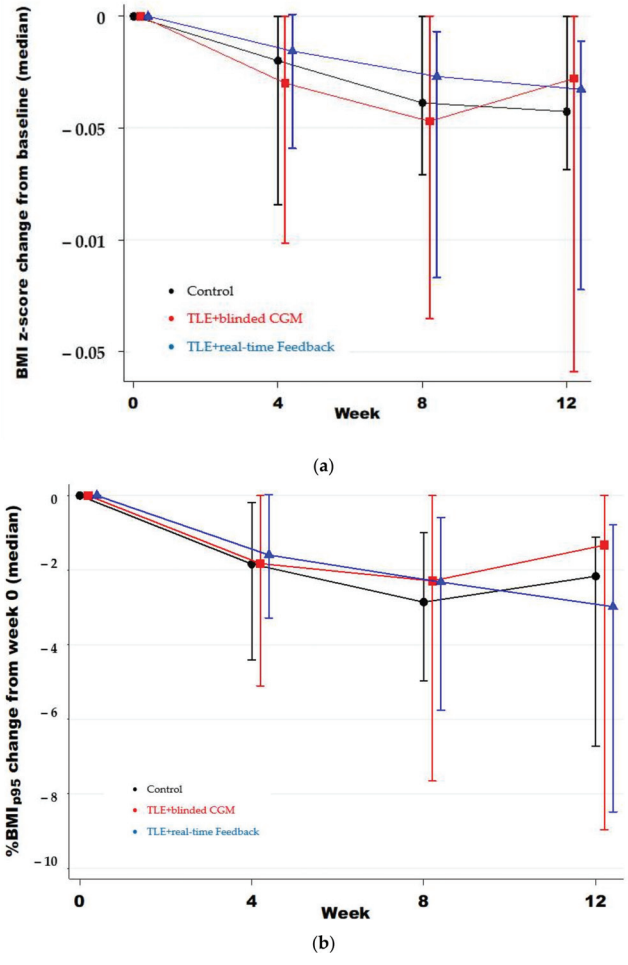


Figure 3. Weight change across the study period by intervention group for (**a**) change in excess percent of the 95th percentile (%BMI_{p95}) and (**b**) change in BMI z-score.

3.2.5. Quality of Life

The overall mean summary score on the PEDsQL scale significantly increased over the study duration (~10% increase, $p < 0.01$), with no significant difference noted between groups ($p = 0.7$. The mean summary (adolescent—β: 3.29, 95% CI: (1.12, 5.46), $p = 0.003$), psychosocial (adolescent—β: 4.50, 95% CI: (1.79, 7.21), $p = 0.001$), and physical health scores (β: 1.39, 95% CI: (−1.50, 4.29), $p = 0.3$) across all three groups significantly increased over time, with no significant difference between groups (all $p > 0.05$). There was no significant change in the mean summary score at week 12 compared to baseline depending on the intervention group (interaction $p = 0.8$). There was no significant effect of eating window (TLE vs. control) or blinded vs. unblinded CGM group (blinded CGM vs. real-time CGM feedback) on the summary score (all p-values > 0.5). On the mixed logistic regression model,

there was no association between weight loss and improvement in the mean summary score (95% CI: −0.02, 30.99, $p = 0.2$)

3.3. Continuous Glucose Monitoring

There was no serious hypoglycemia reported in this cohort. There was no difference in the rates of hypoglycemia between groups (control as reference—TLE + blinded CGM: β = 1.3, 95% CI: (−1.5, 4.2), $p = 0.3$ and TLE + real-time CGM: β = 1.4, 95% CI: (−9.0, 6.1), $p = 0.4$). We evaluated whether adolescents randomized to the TLE + real-time CGM feedback engaged with the glycemic data during the study duration. Although all participants in this group had access to their glycemic data, only nine of the adolescents opened the application to review their glycemic data during the study. Post hoc analysis was completed, and there were no statistically significant differences in weight loss between those who did access the GCM data and those who did not (all $p > 0.05$). There was no difference in the reduction of average glucose levels or percent time in the range between TLE and control (all $p > 0.5$, Table 5).

Table 5. Mixed-effects generalized linear model of the glycemic profile change extracted from CGM data.

Glycemic Profile	β	95% CI	*p*
Average blood glucose			
Visit Week			
0	Ref		
4	2.7	(−4.3, 9.7)	0.4
8	3.3	(−3.6, 10.3)	0.3
12	3.3	(−7.9, 14.6)	0.6
Intervention group			
Control	Ref		
TLE + blinded CGM	−4.2	(−14.7, 6.1)	0.4
TLE + real-time feedback	−7.1	(−20.2, 6.0)	0.2
Estimated HbA1c			
Week			
0	Ref		
4	0.1	(−0.1, 0.3)	0.3
8	0.1	(−0.1, 0.3)	0.3
12	0.1	(−0.2, 0.4)	0.5
Intervention group			
Control	Ref		
TLE + blinded CGM	−0.2	(−0.5, 0.1)	0.2
TLE + real-time feedback	−0.3	(−0.7, 0.1)	0.2

4. Discussion

This is the first study to investigate the feasibility, safety, and preliminary efficacy of TLE in adolescents with obesity. We were able to recruit and retain adolescents in the study, and most participants were able to adhere to the prescribed eating windows. In addition, adolescents reported that TLE was a feasible approach, and it did not interfere with their normal daily patterns and social engagements. Like previous longitudinal monitoring of eating patterns in adults, the eating times in this group at baseline varied considerably [54,55]. Eating events were spread over a wide period of the day for many adolescents (1–24 h). Most participants selected an afternoon/evening eating window regardless of the assignment to the control or TLE groups.

Although we found no between-group difference in weight change, one-third of adolescents in the TLE groups and one-quarter of the control group achieved clinically meaningful weight loss of more than 5% of their baseline weight. One possible explanation for the absence of between-group difference lies in the structured day hypothesis [56–58].

Structured eating has been shown to produce weight loss in adult and pediatric populations [55], and all study participants were provided with a prescribed eating window (i.e., 8-h vs. 12-h eating window). Conceivably, adherence to a controlled eating schedule may help explain weight loss in a subset of adolescents in both study arms [55,59–61], especially considering the unprecedented disruptions created by the COVID-19 pandemic on adolescents' schedules and daily activities [62–66].

The absence of a between-group difference may also be due to the eating window selected by participants assigned to TLE. All adolescents assigned to TLE selected an afternoon eating window. This finding aligns with a previous study done by this group, which showed that the majority of adolescents with obesity prefer an afternoon/evening eating window [67]. Available evidence in animals and humans suggests that early TLE (i.e., tantamount to skipping the evening meal) is more effective than late TLE (i.e., equivalent to skipping breakfast) for weight loss and metabolic benefits [28,56,57,68,69]. These findings have been explained in terms of alignment between central and peripheral circadian clocks involved in energy expenditure and fat oxidation [28,56,57,68,69]. In the present study, we allowed adolescents to select their own eating window to promote compliance, resulting in a late TLE regimen. Studies are needed to examine the feasibility of early TLE in adolescence and to compare the effectiveness of early and late TLE in adolescent and adult populations.

An alternative explanation for the absence of a difference in weight loss across study arms lies in the possible interventional effect of CGM. It is well-documented that wearable technology often results in a short-term weight loss; however, reactive effects are usually short-lived [58,70]. Only one-third of adolescents in the real-time CGM group looked at their data; however, participants' mere knowledge that their glucose was monitored by the study team may have provided accountability, not provided outside the study. Additional work is needed to explore the role of CGM, with and without real-time biofeedback, in dietary intervention trials.

Akin to findings reported in adult cohorts, the assigned eating window (TLE vs. control) did not adversely affect quality of life, physical activity, or eating behaviors [71–73]. In this sample of adolescences, TLE was associated with a modest improvement in quality of life relative to baseline, with no difference compared to the control [73]. It has been widely reported that weight loss has a positive impact on quality-of-life measures after short-term interventions [74,75]; however, improvement in self-reported quality of life was not related to weight loss in the present study. Compared to a prolonged eating window, TLE did not impair physical activity. Interestingly, all adolescents showed an increase in the number of days of physical activity per week over the course of the study. These findings contrast the many reports documenting decreased physical activity during the COVID-19 pandemic [62,64,65,76,77], although not entirely surprising as children were not held to classroom schedules involving long periods of sedentary time [63,65,78].

TLE did not result in any unhealthy compensatory eating behaviors [71,72,79–81]. This finding is important given the concerns that TLE may lead to unhealthy eating behaviors and attitudes. Disordered eating behaviors are prevalent among adolescents with obesity; however, many studies have suggested that monitored intervention programs implemented by trained professionals may decrease eating behavior symptomatology [82–84]. The potential for unhealthy eating should be continuously monitored in future studies [73,83,85].

In most adult trials, TLE inadvertently reduced daily caloric intake and thus lead to weight loss [26,29–31,63,86,87]. Despite no recommendations to decrease caloric intake, there was a 25% reduction in daily caloric intake during the intervention compared to baseline with no difference between groups. As outlined above, this was likely secondary to increased daily eating structure and the consistency of the eating window. Additionally, although there was a wide variability of eating patterns at baseline, most adolescents were eating late into the evening and night and this night-time eating was limited with a structured eating pattern and may have contributed to caloric reduction [73,88]. It remains unknown if limiting night-time eating impacts caloric intake in adolescents; however,

a large longitudinal study in adults with obesity comparing early and late eating periods found that timing of meal consumption was not associated with decreased caloric intake [73,88]. Additional investigation is needed to determine if TLE in adolescents is associated with consistent caloric reduction independent of the timing of the eating window.

Finally, as an exploratory outcome, we examined CGM use in this cohort as both a metric of adherence and an intervention modality. The efficacy of TLE interventions is dependent on accurate assessment of eating vs. fasting windows and therefore in this sample, CGM proved to be a useful tool to monitor the effect of fasting on glycemic profiles. By adding CGM data to self-report and dietary recalls, we were able to better understand the eating and fasting periods of this group and evaluate how the glycemic profiles changed during fasting. Certainly, this method is not without limitations in that glycemic excursions vary significantly based on the dietary macronutrient composition. However, the combination of dietary recall, self-report, and CGM data provides a strategy to evaluate adherence to fasting windows and useful data to inform future studies regarding expected glycemic excursions during fasting in adolescents with obesity without diabetes. No studies to date have investigated whether extended fasting periods increase risk of hypoglycemia in youth with obesity given their potential risk for glucose dysregulation. As a safety metric, we examined the frequency of hypoglycemia reported over time within and between groups in this cohort. We defined glucose based on the threshold of insulin secretion in the fasting condition in otherwise healthy adults as 70 mg/dL. Consistent with reports in adult cohorts without diabetes, there was no hypoglycemia noted during reported fasting periods, suggesting that prolonged fasting periods is not a risk factor for hypoglycemia in this age group. The CGM data also provided the opportunity to monitor average blood glucose and estimate HbA1c over time. Our findings are consistent with those previously report in adults in that we did not see a significant change in glucose regulation over the study period between groups because our cohort had baseline normal fasting glucose levels. In many adult trials, TLE has been associated with a reduction in fasting glucose and insulin sensitivity in participants whose baseline fasting glucose was >100 mg/dL [89,90]. The impact of TLE on glucose regulation may be related to severity of beta cell dysfunction at baseline and therefore further studies are needed in youth with pre-diabetes and type 2 diabetes to understand the impact of TLE on glucose regulation.

Limitations

This study is not without limitations. First, given the COVID-19 research restrictions, our study was conducted entirely remotely. We were neither able to collect and verify all anthropometric outcomes nor collect body composition measures and other metabolic markers as initially intended, which added more variability to our analysis. Second, as this was a pilot study, we had a small sample size and were not powered to evaluate our secondary outcomes. Thirdly, the study was not conducted in a controlled or inpatient setting. We intentionally conducted our study in a real-life setting. We encountered unique barriers to recruitment, brought on by the pandemic, with evolving restrictions and unexpected delays during the research period. Fourthly, given this was a pilot trial, we did not exclude participants with a shorter eating window at baseline and required adolescents to adjust their eating window based on their randomization arm. In addition, our design is also subject to omitted variable bias, such as unmeasured or uncontrolled factors (i.e., impact of COVID-19 during the study period). Fifth, the current study could not evaluate whether TLE is sustainable over the long term given the short study duration. Although adherence was high for the study duration, further investigation is warranted to assess if TLE is more sustainable than other caloric restriction approaches given its simplicity and ability to be implemented in a real-life setting. Finally, our sample strictly included adolescents enrolled in a weight management intervention. Our focus on treatment-seeking adolescents is important to characterize the heterogeneity and specific needs of adolescents who seek obesity treatment.

5. Conclusions

Our results suggest that TLE, combined with CGM, is feasible, acceptable, safe, and can lead to clinically meaningful weight loss. All adolescents in the TLE groups selected an afternoon/evening eating window. TLE did not result in changes in physical activity, quality of life, or compensatory eating behaviors. Further research is needed to determine the effectiveness of TLE + CGM on weight reduction in larger cohorts, over longer intervention periods, and to investigate the optimal timing of TLE to produce the greatest weight reduction and improved health outcomes in this age group.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Re-view Board (or Ethics Committee) of Children's Hospital Los Angeles (CHLA-000193, date of approval—20 December 2019).

Informed Consent Statement: Written informed consent was obtained from all participants and a parent or guardian involved in the study including consent for publication of findings.

Data Availability Statement: The datasets from this study will be available from the corresponding author on written request.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Metabolic Derangement in Pediatric Patient with Obesity: The Role of Ketogenic Diet as Therapeutic Tool

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Abstract: Obesity is defined as a condition characterized by an excessive fat accumulation that has negative health consequences. Pediatric obesity is associated with an increased risk for many diseases, including impaired glycemic and lipidic control that may lead to the development of chronic, and potentially disabling, pathologies, such as type 2 diabetes mellitus (T2DM) and cardiovascular events, in adult life. The therapeutic strategy initially starts with interventions that are aimed at changing lifestyle and eating behavior, to prevent, manage, and potentially reverse metabolic disorders. Recently, the ketogenic diet (KD) has been proposed as a promising dietary intervention for the treatment of metabolic and cardiovascular risk factors related to obesity in adults, and a possible beneficial role has also been proposed in children. KD is very low in carbohydrate, high in fat, and moderate to high in protein that may have the potential to promote weight loss and improve lipidic derangement, glycemic control, and insulin sensitivity. In this review, we present metabolic disorders on glycemic and lipidic control in children and adolescents with obesity and indication of KD in pediatrics, discussing the role of KD as a therapeutic tool for metabolic derangement. The results of this review may suggest the validity of KD and the need to further research its potential to address metabolic risk factors in pediatric obesity.

Keywords: obesity; ketogenic diet; insulin resistance; lipids; diabetes

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1. Introduction

Obesity is a complex, many-faceted status that exposes the pediatric patients to a wide spectrum of inflammatory, metabolic, and endocrine dysfunctions, which influence and enhance each other through biochemical and molecular interactions [1]. Pediatric obesity exposes the affected subjects to a higher risk of short- and long-term complications, such as type 2 diabetes (T2DM), dyslipidemia, nonalcoholic fatty liver disease (NAFLD), obstructive sleep apnea (OSA), asthma, polycystic ovary syndrome (PCOS), musculoskeletal comorbidities [2], and adolescents with obesity are at increased risk of psychological disturbances [3–5].

Early metabolic disorders, including insulin resistance, prediabetes, and dyslipidemia, are metabolic disorders that are often detected in obese children and adolescents [6]. They have a particular relevance because of their strong association with the development of chronic, and potentially disabling, pathologies, such as type two diabetes mellitus

(T2DM) and cardiovascular events, in adult life [7]. Considering the short- and long-term risk complications, pediatricians and specialists should be aware of the frequency and extent of obesity-related comorbidities, and address patients to periodic screening procedures [6]. It is essential to follow the children who are affected by obesity regularly and in a multidisciplinary team. The strategy initially starts with interventions aimed at changing lifestyle and dietary interventions, to prevent, manage, and potentially reverse metabolic disorders [1]. Recently, the ketogenic diet (KD) has been proposed has dietary intervention that may have the potential to promote weight loss, and improve lipidic derangement and glycemic control/insulin sensitivity in adults [8,9].

In pediatrics, a possible therapeutic role in treating chronic inflammatory disease in children has been reported [10].

The purpose of this review is to present metabolic disorders on glycemic and lipidic control in children and adolescents with obesity and indication of KD in pediatrics, discussing the biological plausibility of the mechanisms by which the KD reduces the metabolic derangement that is currently reported for adults. The results of this review could suggest the validity of KD and its potential to address metabolic risk factors in pediatric obesity. A novel non-pharmacological treatment may be useful as an alternative tool in managing children with metabolic derangement. A prompt identification and correction of these abnormalities is fundamental, to significantly decrease the likelihood of adverse events throughout life.

2. Methods

We performed a narrative review [11] according to the English literature in the past 15 years. M.C.P., V.C.M., G.F., E.D.P., E.T., C.F.T. independently identified the most relevant published studies including original papers, metanalysis, clinical trials, reviews. Case reports or series and letters were excluded. Adult and pediatric literature was considered. Regarding pathogenetic mechanism experimental studies were also included. Papers published up to May 2021 in each author's field of expertise were searched with the following keywords (alone or in combination): obesity, adolescents, obesity-related complications, dyslipidemia, glycemic, glucose impairment, insulin resistance, prediabetes metabolic risk, metabolic syndrome, type 2 diabetes, ketogenic diet, ketogenic diet and metabolic syndrome, ketogenic diet and cardiovascular disease, very-low-carbohydrates ketogenic diet, very-low-carbohydrates ketogenic diet and diabetes or insulin resistance or polycystic ovary syndrome. The following electronic databases were searched: PubMed, Scopus, EMBASE and Web of Science.

3. Pediatric Obesity

3.1. Epidemiological Data

Obesity is defined as a condition characterized by an excessive fat accumulation that has negative health consequences, being a risk for many diseases, including a wide spectrum of metabolic and cardiovascular disorders [1]. The condition is extremely diffused worldwide, and represents a major public health problem in both childhood and adulthood [12,13].

Obesity has increased significantly in the last four decades, both in industrialized and developing countries, and it is of major relevance in the pediatric age. The global prevalence of overweight and obesity in males and females aged 5–19 has risen from 4% in 1975 to 18% in 2016 [14]. According to the World Health Organization report, in 2016, more than 340 million children and adolescents worldwide were in a condition of excess body weight. Consistent with the pooled analysis of 2416 population-based studies, performed by Non-Communicable Diseases (NCD) Risk Factor Collaboration (NCD-RisC), obesity has increased by 4.9% in females (from 0.7% in 1975 to 5.6% in 2016) and 6.9% in males (from 0.9% to 7.8%) [15].

Italy is one of the nations with the highest levels of excess weight in children; about one in four children have a weight greater than that expected for their age. The prevalence

of overweight and obesity in 2019 was 20.4% and 9.4%, respectively, with the highest number of cases in the south. Obesity was slightly higher in males (9.9% vs. 8.8% in females) [16].

3.2. Risk Factors for Obesity

Essential obesity is a multifactorial and complex condition, whose fundamental basis is an unbalanced relationship between caloric intake and energy expenditure [17]. This mainly derives from unhealthy eating habits or an excess sedentary lifestyle, and typically results from both. A diet that is rich in highly processed foods, with frequent consumption of sweetened drinks, ready-to-eat snacks, and fast-food preparations, constitutes an important contribution to the development of childhood obesity [4]. On the other hand, nowadays the tendency to enroll children at a sport activity has been reduced, simultaneously, the time spent in sedentary activities, such as use of the television and video games, has increased [5]. The changes in lifestyles create an "obesogenic" environment, which contributes to explaining the high prevalence of the condition.

Other risk factors are involved, as follows: there is a strong genetic susceptibility, confirmed by the strong association between obesity in first-degree relatives and the risk for the child to become overweight. Moreover, twins tend to have similar BMI [18]. Genetics would therefore play a permissive role, interacting with environmental factors that promote obesity.

In addition to those already mentioned, the risk factors for obesity include the following: low socio-economic level, urban area of residence compared to rural, Hispanic and South Asian ethnicity, psychosocial and emotional factors (where food becomes a mean to suppress feelings and negative moods), intestinal bacterial flora composition (for example, the relationship between Firmicutes and Bacteroidetes), and a poor quality and duration of sleep [17]. Sleep may also be related to glycemic homeostasis and insulin sensitivity, as described in a cross-sectional observational study that was conducted by Koren and co-authors, between 62 obese adolescents. From the study emerged a significant correlation between sleep duration, HbA1c values, and glucose levels, with the oral glucose load test (OGTT), regardless of gender, degree of obesity, and pubertal stage [19]. Insufficient and excessive sleep was associated with short-term and long-term hyperglycemia in the obese study group. Decreased slow-wave sleep (phase N3) was associated with reduced insulin secretion [19].

Other conditions that are associated with a higher risk of excessive adiposity are intrauterine exposure to the mother's excess adiposity, gestational diabetes, and small-for-gestational-age (SGA) newborns, who show a subsequent early recovery of growth [17]. During pregnancy, the differentiation of fetus hypothalamic hunger and satiety centers occurs, and the number of adipocytes increases. Accordingly, an overstimulation of these centers, during the intrauterine life, predispose to obesity.

3.3. Diagnosis of Overweight and Obesity

The diagnosis of overweight and obesity in children up to two years of age is based on the weight-to-length ratio, using the WHO child growth standards reference curves for age and sex [6,20]. After two years of age, the diagnosis is made using the parameter body mass index (BMI), calculated by dividing the child's weight, expressed in kilograms, by the square of their height, measured in meters (kg/m^2) [6]. Even though BMI in pediatrics is calculated using the same formula as adult BMI, it is interpreted differently. In adults, BMI is interpreted using standard weight status categories. On the contrary, at pediatric age, the BMI calculated is compared with standard reference curves that are gender-, age-, and population-specific, because the amount of body fat changes with age, and the amount of body fat differs between girls and boys. The adoption of reference curves with normative BMI percentiles considers that children and adolescents are constantly growing, therefore it is not possible to assume a single BMI value as a cut-off to define overweight and obesity, as happens for the adult population.

Several reference standards are available. The International Obesity Task Force has proposed standard centile curves based on pooled international data for body mass index [21]. National growth curves for the Italian population are also available (Italian Society for Pediatric Endocrinology and Diabetes (SIEDP) 2006 growth charts) [22]. However, the most used growth charts are those published by the Centers for Disease Control and Prevention (CDC) for children who are 2 to 20 years of age [23]. The CDC BMI growth charts are the standards that are recommended to diagnose overweight and obesity in children and adolescents $\geq$ 2 years of age, by the Endocrine Society Clinical Practice Guideline [3].

BMI is the accepted clinical standard measure to diagnose overweight and obesity. Nevertheless, it is not a direct measure of body fat, it does not predict the body distribution of fat, and it does not allow the distinction between fat mass and lean mass. Therefore, it could sometimes overestimate adiposity in particularly muscular and athletic children and, conversely, underestimate fat mass in children with reduced muscle mass, such as particularly sedentary patients [17].

For the mentioned reasons, during the clinical evaluation, it is important to integrate BMI with other anthropometric parameters. Those include the following:

- Waist circumference (cm) and waist circumference/height ratio (CV/h); this is particularly useful for investigating visceral obesity. A waist/height ratio > 0.5 is indicative of visceral obesity. This parameter is recognized as a better predictor of insulin resistance and metabolic risk in youths [24,25].
- Skinfold thickness; this measure is obtained using a skinfold meter, and evaluates the subcutaneous body adipose tissue by detecting the thickness of the raised skin fold. The triceps skinfold thickness is usually measured [26].

More recently, additional adiposity indices have been proposed, including the body shape index (ABSI), which indicates the abdominal-to-peripheral adiposity ratio, and it highlights the importance of waist circumference in obesity, related to metabolic and cardiovascular complications [27]; the triponderal mass index (TMI), which has been suggested for body composition evaluation and as predictor of MetS [27]; the visceral adiposity index (VAI), identified as a new cardiometabolic risk marker reflecting abdominal fat distribution and dyslipidemia; the conicity index (C-Index), which was recently proposed as a useful tool to screen for MetS and alterations in the lipid profile of adolescents [28].

Bioelectrical impedance, magnetic resonance imaging, and dual-energy X-ray absorptiometry (DEXA), are more technical measurements of body composition assessment. However, such tools are expensive and used much less in routine clinical practice.

3.4. Obesity-Related Complications

The obesity-related complications can occur both in the short term and in the long term. With the global increasing prevalence and severity of pediatric obesity, these overt comorbidities and several initial alterations are beginning to be found in children as well [29].

Obesity is associated to a chronic low-grade inflammatory state, whose effects affect almost all organ systems, with an increased risk of endocrine, cardiovascular, gastrointestinal, reproductive, pulmonary, musculoskeletal, and psychological complications [17,30]. They include hypertension, dyslipidemia, hyperinsulinemia, type 2 diabetes mellitus, cardiovascular disease, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), cholelithiasis asthma, sleep apnea, osteoarthritis, hyperandrogenemia, polycystic ovarian syndrome (PCOS), foot and lower limb pain, and musculoskeletal comorbidities [2,31,32].

Obesity is also often related to psychosocial issues, depression symptoms, reduced quality of life, and social isolation [4].

Due to the direct consequences that can occur at pediatric age, it should be added that a high percentage of obese children will carry their excess adiposity into adolescence and adulthood, with a significant increase in the long-term risk of adverse outcomes, especially cardiovascular and metabolic (atherosclerosis, coronary heart disease, metabolic syndrome) [33,34].

The details on the metabolic derangement in a pediatric patient with obesity are detailed in the next sessions.

The obesity-related complications are summarized in Figure 1.

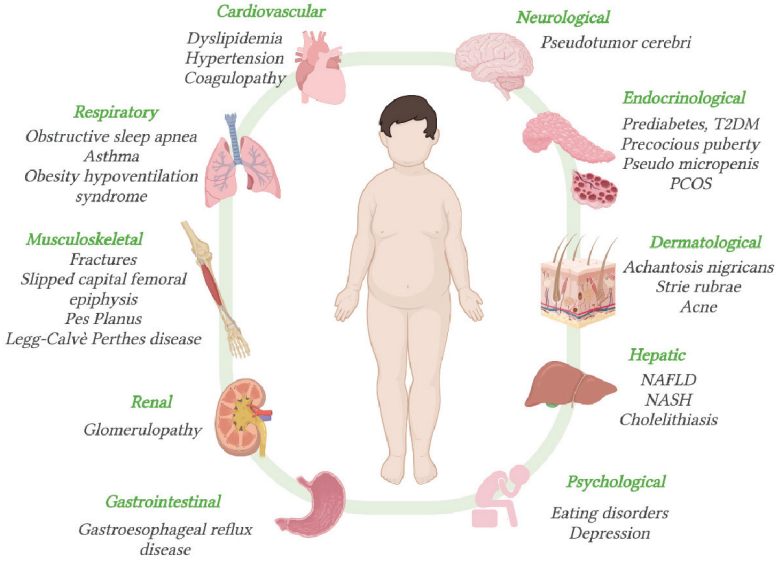

Figure 1. Obesity-related complications. T2DM = type 2 diabetes mellitus; PCOS = polycystic ovarian syndrome; NAFLD = nonalcoholic fatty liver disease; NASH = nonalcoholic steatohepatitis.

4. Obesity and Metabolic Disorders

4.1. Pediatric Obesity and Glucose Disorders

Glucose imbalances are one of the main endocrinologic comorbidities of obesity. Among the glucose dysmetabolisms affecting overweight or obese patients during childhood, it is possible to characterize the following three main entities: insulin resistance (IR), the so-called "prediabetes" state, and T2DM [35,36].

4.1.1. Insulin Resistance

Insulin resistance, in children as in adults, is defined as a state in which insulin produces a subnormal biological response [37]. Thus, the ability of insulin to promote glucose use, by muscle and adipose tissue, is lower than expected, as is its ability to suppress glucose production by the liver [38,39].

IR is considered the most common metabolic alteration related to obesity [40]. Recently, a population-based study on IR epidemiology in children has been performed, and the overall prevalence rates of IR ranged between 3.1 and 44%. This difference is mainly due to the different methods that were used to diagnose IR in the studies analyzed, as unanimous diagnostic criteria for this condition are lacking [41]. Among obese children, the prevalence of IR is estimated to be around one third, but it is highly variable, also depending on the different degrees of obesity, racial and ethnic variation, and age of the sample studied [42,43].

Fat tissue itself has a key role in IR development, through the action of different metabolites, adipocytokines, and hormones [37,39]. Among these, adiponectin, one of the main cytokines produced by fat cells, with a fundamental insulin-sensitizing effect, is decreased in obese children [44]. Moreover, it was found that the adipose tissue of obese children is characterized by macrophage infiltration, further highlighting the link between fat tissue inflammation and IR [45,46].

Different inflammatory cytokines and hormonal factors, altered in obese individuals, have been shown to interfere with the insulin signal transduction pathway, leading to IR. Specifically, the cytokines involved seem to be the nuclear factor kb (NF-kB) [47], tumor necrosis factor-α, and interleukin-6 [48]. Some studies also correlated IR to leptin levels [49] in obese children, retinol-binding protein 4 (RBP4) in obese adults [50], and resistin, another adipose tissue molecule, in animal models [48]. Finally, in the development of IR in obese patients, the role of growth factors seems relevant. Indeed, insulin-like growth factors (IGF1 and IGF2) and IGF-binding proteins have been recently correlated to the insulin resistance state in obese adolescents [51].

Further studies are needed to completely understand the precise molecular interactions between these factors that result, initially, in decreased insulin sensitivity, then in overt diabetes. Indeed, IR has been demonstrated to be an important risk factor for the development of DMT2 [39,52], together with β-cell dysfunction [45,53].

4.1.2. Prediabetes

The term 'prediabetes' indicates individuals whose glucose levels do not meet the criteria for diabetes, but are too high to be considered normal. The prevalence of prediabetes is, instead, more difficult to determine; it was shown to be around 18% of adolescents, based on data from the National Health and Nutrition Examination Survey [54]. The association of this condition with obesity has been well documented and its incidence was also linearly correlated to obesity severity [55]. Furthermore, overweight children and adolescents have impaired insulin sensitivity, coupled with a declining beta cell function, below the thresholds that are used to define prediabetes states [56,57].

In the spectrum of this condition, the following two different entities are recognized: impaired glucose tolerance (IGT) and impaired fasting glucose (IFG). These are considered to be intermediate stages from the normal carbohydrate metabolism and the overt condition of diabetes mellitus [58].

It is important to highlight that individuals who, according to these criteria, are classified as having an IGT or an IFG, may have glycemic levels in the normal range in daily life, but the importance of these conditions relies in the fact that they are associated with an increased risk of progression to diabetes during adulthood [59].

In this context, it is necessary to underline the fact that prediabetes is often transient during adolescence, as it has been shown that around 60% of prediabetic adolescents return to normal glucose levels in 2 years after puberty. This phenomenon seems to be related to transient insulin resistance, which is a normal component of pubertal development, related to the physiological elevation of the IGF-I/GH axis; plasma IGF-I levels are primarily regulated by GH, which is a counterregulatory hormone that is known to be a potent insulin antagonist [60]. An important risk factor for the persistence of the prediabetic state, and the following development of diabetes, is persistent weight gain [61], and important strategies to prevent this progression are decreased caloric intake and increased physical activities [62].

4.1.3. Type 2 Diabetes Mellitus

Type 2 diabetes (T2D) develops in people with insulin resistance, when the pancreatic β-cell becomes unable to produce sufficient insulin to compensate for the decreased insulin sensitivity. The greater the insulin resistance, the higher the probability to develop overt type 2 diabetes [63]. In obese individuals, blood insulin levels have been shown to be increased in the first phases, and tend to decrease, at sub-normal levels, later in the course of the disease, when β-cell dysfunction progresses [63]. Consequently, at the beginning, the disease progresses silently, evading possible therapeutical interventions, until the deterioration of pancreatic β-cell becomes irreversible and therapy, with medications and/or insulin, is necessary [64].

Many studies have shown that T2D, in the last decades, has tremendously risen in children and adolescents throughout the world [65,66].

The reported prevalence varies according to the different studies, having a range of 1–51/1000, depending upon ethnicity, with higher incidence in Native American, Black, Hispanic, and Asian or Pacific Islander children, with respect to White ones. This trend coincides with the rise in severe obesity in these groups [43,65].

Interestingly, before the mid-1990's, among children affected by diabetes, only about 1–2% were affected by T2D. Instead, in the last decades, the incidence of T2D has increased to 25–45% of all youths diagnosed with diabetes [67], mirroring the increased incidence of obese children [65].

Overt T2D in children and adolescents is diagnosed according to the ADA criteria [58], with the presence of the typical symptoms of diabetes (polyuria, polydipsia, nocturia, unexplained weight loss) and increased plasma glucose levels, specifically as follows:

- FPG $\geq$ 7 mmol/L ($\geq$126 mg/dL);
- Post OGTT 2 h plasma glucose $\geq$11.1 mmol/L ($\geq$200 mg/dL);
- A random plasma glucose $\geq$200 mg/dL (11.1 mmol/L);
- HbA1c $\geq$ 6.5% (48 mmol/mol).

The screening for T2DM is only recommended in children and adolescents with specific risk factor for diabetes, of which obesity is the most correlated [58,64].

The long-term prognosis of youth with T2D is not precisely known, but it is estimated that, depending on their glycemic control, these youths may have a decreased life expectancy, by up to 15 years [68]. It seems that glycemic imbalances in young individuals start within two years after diagnosis [35,68] and progress rapidly, increasing the risk of the development of serious health complications, as microvascular and macrovascular diseases, such retinopathy, neuropathy, nephropathy, and cardiovascular disorders [69,70], and the other metabolic comorbidities, including hypertension, dyslipidemia, and fatty liver [70].

4.2. Pediatric Obesity and Dyslipidemia

Disorders in the lipid profile are often present in pediatric patients with obesity. According to the population studied, 28% to 46% of overweight and obese children have dyslipidemia [71–74]. The severity of obesity may influence the prevalence of an abnormal lipidic profile in a given cohort. The risk for an obese child to have dyslipidemia was calculated to be 2.8$\times$ higher than for a child with a normal BMI [71].

The most common dyslipidemic pattern that is associated with childhood obesity is secondary combined dyslipidemia, known as combined dyslipidemia of obesity (CDO). The disorder presents a combination of moderate-to-severe elevation in triglycerides and non-high-density lipoprotein cholesterol (non-HDL-C), mild elevation in total cholesterol (TC) and LDL-cholesterol, and low HDL-C level [71,75,76].

When evaluating a child for combined dyslipidemia, it is useful to add the following two significant measures to the routine lipid panel: non-HDL-C and serum triglyceride-to-HDL cholesterol ratio (TG/HDL-C ratio) [76].

Non-HDL-C proved to be a good predictor of subclinical atherosclerosis in asymptomatic younger adults [77].

The TG/HDL-C ratio has been shown to be solidly related to the atherogenic lipid profile, coronary disease development risk, and extent of coronary disease in adults [78]. TG/HDL-C ratio > 2.2 (corresponding to the 75th percentile) can be considered a useful and simple marker of atherogenic dyslipidemia and an altered cardiometabolic risk profile in Italian children with obesity [6,78].

As reported by the Princeton LRC Follow-up Study, 14.6% of subjects with hypertriglyceridemia in both childhood and adulthood had CVD, compared to 2.9% of subjects with high triglycerides (TG) only during adulthood, and 1.9% of subjects with high TG when they were children and then normalized in adulthood. Instead, the incidence of CVD was 1% in the group without a story of past or present hypertriglyceridemia [79].

The dyslipidemic pattern has been shown to be linked to the initiation and progression of atherosclerotic lesions in children and youths [80]. The Bogalusa Heart Study clearly

pointed out how fatty streaks, an early sign of atherosclerosis, are commonly found in the aorta and coronary arteries autopsies of children aged 2–15 years, who did not die from cardiovascular causes [81].

It is known that excess circulating low-density lipoproteins (LDL) accumulate in the vascular internal wall, and are oxidized (oxLDL) and phagocytized by macrophages, which transform themselves into foam cells and give origin to the fatty streaks. However, other factors are involved in atherosclerosis too, and inflammation has a key role [82].

A Bogalusa Heart Study analysis also showed that the extent of fatty streaks covering the intimal surface of the vessels increased in young people with multiple risk factors (high BMI, systolic blood pressure, LDL-C and triglycerides), supporting the notion that risk factors exert a synergistic effect on cardiovascular events [81].

Obesity is a state of chronic low-grade inflammation and is typically characterized by dysfunctional changes in the adipose tissue, in terms of both the adipocytes' size and profile secretion [83]. Under the obese status, the hypertrophied adipocytes show altered adipocyte-derived cytokine production and metabolic derangements.

For instance, obese patients have lower circulating levels of the anti-inflammatory adiponectin and omentin [84–87], and increased circulating levels of both various proinflammatory cytokines and hormones (such as leptin, resistin, IL-6, IL-10, tumor necrosis factor α TNF-α) [86,88,89]. The unbalanced adipokines secretion may affect the cardiovascular system, thus contributing to explaining the obese-related cardiometabolic disorders [83]. Adipose tissue from obese individuals is in M1-like macrophages, which secrete large amounts of TNF-α and other pro-inflammatory molecules. Conversely, the expression of adiponectin is reduced. Adiponectin has vascular protective functions, mainly mediated through the activation of endothelial nitric oxide synthase (eNOS), and insulin-sensitizing properties [90].

Obese patients often have signs of insulin resistance (IR). Thus, insulin antilipolytic action is impaired and IR promotes free fatty acid (FFAs) release from the adipose tissue into the portal circulation, excess FFAs flux to the liver and undergo hepatic accumulation [91]. It has been proposed that high FFAs levels and inflammatory cytokines diminish nitric oxide (NO) production by eNOS, leading to endothelial dysfunction. Other alterations, such as increased expression of plasminogen activator inhibitor-1 (PAI-1, a prothrombotic molecule) and increased vascular smooth muscle cell proliferation, follow the decreased NO levels, and contribute to creating the proinflammatory environment that promotes atherosclerosis [92,93].

Moreover, the increased FFA flux to the liver increments hepatic triglycerides (TG) synthesis and the secretion of VLDL. Insulin is a stimulator of lipoprotein lipase (LPL), which is the main enzyme involved in the hydrolysis of TGs. Consequently, LPL action is impaired in the insulin-resistant status. Excessive TG deposition in the liver and peripheral tissues promotes, in turn, insulin resistance, in a vicious circle.

Lastly, elevated TG concentrations are associated with increased levels of proatherogenic small, dense LDL particles and reduced levels of HDL-C [91,94].

The management of children with a dyslipidemic pattern should start with early detection.

As for dyslipidemia, the Consensus Position Statement of the Italian Society for Pediatric Endocrinology and Diabetology recommends assessing cholesterol, HDL-cholesterol, and triglycerides, in all children and adolescents with obesity since the age of six. The measurements should be repeated every three years, or sooner if there are abnormalities or the child develops comorbidities or rapid weight gain [6].

The American Academy of Pediatrics and the American Heart Association endorse the National Heart, Lung, and Blood Institute (NHLBI) expert panel guidelines, which recommend universal routine lipid screening for all children and adolescents of 9–11 and 17–21 years. Targeted screening is strongly suggested for children of 2–8 years and 12–16 years, with risk factors for dyslipidemia (including diabetes, hypertension, smoking,

BMI ≥ 95th percentile for age and sex if 2–8 years, BMI ≥ 85th percentile for age and sex if 12–16 years, positive family history for cardiovascular diseases) [75].

The diagnosis of dyslipidemia is based on the criteria advanced by the NHLBI expert panel, adopted by the Italian Consensus Position Statement too. The references values that are considered acceptable, borderline-high, and high, are listed in Table 1 below [75].

Table 1. Reference values of lipidic pattern in children and adolescents. LDL = low-density lipoprotein; HDL = high-density lipoprotein.

Category	Acceptable	Borderline-High	High
Total cholesterol (mg/dL)	<170	170–199	≥200
LDL cholesterol (mg/dL)	<110	110–129	≥130
Non-HDL cholesterol (mg/dL)	<120	120–144	≥145
HDL cholesterol (mg/dL)	>45	40–45	<40
Triglycerides (mg/dL)			
0–9 years	<75	75–99	≥100
10–19 years	<90	90–129	≥130

The lifestyle interventions focused on diet and physical activity, proposed as the first-line dyslipidemia treatment in childhood [75].

5. Therapeutic Strategies for Pediatric Obesity: The Lack of Effectiveness of Current Treatments in Weight Loss and Dysmetabolism

The prompt treatment of overweight/obese children and adolescents is extremely important, as this condition affects many systems and has negative consequences both at the physical and at the psychological level. Thus, the therapy should have a multidisciplinary approach [1,95]. The therapeutic strategies can be divided into the following three main groups: non-pharmacological treatment, pharmacological ones, and, lastly, bariatric surgery [95].

5.1. Non-Pharmacological Treatment

The fundamental pillar of the non-pharmacological treatment is education. Indeed, both the patients and their families should be instructed to correct their lifestyle changes, mainly focused on a healthy diet and an increase in physical activities [5].

Before the eventual start of a personalized diet, families are educated to a heathy diet, with reduced sugary and industrialized foods or beverages, as well as products that are high in fats, of animal origin. Moreover, an increased dietary intake of fruits and vegetables is promoted, as these strategies have shown a relevant effect in weight loss [5]. A balanced and high-fiber diet, during childhood and adolescence, has also been correlated to improvements in the glycemic control later in life, as it increased peripheral insulin sensitivity and lowered fasting glucose levels [96,97]. Importantly, these nutritional habits must be linked to daily moderate physical activity, tailored on the patient's age, tolerance, and preference, to increase the compliance as much as possible [98,99].

The recommended amount of physical activity is a minimum of 20 min per day [3], of moderate-intense activity, correlated to an increase in insulin sensitivity, independently from of the body fat percentage [100]. Regular vigorous physical activity was shown to influence insulin action on skeletal muscle glucose and fat metabolism [101], but the mechanisms behind the decreased IR have not been clearly elucidated yet [102].

The amount of weight loss is individualized according to the patient's age, obesity severity, and eventual comorbidities [103]. Moreover, family-based behavioral approaches are recommended, as shown, to give better results [5,104].

There is still lack of consensus concerning the best structured diet for weight reduction in overweight children and adolescents. Low-carbohydrate and low-glycemic index (GI) regimens have been shown to be comparable, in terms of short-term weight loss, to standard portion-controlled diet [105,106].

It was recently demonstrated that in obese/overweight adolescents, the lack of effect of a high-protein/low-glycemic index diet on BMI reduction and IR was mainly due to insufficient dietary compliance after 2 years [107].

Because of the scarce results that have been obtained with lifestyle interventions, which rarely result in long-term weight loss and resolve obesity-associated comorbidities [108], the use of a pharmacological approach may be needed [1,5].

5.2. Pharmacological Treatment

The pharmacological treatment of childhood obesity is still a debated theme [5,109]. Importantly, pharmacological management is suggested only after the unsuccess of a formal program of lifestyles changes [1]. Indeed, the main drugs that are used in this field are orlistat, approved for obese adolescents $\geq$ 12 years old by the Food and Drug Administration [110], phentermine, a sympathomimetic amine, used for the short-term management of obesity in individuals > 16 years of age [111], liraglutide, an agonist of the glucagon-like peptide-1 receptor, and metformin, approved for the treatment of children $\geq$10 years old, who are affected by DMT2, and used off-label for weight managements in pediatrics [112,113].

In more detail, orlistat is a potent lipase inhibitor, which acts at the gastrointestinal level, blocking up to 30% of the absorption of fats from the diet. Unfortunately, the efficacy of this drug, in terms of loss of weight, is only modest, and it has several gastrointestinal adverse effects, such as diarrhea, flatulence, and fatty stools [110]. These aspects, coupled with the risk of fat-soluble vitamin deficiencies and the consequent need of multivitamin supplementations, limit its use [109].

Metformin, instead, is the drug of choice for diabetic children and obese adolescents. This drug decreases the glucose production from the liver, increasing peripheral insulin sensitivity, and thus decreasing IR [62,99]. Metformin is extremely useful and effective in the management of glycemic metabolism [58]. Indeed, it was shown to improve glucose homeostasis in obese insulin-resistant children, and delay the appearance of impaired glycemic metabolism in children who were at high risk for DMT2 [114], but when administered to promote weight loss, it only resulted in modest reductions in BMI [115].

The pharmacological treatment of choice for lipid dysmetabolisms in obese children are statins, HMG-CoA reductase inhibitors. These drugs are recommended, according to the American Association of Pediatrics (AAP), in addition to lifestyle changes, only in patients $\geq$8 years old with LDL cholesterol $\geq$ 190 mg/dL, or $\geq$ 160 mg/dL with risk factors. In the case of the presence of DM, therapy can be started with LDL cholesterol $\geq$ 130 mg/dL. According to the National Heart Lung and Blood Institute (NHLBI), instead, therapy can be started in children $\geq$ 10 years old with LDL cholesterol consistently $\geq$ 190 mg/dL. In children $\leq$8 years old, instead, the pharmacological therapy is started only in the case of an LDL cholesterol higher than 500 mg/dL, according to the American Association of Pediatrics [75], and in children $\leq$ 10 years old, who are affected by severe primary hyperlipidemia or a high-risk condition associated with severe medical morbidity, according to NHLBI [116].

5.3. Surgical Treatment

The last option for obesity management is bariatric surgery. Specifically, bariatric surgical procedures are Roux-en-Y gastric bypass, biliopancreatic diversion with duodenal switch, adjustable gastric banding, and laparoscopic sleeve gastrectomy [117]. The vertical sleeve gastrectomy is the procedure of choice for severely obese adolescents, according to the Pediatric Metabolic and Bariatric Surgery guidelines [118,119].

This approach, in the adult population, resulted in a significant and sustained BMI decrease and was also correlated to improvements in obesity-related comorbidities (such as DMT2 and lipidic dysmetabolism), as well as a reduction in mortality [120,121].

Bariatric surgery, in the pediatric patients, is reserved only for cases in which lifestyle changes and pharmacological treatment approaches are not effective in weight loss and

the control of comorbidities [122]. Importantly, for patient selection, strict criteria must be respected, according to the American Society for Metabolic and Bariatric Surgery [118].

The surgical approach in adolescents, independently from the surgical techniques, was shown to be beneficial, in terms of metabolic unbalances, as dyslipidemia and DMT2, and BMI reduction [122].

Bariatric surgery leads to more significant weight loss in severely obese adolescents, with respect to lifestyle interventions [119]. Moreover, it was correlated to an improvement in the quality of life [123,124].

Although effective, both in terms of weight loss and cardiovascular risk factors reduction [122], surgical procedures are invasive and have important complications both in the short term (such as wound infections, anastomosis leakage, and bowel obstruction) [125] and in the long term (deficiencies in thiamine, vitamin b12, iron, and vitamin D) [126]. Thus, lifelong vitamin and mineral supplementation is recommended, but, unfortunately, also in this case, the adherence to recommendations is scarce [5].

The long-term success of the current treatments is still limited, as is that of the current prevention strategies [127,128], thus the negative impact of obesity-related comorbidities remains an extremely important health issue [129].

A new approach is indeed needed to face childhood obesity, which is defined as one of the most important public health problems in the world [5].

6. Ketogenic Diet: Indications in Infants and Children

The ketogenic diet (KD) is an established non-pharmacological treatment that is used for infants and children with drug-resistant epilepsy (DRE) [130–132]. It consists of a high-fat, low-carbohydrate and adequate-protein diet, designed to mimic the effects of fasting on the organism [132]. Fatty acids are used as the main energy source, through the production of ketones, resulting in improved inhibitory neurotransmission and decreased seizure frequency [133].

6.1. Ketogenic Dietary Therapies in Infants and Children

A 2016 Cochrane review reported that KD is a viable option in patients with intractable epilepsy or who are unsuitable for surgery [134].

KD was traditionally not recommended in children under 2 years of age. Although, KD has been reported to be a safe, effective, and a practical management modality in breastfeeding infants and children under 2 years of age, with drug-refractory epilepsy [130,135,136].

In recent years, there has been a great increase in the interest and research suggesting similar results in the use of dietary therapies for adults [137].

Ketogenic dietary therapies are the treatment of first choice for some metabolic disorders and types of epilepsy (Table 2). Nevertheless, KD is contraindicated in several specific inborn errors of metabolism that could lead to a severe metabolic crisis in children. Relative and absolute contra-indications should, therefore, be ruled out before starting the diet (Table 2) [130,131,138].

The implementation of KD is challenging, but the primary outcome of crisis reduction is generally achieved [139,140].

Acceptance of this modality, finding the essential tools, and adhering to a dietary regime, can affect the quality of life (QoL), and this is the major disadvantage of this new therapeutic frontier. Close support and motivation for the family is required. A systematic review of the literature assessed the effect of KD on the QoL of their immediate family members [141].

Other negative features and reasons for discontinuation are major adverse effects. Gastrointestinal adverse effects, particularly vomiting, diarrhea, and constipation, are the most common, and occur in 30% of patients during the initiation phase. Overall, they responded to dietary adjustments and medication, and therefore this did not lead to discontinuation of the dietary treatment [133]. Up to 7% of children on KD therapy may develop kidney stones [142]. QT interval prolongation and cardiomyopathy have

been found during prolonged KD, with unclear causal mechanisms [143]. Osteoporosis and vitamin D deficiency may be observed in children on KD, especially when these children are on multiple anti-seizure medications [144]. Biochemical alterations that may be observed with KD include hypercholesterolemia, hypertriglyceridemia, and depressed levels of low-density lipoprotein (LDL) [145].

Table 2. Indications and contra-indications of ketogenic diet in infants.

Indications for Dietary Therapy	Absolute and Relative Contra-Indications
Epilepsy:	Absolute:
- Medically refractory epilepsy, after use of 2 anti-epileptic drugs (AEDs) - West syndrome - Ohtahara syndrome - Febrile infection-related epilepsy syndrome (FIRES) - Severe intolerance to AEDs - Severe myoclonic epilepsy of infancy (Dravet syndrome) - Epilepsy with myoclonic-atonic seizures (Doose syndrome)	- Fatty acid oxidation deficiencies (LCAD, LCHAD, MCAD, OCTN2, CPT1, CPT2) - Pyruvate carboxylase deficiency and other gluconeogenesis defects (fructose 1,6 diphosphatase deficiency) - Glycogen storage diseases (except type 2) - Ketolysis defects - Ketogenesis defects - Porphyria - Prolonged QT syndrome or other cardiac diseases - Liver, kidney or pancreatic insufficiency - Hyperinsulinism
Metabolic and genetic disorders:	Relative:
- Glucose transporter protein 1 (GLUT-1) deficiency - Pyruvate dehydrogenase deficiency (PDHD) - Mitochondrial respiratory chain complex disorders - Angelman syndrome - Tuberous sclerosis complex	- Inability to maintain adequate nutrition - Surgical focus identified by neuroimaging and video-EEG monitoring - Parent or caregiver non-compliance - Growth retardation - Severe gastrointestinal reflux - Familial hypercholesterolemia - Propofol concurrent use

Modified from [130,138]. LCAD = long-chain acyl-CoA dehydrogenase deficiency; LCHAD = long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency; MCAD = medium-chain acyl-CoA dehydrogenase deficiency; OCTN2 = organic cation/carnitine transporter 2; CPT1 = carnitine palmitoyltransferase type 1 deficiency; CPT2 = carnitine palmitoyltransferase type 2 deficiency.

Clinical evaluation and screening laboratory studies should be obtained prior to starting KD, in order to identify the seizure type, rule out metabolic disorders, and evaluate for complicating comorbidities [138,146]. Dietary therapy should be provided for at least 3 months before considering that the therapy is ineffective. All the children should receive a daily multivitamin, calcium, and vitamin D intake. Oral citrates appear to prevent kidney stones; however, there is still no unanimous consensus on its use. There is no recommendation for the empiric use of antacids, laxatives, probiotics, exogenous ketones, additional selenium, or carnitine, with the KD currently [138].

6.2. Ketogenic Diet: Nutritional Composition

The ketogenic diet is a nutritional protocol, with high fat (70–90% energy), carbohydrate restriction (4–19%), and an adequate amount of protein to support growth [130]. The ketogenic diet is calculated in grams of fat to grams of proteins plus carbohydrates. This "ketogenic ratio" varied among different types of KD and may range from 1:1 to 4:1 [131]. Therefore, the permitted quantity of carbohydrate follows from the calculation of energy and protein requirements, and the establishment of the necessary quantity of fat [130]. Reaching an adequate protein intake is recommended in infants, which the daily RDA have to be respected in order to sustain growth [130,131]. Nowadays, five types of KD, with different compositions, are currently prescribed in clinical practice [147] (Figure S1 in Supplementary Materials).

The classical version of KD is based on a 4:1; this means that for every 4 g of fat, there is 1 g of combined protein and carbohydrates. In this classic protocol, 90% of the energy comes from fats, usually long-chain triglycerides, and only 10% from protein and carbohydrates [138,148]. The traditional method of initiating KD involves a period of fasting (12–48 h), after which food may be progressively administrated. Fasting can result in hypoglycemia, acidosis, dehydration, and lethargy, therefore the classic KD protocol was usually instituted in hospitals [131]. Nowadays, fasting is no longer required and recommended as well as initial fluid restriction; indeed, a gradual initiation protocol offers the same seizure control compared to a traditional KD protocol [131,147].

Moreover, a 3:1 ratio can be used alternatively, to increase the protein and carbohydrate intake [130]. This ketogenic ratio is more appropriate in infants, not only for diet initiation, but also in order to meet the protein requirements [130]. Different studies showed that a lower ketogenic ratio is as effective as a 4:1 ratio, and less side effects can be observed [133,149,150].

Particularly in young infants (<12 months), diet initiation should be undertaken without fasting and with a stepwise start, starting with a 1:1 ratio and progressively reaching the level of ketosis required. In addition, a KD formula with ratio 3:1 can be used purely or combined with breast milk [130,138].

Despite being the conventionally prescribed diet, the classic KD has an highly restrictive nature, which may require hospitalization at the outset [147]. Moreover, classic KDs are associated with poor compliance, thus lowering the ratio may improve the compliance, reducing the difficulties to follow this approach [138].

Another approach is the KD with medium-chain triglycerides (MCT), which comprises 60% of the energy from MCTs. However, the use of MCT in infants is limited. Older infants can use 50% MCT mixed with a low-fat milk product, or, alternatively, a low-amount (20% MCT) emulsion drink. Usually the tolerability of the total MCT in infants is 10–25% of the daily energy intake. Indeed, a high level of these fats can cause common adverse effects, such as abdominal discomfort and bloating, which lead to the discontinuation of therapy in most children [130,147,151].

This protocol has an increased ketogenic potential, as MCT are absorbed more rapidly than long-chain triglycerides [152]. As a result, an MCT-based diet yields more ketones per calorie of energy, resulting in a less total fat requirement compared to classic KD, thus allowing consumptions of more carbohydrates and proteins [131,152,153]. It is comparable to the classical KD in efficacy and tolerability, with it also being less restrictive and more palatable [154].

The modified Atkins diet (MAD) is a less restrictive KD, in which fat provides approximately 65% of the calories, with approximately a 1:1–1.5:1 ketogenic ratio [155]. The advantages of no limitation on protein, fluids, or calories, make this protocol easier to follow [138], in which the initiation does not require prior hospitalization and restriction of other micronutrients [131,153]. MAD is effective in children with refractory epilepsy, over 2 years of age and with Lennox-Gastaut syndrome [156,157]. However, classical KD was significantly more successful in children under two years of age [157]. Currently, it is a promising choice for resource-limited settings, but in developed settings it is predominantly used in adolescents and adults [147].

Finally, low-glycemic index (LGI) diets are based on a nutritional protocol that is focused on lowering the glycemic index of foods in the diet. LGI diets aim to flatten the postprandial blood glucose and insulin curves, and their positive effect has been proved in obesity, diabetes, and other non-communicable diseases [158–160]. This model is also suitable to the ketogenic diet, which is a less restrictive form of ketogenic therapy (LGI ketogenic diet), composed of 60% fats, 10% carbohydrates, and 30% proteins [161]. The amount of carbohydrates intake is approximately 40–60 g/day, but of low-glycemic index indices (<50). An LGI-based diet produces minimal ketosis compared to classic KD, with equivalent anti-seizure efficacy and a better safety profile. This variant of KD has

been found to be particularly effective in controlling seizures in patients with Angelman syndrome [147,162].

The dietary choices for children and adolescents following a ketogenic diet are summarized in the "ketogenic plate" (Figure 2).

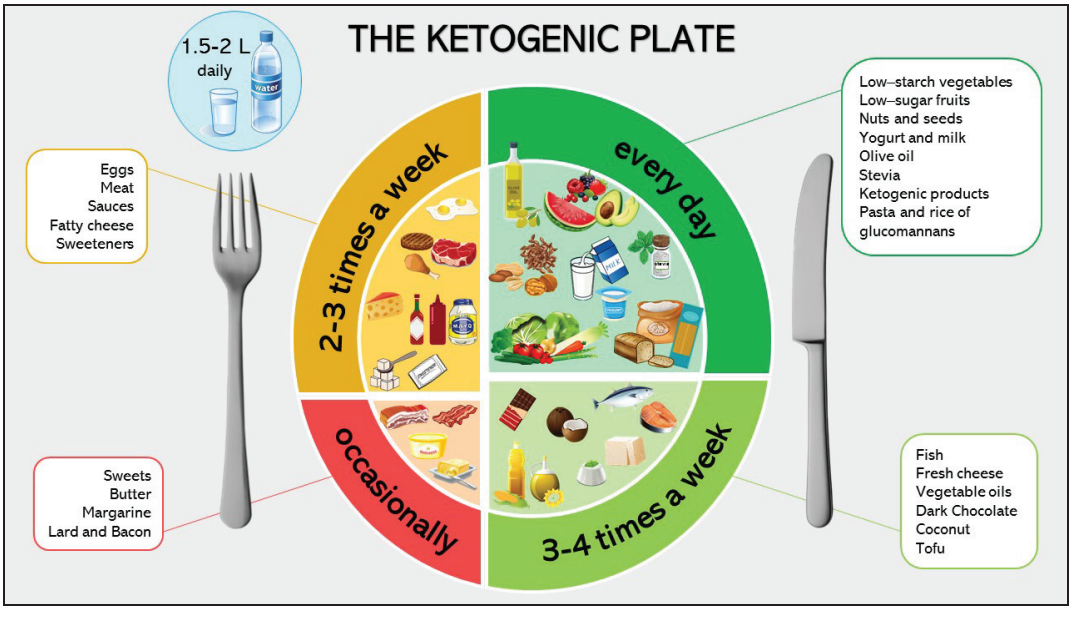

Figure 2. The ketogenic plate.

6.3. Ketogenic Diet: Mechanism of Action

Nutritionally induced ketosis is the condition where fatty acid oxidation is diverted to ketone production in the liver, because of low tissue glycogen levels [163]. When the glucose reserves run out, the central nervous system (CNS) is not able to use fatty acids as an energy source, thus, ketone bodies, produced from acetyl-CoA, are produced as alternative energy sources. Since, in postnatal life, these molecules are essential during brain formation, acting as lipid precursors and sparing glucose [148]. Although rapid, the ability of the brain to switch from one energy source to another requires significant metabolic adaptation, including the changing of ketone bodies transporters in response to ketone level variations [148,164]. Moreover, KD is significantly effective in children, due to the greater permeability of the blood barrier.

Many cell types in the CNS are able to use ketone bodies as a glucose substitute, and are spared the damaging effects of glucose deprivation. In particular, beta-hydroxybutyrate (βHB) is the primary energy source for neurons, if glucose is compromised [148,165,166]. However, according to studies that examined the effects of ketone body supplementation to glucose, under cell culture conditions, ketone bodies are preferentially used in lipid synthesis, while glucose remains the primary energy source in the CNS, when feasible [148,167].

There is supportive evidence that the ketogenic diet exerts an anti-seizure effect, through different mechanisms, having the CNS as the main focus of action. Although the mechanisms underlying the effects of KDs on weight loss are still a subject of debate [168]. There are multiple potential mechanisms through which the ketogenic diet may affect the obesity phenotype and status (Figure 3).

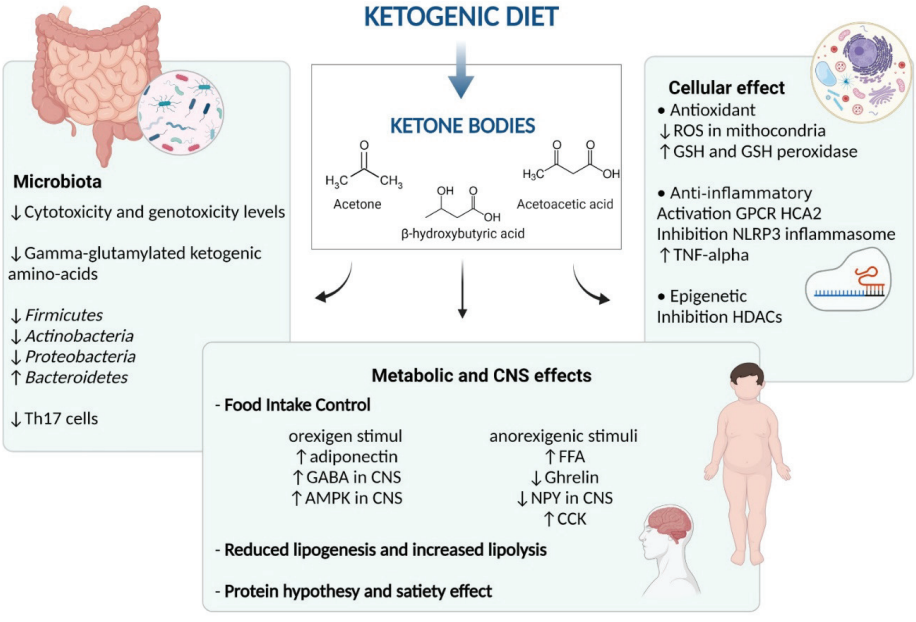

Figure 3. Mechanisms of ketogenic diet action. ROS = reactive oxygen species; GABA = gamma-aminobutyric acid; GSH = glutathione; G-protein-coupled receptor (GPCR); HCA2 = hydroxycarboxylic acid receptor 2; HDAC = histone deacetylase; Th = T helper; TNF-α = tumor necrosis factor alpha; NPY = neuropeptide Y; CCK = cholecystokinin; CNS = central nervous system; AMPK = AMP-activated protein kinase.

Gibson et al., in their metanalysis, reported that the clinical benefit of a ketogenic diet relies on/stays in preventing an increase in appetite, despite weight loss, although individuals may indeed feel slightly less hungry (or more full or satisfied). Ketosis appears to provide a plausible explanation for this suppression of appetite [169]. A possible explanation underlying this mechanism of action is the suppressant action of ketone bodies on appetite or modifications in hormone control.

In more detail, ketosis might have a direct or indirect effect on the secretion of appetite-related hormones, as they seem to exert an action on both orexigen and anorexigen signals, mainly (βHB) [163,170]. In the orexigen mechanisms, KD increases the circulating levels of adiponectin, while acting in the CNS, regulating food behavior, via enhancement of the brain gamma-aminobutyric acid (GABA) and AMP-activated protein kinase (AMPK) phosphorylation. The anorexigenic mechanism implies a rise in the circulating free fatty acids after meals, and a subsequent reduction in NPY, which is a neuropeptide that is essential in the food intake control, acting on the arcuate nucleus (ARC) of the hypothalamus. Moreover, KD decreases the circulating ghrelin levels (the appetite hormone) and maintains the CCK post-prandial anorexigenic response after weight loss. Thus, the net balance of the contrasting stimuli results in a general reduction in perceived hunger and, consequently, lower food intake [163,170].

Following this, KD modifies the metabolic pathways of the subject, by reducing lipogenesis and increasing lipolysis, thus influencing the adipose tissue and dyslipidemia status. Moreover, another hypothesis suggests the positive role of protein intakes, of which the utilization by the body requires an "expensive" process that increases energy expenditure compared to other diet protocols. In fact, glucose production during KD is obtained from the gluconeogenesis pathway, an energy-demanding process, which relies on dietary or tissue-origin proteins [168]. Together with this, the protein hypothesis

states possible mechanisms for higher weight, due to the higher satiety effect of this nutrient [8,168].

The ketogenic diet has beneficial effects on the obese phenotype, which are broader than simply fat and weight loss. Obesity is a recognized systemic low-grade inflammatory state, in which adipose tissue iper-proliferation alters the signaling between the adipocytes, immune cells, and epithelial cells. Thus, resulting in a secretion of active molecules, such as cytokines, TNF-alpha, and IL-6, which increase insulin resistance and stimulate inflammatory processes [171]. Given that KD is a potential tool in counteracting inflammation, for example, by reducing TNF-alpha after treatment [172].

Furthermore, ketone bodies are useful antioxidative and anti-inflammatory molecules at the cellular level. In fact, the ketogenic diet has been associated with antioxidant effects, producing lower amounts of ROS (reactive oxygen species) in the mitochondria, and increasing glutathione (GSH) and glutathione peroxidase activity in animals [165]. Recently, βHB has been found to modulate inflammation through the following two mechanisms: via activation of Gi-protein-coupled receptor HCA2, contributing to the neuroprotective effect and via the inhibition of NLRP3 inflammasome, which mediates IL-1β and IL18 production in human monocytes [173,174].

Lastly, ketone bodies could exert epigenetic cellular effects, involving the inhibition of histone deacetylases (HDACs) enzymes, and consequently affecting transcription differently and upregulating some genes encoding for bioenergetic enzymes [165,174,175].

6.4. Ketogenic Diet and Gut Microbiota

The gut microbiota and their metabolites have gained attention as possible players that directly modulate host health [147,176]. SCFA, namely, butyrate, acetate, and propionate, are gut metabolic end-products that exert multiple beneficial effects on human metabolism, particularly in obese individuals [176,177]. Recently, studies [10] have shown changes in the gut microbiota, after adopting the ketogenic diet, suggesting a possible role of ketone bodies in altering the intestinal microbiome. Interestingly, recent preclinical data underline the possible pathogenetic role of the gut microbiome on the benefits of the KD [178].

In mouse models receiving KD, an increase in *Akkermansia muciniphila* and *Parabacteroides merdae*, in the intestinal population, has been noted. These bacteria reduce gamma-glutamylated ketogenic amino acids, both in the gut and blood. This fact, in turn, had the effect of rising the ratio of GABA-to-glutamate in the brain of mice. Therefore, the reduction in GG amino acids, displayed by the gut microbiota, might be linked with neurotransmission at the CNS level [179].

Moreover, recent studies suggested that ketone bodies inhibit the reduction in the levels of proinflammatory Th17 cells in the intestine, therefore acting as possible modulators of inflammatory conditions [132,180].

Clinical evidence on the children population is still limited to epileptic patients. For example, a group of 14 epileptic children, evaluated before starting the KD diet, experienced an imbalance in the gut microbiota compared with healthy controls, harboring a higher amount of pathogenic bacteria and decreasing beneficial bacteria. After a KD treatment, a healthier microbiota was observed, by decreasing the *Protobacteria* (from 24.34% to 10.77%) and enhancing the *Bacteroidetes* phylum (from 26.75% to 38.71%) [181]. According to these, Zhang et al. also found a selective increase in *Bacteroidetes*, and a decrease in *Fimicutes* and *Actinobacteria*, after KD treatment in epileptic children [182].

Interestingly, applying KD in a group of obese adults, Basciani et al. observed a changed microbiota pattern, which resembled the ones observed in children who are affected by refractory epilepsy, treated with KD, i.e., *Fimicutes* significantly diminished and *Bacteroidetes* increased. Moreover, they found divergent responses on the gut microbiota, according to a protein source during KD, experiencing a healthier microbiota composition with whey or vegetable protein sources in the diet [183].

Due to the low fermentable carbohydrate intake during KD, gut metabolites significantly reduce during the ketogenic diet treatment. Therefore, Ferrari et al. studied the

impact of this high-fat diet protocol on gut health, having expected an increase not only in the bile acid secretion, but also in the secondary bile acid detrimental effects. Surprisingly, the KD diet, even though it is a high-fat protocol, showed a decrease in cytotoxicity and genotoxicity levels. The absence of adverse effects on fecal water toxicity after KD treatment, has therefore been assigned to the better health conditions of patients after the KD diet [184]. The hypothesis of a multistep impact of KD on human health, besides the gut environment, to a systemic level, is still open.

Given that obesity is a well-known condition, in which the Firmicutes/Bacteroidetes ratio is altered, KD is gaining attention as a possible therapeutic strategy, to ameliorate the inflammatory condition by changes in the gut microbiota species and metabolites [176,182,185,186].

7. Ketogenic Diet and Metabolic Disorders in Adults and Children: State-of-the-Art

Indications and contraindications for the use of VLCKD diets in adults, are shown in Table 3 [187,188]. VLCD and VLCKD diets are administered orally and often using commercial products that contain nutrients of high biological value, and may be in solid, liquid, or powder form. Recently, EFSA expressed a scientific opinion about the correct amount of macronutrients, establishing a minimum content of protein (75 g/day), carbohydrates (30 g/day), linoleic acid (11 g/day), α-linoleic acid (1.4 g/day), and not less than 600 kcal/day, until 800 kcal/day. In addition, VLCKDs can be followed for 12 consecutive weeks, under medical supervision [189].

Table 3. Indications and contra-indications of ketogenic diet in adults.

Indications	Contra-Indications
- Obesity (BMI > 30 kg/m^2) - Overweight with co-morbidities (hypertension, non-insulin dependent diabetes mellitus, dyslipidemia, obstructive sleep apnea syndrome, metabolic syndrome, severe osteopathy or arthropathy, NAFLD, PCOS) - Patients eligible for bariatric surgery	- Pregnancy and breastfeeding - Mental and behavioral disorders - Alcohol and other substance abuse - Type 2 diabetes mellitus with significant glucometabolic decompensation and in subjects treated with SGLT2 inhibitors - Severe liver failure (chronic active hepatitis, cirrhosis of the liver) - Renal failure - Acute myocardial infarction within the previous three months, heart failure, unstable angina and arrhythmia - Stroke within the previous three months

Modified from [187]. BMI = body mass index; NAFLD = nonalcoholic fatty liver disease; PCOS = polycystic ovarian syndrome; SGLT2 = sodium–glucose cotransporter 2.

In recent years, VLCDs had been explored for the treatment of several diseases in adults, proving a number of potential therapeutic impacts on the gut microbiota, cancer, diabetes, weight loss, and cardiovascular diseases [190].

The condition that sets in, following a few days of adherence to VLCKD, is known as 'physiological ketosis', which, unlike ketoacidosis that is caused by metabolic decompensation, maintains a physiological pH and low levels of ketones [191]. The subjective sensation of appetite is greatly reduced during this diet, probably in relation to the suppression of ghrelin secretion, which is the main gastrointestinal estrogen hormone [192]. Finally, no clinically relevant changes in liver function have been reported during VLCKD, and this diet can be considered safe [193]. The most frequently reported side effects are headache, halitosis, constipation, alternating with diarrhea, electrolyte disorders, and muscle cramp [187].

After the most restrictive phase, patients can gradually reintroduce different food groups (starting with those with a lower glycemic index, then moderate, and, finally, high),

and in the meantime they follow a nutritional re-education program, to promote a change in their eating habits and to maintain weight loss in the long term [191].

7.1. Ketogenic Diet and Obesity

A 2014 systematic review and meta-analysis of 20 RCTs, conducted on adult patients aged 28–48 years, with a BMI between 27.9 and 41.9 kg/m^2 (from overweight to severe obesity), found significant weight reduction during the VLCD and low-calorie diet (LCD) adherence period. In contrast, in the maintenance phase, and up to 22 months after following the diet, less weight regain was observed in the anti-obesity drugs group or meal replacements group, compared to a protein-rich diet group or exercise group [194].

In addition, a 2017 review and meta-analysis of five rcts, assessing weight loss in diabetic and non-diabetic patients after following VLCD (<800 kcal/day) or low-energy liquid-formula diets (LELD) (>800 kcal/die), found that the weight loss was the same in both the diabetic and non-diabetic patients, ranging from 8 to 21 kg, over a period of 4 to 52 weeks.

Moreover, a recent systematic review and meta-analysis of 15 studies (7 noncontrolled, 2 controlled, and 6 randomized controlled studies) shows significant reduction in body weight and BMI at 1, 2, 4–6, 12, and 24 months, and this and appears to be associated with larger weight loss rates compared to other diets with a different energy content (i.e., LCD and VLCD) of the same duration [187]. They also found a reduction in waist circumference and a loss of lean mass, comparable to that found in subjects who follow other dietetic interventions [187].

A recently published narrative review explores the various positive effects of the ketogenic diet [190]. In particular, on weight loss, they report a study [195] that was conducted on 322 moderately obese patients, over a period of two years, following a low-fat restricted-calorie diet (LFD), a Mediterranean restricted-calorie diet (MD), and low-carbohydrates non-restricted calorie diet (LC). The LC group was instructed to reduce carbohydrates (<20 g/day) for the first two months, and then increase to up to 120 g/day of carbohydrates. The weight loss after 3 months was greater in the low-carb group, but when carbohydrates were reintroduced, the weight was similar to the MD group. A similar study [196] monitored, for two years, weight loss and changes, in visceral fat, using DEXA. The participants were divided into the VLCKD group and LCD group. The weight loss, in kilograms, in the VLCK diet was double that of the LC diet throughout most of the study, and remained significant at the end of the trial. The amount of visceral fat loss in the VLCK diet group was three times greater than the control group, while preserving lean body and skeletal bone mass [196]. This evidence, regarding the maintenance of lean mass, is a fundamental point, because when rapid weight loss occured, the basal metabolic rate (BMR) decreased, leading to weight regain, due to increased hunger and lower energy expenditure.

7.2. Ketogenic Diet, Insulin Resistance, Type 2 Diabetes and Polycystic Ovary Syndrome

Correct nutrition should be considered to be an integral part of the metabolic management of diabetes, and the ketogenic diet could at least be offered as a treatment option [197]. The rapid reduction in carbohydrate intake reduces glucotoxicity and insulin resistance, improves pancreatic beta-cell function, and leads to better glucometabolic control [198].

In patients undergoing a VLCKD, hepatic gluconeogenesis maintains stable glycaemia through basal insulin secretion. In fact, the preserved insulin secretion prevents the onset of pathological ketoacidosis. The differences between the normal diet, the ketogenic diet, and diabetic ketoacidosis, are summarized in Table 4 [168].

Over the years, several scientific societies have expressed positions on the role of the ketogenic diet in diabetes. In 2019, a consensus was published that considering reducing the overall carbohydrate intake, with low-carbohydrate (LC) or very-low-carbohydrate (VLCD) meal plans, is a feasible approach for adults with type 2 diabetes, who are not achieving their glycemic targets or where reducing hypoglycemic medication is a priority [199]. The

American Diabetes Association's Position Statements 2020 also suggested the use of a low-carbohydrate meal plan for people with prediabetes, stating that further research is needed to establish its usefulness [200,201]. Numerous systematic reviews and meta-analyses of RCTs on patients with DMT2, show that both VLCD and VLCKDs induce greater weight loss in the short term (<6 months) than standard hypocaloric diets. The most important aspect, however, seems to be the duration of the positive effects on glucometabolic set-up. Steven et al. confirmed that recent-onset TMD2 can be considered to be characterized by a reversible, altered β-cellular response, and thus that the first phase of insulin secretion can be recovered. VLCD was particularly effective in patients with a short duration of diabetic disease and a preserved insulinemic response. The patients who followed VLCD for 2 months showed weight reduction and stable insulinemic levels at 6 months. After returning to an isocaloric regimen, a lower fasting blood glucose was observed without the use of hypoglycemic drugs. [202]. A recent systematic review, using American Diabetes Association remission definition (<6.5% HbA1c threshold), found that patients who adhered to VLCD resulted in 32% increase rates of remission of diabetes at 6 months, compared with diets that are commonly recommended for DMT2 management [203]. In terms of body composition, to support the efficacy of this treatment, lean mass and fat mass were assessed by X-ray densitometry (DXA), basal metabolism by indirect calorimetry and biochemical analyses for metabolic balance, in 25 subjects with TMD2, who followed VLCKD for 8 weeks. The results show reduced abdominal fat mass, maintenance of resting energy expenditure (REE), and restoration of metabolic function [204].

Table 4. Blood levels in normal diet, the ketogenic diet and diabetic ketoacidosis.

Blood Levels	Normal Diet	Ketogenic Diet	Diabetic Ketoacidosis
Glucose (mg/dL)	80–120	65–80	>300
Insulin (mU/L)	6–23	6.6–9.4	around 0
Ketone bodies (mM/L)	0.1	7–8	>25
PH	7.4	7.4	<7.3

Modified from [168].

The different studies show that the duration of the treatment and the gradual transition to the different regimes is crucial for positive results. After the planned weeks of VLCKD (600–800 kcal/day, CHO 20–60 g/day), the transition phase begins, during which carbohydrates are gradually reintroduced. Foods with a lower glycemic index (fruit and dairy products) are the first to be reintroduced, followed by foods with a moderate glycemic index (legumes), and finally foods with a high-glycemic index (bread, pasta, and cereals). During the transition phase, the quantity of carbohydrates should not exceed 90 g/day and the daily calorie intake should not exceed 1500 kcal. In the subsequent maintenance period, the quantity of carbohydrates should not exceed 130 g/day and the calorie intake should be between 1500 and 2000 kcal/day. The main purpose should be to maintain weight loss and promote a healthy lifestyle, as close as possible to the fundamentals of the Mediterranean diet [205,206].

Insulin resistance and obesity are common signs of polycystic ovary syndrome (PCOS). Hyperinsulinemia contributes to hyperandrogenism in women with PCOS, which, in turn, is responsible for increased visceral and subcutaneous body fat [207]. Interestingly, low-grade inflammation, with an excess of carbohydrate intake, acts with insulin resistance and hyperandrogenism, to enhance the metabolic phenotype of PCOS [208]. Acute hyperglycemia produces reactive oxygen species (ROS), and increases oxidative stress and inflammation [209]. Blood glucose levels are influenced by carbohydrate intake and insulin secretion, so VLCDs have been widely proposed as a valid alternative to hypocaloric diets, in terms of improving the outcomes in women with PCOS [210].

A randomized-controlled trial demonstrated that the KD significantly reduces anthropometric parameters and body composition. A VLCD, with an adequate supply of protein intake, preserves the lean body mass. In addition, the observation of a significant reduction in the liver function markers emphasizes VLCD as a treatment of fatty

liver, compared to the traditional pharmacological treatment [211]. Zhang et al. also demonstrated a significant reduction in glucose and insulin blood levels, with a significant improvement in insulin resistance. An improvement of the lipid profile was observed, with a significant decrease in triglycerides, total cholesterol, and low-density lipoprotein (LDL), and an increase in high-density lipoprotein (HDL). The LH/FSH ratio, LH total, free testosterone, and dehydroepiandrosterone sulfate (DHEAs) blood levels, were also significantly reduced [212].

An important limitation is the high dropout rate among women with low evidence of a long-term effect, which led to the consideration of VLCDs as safe for short dietary cycles. It could be suitable to shift to a Mediterranean dietary pattern, with physical activity, to achieve results in the long term [213].

7.3. Ketogenic Diet on Cardiovascular Risk and Dyslipidemia

Regarding the possible role of VLCKDs on cardiovascular risk, markers are beginning to emerge. In a clinical trial, conducted on 30 adults with metabolic syndrome (MetS) diagnosis and prediabetes or diabetes, and BMI > 25 kg/m^2, compared the effects of the ketogenic diet (KD), standard American diet (SAD), and standard American diet plus exercise, on health outcomes. The results showed that the KD group had a decrease in weight, body fat, BMI, HA1c, triglycerides, and a higher resting metabolic rate (RMR), after 10 weeks [214]. One study compared a low-carb diet (<30 g/day) to low-fat diet in obese adults. After 6 months, the results showed a drastic decrease in TG for the low-carb diet group, but no significant difference for the total cholesterol (TC), HDL, or LDL [215]. In a study of overweight patients who were followed for two years, divided into groups according to diet (Mediterranean diet, low-fat diet, or low-carb diet), a significant decrease in triglycerides, but also in TG/HDL ratio, was found in the low-carb diet group, and there was an increase in the HDL levels in all the groups. In the low-fat diet group, the decrease in TG/HDL ratio was 12%, while in the low-carb diet group it was 20% [195]. Choi et al. found an improvement in the blood lipid profile in the KD group of obese adults [216]. Lastly, in a 6-month study of obese patients, the KD and low-calorie diet were compared, and showed a decrease in TG, total cholesterol, and LDL, and an increase in HDL [217]. In conclusion, we can say that there are many preliminary studies showing the important effects that the ketogenic diet could have on CVD outcomes. Although a recommendation, different from the current, on fat intake, to prevent the onset of cardiovascular disease, has not yet been firmly established, a good attempt would be to establish a dietary pattern that was able to reduce the increasing incidence of diabetes and obesity, which are both linked to cardiovascular risk.

7.4. Ketogenic Diet Metabolic Impact in Children and Adolescents

Recent evidence suggests that more intensive dietary approaches may have benefits, especially for adults with severe obesity and obesity with comorbidities. Based on the efficacy of these approaches in adults, very-low- and low-carbohydrate approaches have been suggested to be beneficial for young people with prediabetes, insulin resistance, or nonalcoholic fatty liver disease [218,219]. Several studies have suggested a possible role for ketogenic diets in obesity in children, but the effects on metabolic parameters in children have been incompletely assessed. Partsalaki et al. compared the efficacy and metabolic impact of ketogenic and low-calorie diets in obese children and adolescents, and showed that the KD produced a greater improvement in weight loss and metabolic parameters than the low-calorie diet. An improvement was observed in the adiponectin concentrations, lipid profile, and metabolic parameters related to insulin sensitivity and resistance in obese children and adolescents [220]. Krebs et al. [221], in an rct on 33 obese adolescents (aged 14.2 $\pm$ 0.4) divided in two groups (group 1 high-protein, low-carb (<20 g/day) diet and group 2 low-fat diet). A significant reduction in (BMI-Z) was achieved in both the groups during the intervention, and was significantly greater for the high-protein, low-carb diet group. Both the groups maintained significant BMI-Z reduction at the follow-up. Although

no adverse effects were observed in the metabolic profile or cardiac function, a loss of lean mass was also observed in group 1. In another clinical trial, on six obese adolescents aged 12 to 15 years, with a BMI average of 50.9 kg/m^2 (39.8–63.0 kg/m^2), following a ketogenic diet for 8 weeks (650 to 725 kcal/day), the authors concluded that a ketogenic diet can be used effectively for rapid weight loss in adolescents with morbid obesity. The loss in lean body mass is blunted, blood chemistries remain normal, and sleep abnormalities significantly decrease with weight loss [222]. Another randomized, controlled 12-week trial, on 30 overweight adolescents, divided into a low-carb diet (LC) group and low-fat diet (LF) group, found an improvement in non-HDL cholesterol levels and greater weight loss in the LC group, and an improvement in LDL cholesterol levels in the LF group. There were no adverse effects on the lipid profiles of the participants in either group. Therefore, this new dietetic frontier may provide additional benefits for young people with obesity, with severe form and with comorbidities [223–225]. Lastly, Willi et al. demonstrated rapid weight loss, and less dependence from insulin injections and other antidiabetic drugs, in 20 children (mean age 14 $\pm$ 0.4) with T2DM. A reduction in blood pressure has also been demonstrated. In addition to these acute clinical improvements, this treatment option appears to have more lasting benefits on BMI [226].

7.5. Ketogenic Diet: Lights and Shadows

Changes in lifestyle (dietary pattern and exercise) are currently recommended as "first-line" therapies in pediatric obesity [5]. However, the poor adherence to this type of treatment raises the question of what other dietary interventions can be effectively applied in children with obesity and metabolic derangement. Although the evidence reported in this review makes it clear that the use of the KD in adult patients with obesity, metabolic syndrome, and T2DM, is now routine, with positive effects in reducing metabolic disorders [187], few studies were found to be conducted on children on this topic in the literature [220–222,226]. The reasons limiting this type of study are both the difficulty to apply this type of diet in a child's daily life (lack of acceptance of certain foods, poor palatability, meals outside the home to be managed by parents), which would lead to scarce compliance in the long term, with consequences on the child's social sphere, but also the impact of the cost of the diet on the family. Given the selectivity of the diet, another limitation may be the risk of an eating disorder after the restoration of a balanced diet. Moreover, this type of treatment also presents undesirable effects [187], after the first 3–5 days of the regimen, which could lead to a high drop-out rate, even if the appetite-suppressing effect of ketosis could contribute to the success of this diet.

8. Conclusions

Pediatric obesity is associated with systemic low-grade inflammation, which has been acknowledged as one of the major drivers of chronic degenerative pathologies, including T2DM and cardiovascular diseases in adult life [1,3–5]. The effects of obesity on health, and the costs on health care systems [227], clearly dictate the need to provide nutritional interventions for preventing and treating its complications during childhood. The KD represents a promise therapeutic tool for the treatment of metabolic and cardiovascular risk factors related to obesity in adults. In this review, we explain the hypothesized mechanism of action of KD. The literature also supports a potential role in chronic inflammatory diseases in children [10] and in adolescents who are eligible for bariatric surgery and are affected by PCOS, this treatment is currently proposed. However, clinical studies are needed to evaluate KD as a promising therapeutic tool for the treatment of metabolic and cardiovascular risk factors also in pediatrics.

The direction for future research should be to identify a specific subset of obese children, starting with those aforementioned, who would be less at risk of developing the difficulties described above, which could represent a milestone in clinical studies on the KD in pediatrics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13082805/s1, Figure S1: "Nutritional composition of different type of Ketogenic Diet compared to Mediterranean Diet".

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Article

Educational Intervention of Healthy Life Promotion for Children with a Migrant Background or at Socioeconomic Disadvantage in the North of Italy: Efficacy of Telematic Tools in Improving Nutritional and Physical Activity Knowledge

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Abstract: The aim of the "Smuovi La Salute" ("Shake Your Health") project was to implement an integrated and comprehensive model to prevent and treat overweight and obesity in low socioeconomic status (SES) and minority groups living in three different districts in the north of Italy. An app and a cookbook promoting transcultural nutrition and a healthy lifestyle were developed, and no-cost physical activities were organized. Healthy lifestyle teaching was implemented in 30 primary school classrooms. Learning was assessed through pre- and post-intervention questionnaires. At the Obesity Pediatric Clinic, overweight and obese children of migrant background or low SES were trained on transcultural nutrition and invited to participate in the project. Primary school students increased their knowledge about healthy nutrition and the importance of physical activity (p-value < 0.001). At the Obesity Pediatric Clinic, after 6 months, pre–post-intervention variation in their consumption of vegetables and fruit was +14% ($p < 0.0001$) and no variation in physical activity habits occurred ($p = 0.34$). In this group, the BMI z-score was not significantly decreased (-0.17 ± 0.63, $p = 0.15$). This study demonstrates the feasibility and efficacy of telematic tools and targeted community approaches in improving students' knowledge with regard to healthy lifestyle, particularly in schools in suburbs with a high density of migrants and SES families. Comprehensive and integrated approaches provided to the obese patients remain mostly ineffective.

Keywords: healthy lifestyle; education; migrant background; socioeconomic disadvantage; overweight

1. Introduction

The prevalence of childhood overweight and obesity [1,2] as well as the consequent chronic diseases during adulthood are growing worldwide [3–7]. In Italy 20.4% of children aged 8–9 years are overweight and 9.4% are obese [8]; among adolescents aged 11–15 years, 16.6% are overweight and 3.2% are obese [9].

Low socioeconomic status (SES) and minority ethnic groups are disproportionately affected by overweight and obesity [10–14]. SES is strictly correlated with people's level of physical activity (PA), sedentary behavior, and body mass index [15]. Children from low-SES families spend more time engaging in sedentary behavior and show a trend of higher BMI and lower PA levels than children from higher SES families [15].

After migrating to Western societies, families frequently abandon their traditional food habits to adopt Westernized dietary patterns containing higher levels of fat, sugar and salt. Accordingly, they often reduce their consumption of fruits and vegetables [16,17]. Moreover, the lack of a health-conscious exercise culture and the difficulties they encounter in accessing sport facilities in their new country represent another major predisposing factor to the onset or worsening of overweight and obesity; the sport activities offered often do not meet their traditions [14], are highly expensive, or difficult to find or to reach.

Recently, integrated and comprehensive approaches have been proposed in adults at different levels (individual, community, society) to overcome these barriers in low-SES and minority ethnic groups [18]:

(i) Individual level—there is evidence of the effectiveness of primary care-delivered tailored weight loss programs among deprived groups and an example is the *mosaic clinic*, a clinic specialized in ethnic-cultural diversity that provides individualized or group consultation with operators of the same ethnicity [19].

(ii) Community level—community-based behavioral weight loss interventions also have evidence of efficacy, at least in the short term. As an example, comprehensive school health (CSH) is an internationally recognized school-based health promotion approach that integrates multiple aspects to support students' development as learners and as healthy and productive members of society. This approach has been shown to be effective in increasing levels of physical activity, improving dietary habits, and decreasing rates of obesity among children [20,21]. These results have also been confirmed in primary schools with a high proportion of migrant children [22,23]; among children of Mexican origin, a reduction in BMI growth was seen among obese boys in the intervention community (ß-coefficient = −1.94, $p = 0.05$) [23].

(iii) Society level—the Italian Society of Pediatrics recently proposed the diffusion of the "transcultural" food pyramid, which allows families of migrant background to rediscover their tastes and flavors in their diet in the context of nutritional balance proposed by the Mediterranean diet (Supplementary Materials Figure S1).

Based on available evidence, we developed the "*Smuovi La Salute*" project, which is an integrated and comprehensive model of prevention and treatment of childhood overweight and obesity. This action–research project involves the population living in three different districts in the north of Italy and had three main aims:

- To spread knowledge on healthy lifestyles through a universal and community-targeted approach, offering tools to reduce socio-cultural and economic barriers;
- To educate children on healthy lifestyles and to measure the variation in the level of knowledge achieved in schools in suburbs with a high density of migrants and/or low-SES families;
- To prevent and treat overweight/obesity in primary care settings and treat obesity at the second and third levels of care through individual approach, with tools targeting subjects of low-SES or with a migrant background. In these groups, we evaluated lifestyle changes and variation in BMI z-scores.

2. Materials and Methods

The project "*Smuovi la Salute*" was planned by a scientific panel of professionals: pediatricians, experts in the fields of health promotion, physical education and nutrition, and cultural mediators. The project was targeted at three different levels (society, community, and individuals) and was implemented in three economically developed districts in the north of Italy (provinces of Bolzano, Trento and Verona), in suburbs with a high density of migrants and/or low-SES families.

This action–research project lasted 30 months and included the following three phases:

- **2.1. Phase 1:** recruitment and training of operators (November 2017–May 2019);
- **2.2. Phase 2:** development of tools for healthy lifestyle promotion (May 2018–April 2019);
- **2.3. Phase 3:** enrolment of the target population and implementation of the project (November 2018–May 2020).

This project was funded by the Italian National Institute for Health, Migration and Poverty (INMP), a public entity under the Italian Ministry of Health and a Collaborating Center of WHO/Europe (https://www.inmp.it/ita/Archivio-Pubblicita-Legale/Archivio-Delibere/Delibere-2017/Delibere-2017-101-208, accessed on 13 October 2017).

2.1. Phase 1: Recruitment and Training of Operators

All the operators involved in the project were recruited and trained by highly specialized professionals who were part of the scientific panel:

- Family pediatricians and general practitioners followed a one-day course on healthy multi-ethnic nutrition from weaning to adulthood, respecting the cultural and religious needs of the children and their families;
- Cultural mediators of the four most represented ethnic groups in the three districts (Arab, Chinese, Urdu and Russian), students in nursing sciences from different countries and volunteers from foreign associations were trained during a 3-day course to provide advice and dietary recommendations according to the transcultural food pyramid;
- Cultural facilitators and sport operators of various disciplines followed, respectively, a 14 and 12 h course run by members of the scientific panel and experts in interculturalism to better understand the possible barriers to access to sport and recreational activities for immigrants, and thus facilitate their participation. After the course, sport activities in urban spaces (parks, green areas, squares) in some contexts with a high density of migrant residents were organized. The Italian Union Sport for all (UISP), a sport promotion body recognized by the Italian Ministry of Labor and Social operating in many Italian cities, was in charge of organizing these activities.

2.2. Phase 2: Development of Tools for Healthy Lifestyle Promotion

2.2.1. Development of a Transcultural Healthy Eating App

A research foundation, Fondazione Bruno Kessler (FBK), in collaboration with the scientific panel of the project, developed the "*Smuovi la Salute*" app. The aim of this app was to help teenagers and their parents make correct and healthy food choices, from shopping at the supermarket to meal preparation. It guided families towards choosing the right food quality and quantity, suggesting the frequency of weekly consumption, thus trying to raise the awareness of parents and children regarding healthy eating through a process of "virtual coaching". The app, free to download, was prescribed and distributed by health care professionals to families and followed the principles of the transcultural pyramid. Table 1 reports the main features of the app.

The app was only developed in Italian to encourage integration into Italian communities, as suggested by the cultural mediators. Even in migrants, the level of literacy was appropriate and children helped their parents with the app.

2.2.2. Development of a Cookbook Containing Healthy Multicultural Recipes

Through the direct involvement and participation of 67 persons coming from various foreign communities present in the area, recipes for healthy and simple dishes from different culinary traditions were collected. The scientific panel, with the collaboration of a nutrition specialist, evaluated and revised the recipes before publishing them in a book. The book "Healthy Cooking within everyone's reach", was presented through public seminars and events and disseminated through the Internet, social media, and the delivery of printed copies. The book is available online and in PDF format (Supplementary Materials

Figure S2). The cookbook was only developed in Italian to encourage integration into Italian communities, as suggested by the cultural mediators.

Table 1. Main features of the app "Smuovi la Salute".

Main Features of the App "Smuovi la Salute"
Meal tracking through dialogue with the chatbot or with a food diary comprehensive of international foods and recipes
Tracking the achievement of the weekly goal, through messages of encouragement and reminder by the chatbot
Feedback on adherence to the transcultural pyramid through graphic reports and informative messages and suggestions
Request for nutritional information through free text dialogues
Gamification: game interface that develops step by step with the achievement of weekly goals
Periodic suggestions of multicultural recipes taken from the healthy recipe book
Final satisfaction questionnaire. A scale of values from 1 (very little satisfied) to 5 (very satisfied) was given by the subjects.

2.2.3. No-Cost Physical Activities in Urban Spaces

UISP organized no-cost physical and sport activities in urban spaces (parks and urban green spaces) in districts with a high density of migrant residents of the three provinces. UISP sport operators, previously trained, welcomed both patients with overweight and obesity problems sent by the Obesity Pediatric Clinic, and normal-weight children coming from low-SES families or from a migrant background. Children participating in the activities were 7–13 years old. Due to the difficulties in enrolling and keeping them regularly committed to the activities, sport activities were organized as after school activities for students, in suburbs with a high density of migrants and/or low-SES families.

2.2.4. Nutritional and Video Laboratories at School

Nutritional laboratories were prepared and integrated during school time. These laboratories were led by pediatricians with the support of trained mediators and at least one teacher for each class. Children were placed in a "didactic atelier" setting where they could follow a frontal lesson on the basic principles of healthy nutrition and physical activity. They were involved in the discussion, raising doubts or questions, and worked together on nutritional exercises. The first part focused on some basic nutritional aspects such as the different nutrients, the food pyramid and how to eat a healthy breakfast, and on the benefits of practicing regular physical activity. The second part, which took place approximately one month later, consisted of a follow-up lesson on healthy nutrition and correct lifestyles. Moreover, students were encouraged to share what they had learned with their family, to offer them an opportunity to increase their knowledge and to motivate them to help their children improve their lifestyle.

Due to the spread of the SARS-CoV-2 pandemic, all teaching materials realized for the nutritional laboratories were collected in video-laboratories, with the aim of providing teachers with an easy instrument to continue the activity during online teaching. The video format consisted of a cartoon, set in a typical day of school with a group of primary school students. The video is comprised of 8 clips, one for each different topic, which are linked to each other, but at the same time independent. Attached to each clip there is an information sheet addressed to teachers, containing some details on healthy nutrition and lifestyle, a summary of the topic and exercises for the students. All videos were previously tested with a control group of teachers and students receiving positive comments. All school leaders involved in the laboratories expressed interest in pursuing with the educational nutritional program.

2.2.5. Policy Environment

Brochures with the project details were prepared. They contained advice on healthy eating and were distributed to students, families, religious communities, associations, pediatricians, general practitioners, and other health professionals (Supplementary Materials Figure S3).

2.3. Phase 3: Enrolment of the Target Population and Outcomes

2.3.1. Universal Approach

Meetings and conferences for the local community and for the different religious communities of the districts (Muslim, Chinese and Orthodox) were organized to present the tools developed to promote healthy transcultural nutrition and healthy lifestyle. Trained operators (including cultural mediators) were invited to moderate these meetings. The reason for needing to change behavior was presented as the necessity of preventing obesity, considered a disease associated with reduced self-esteem, depression, nocturnal sleep apnea, physical exercise intolerance, short-term orthopedic problems, as well as metabolic and cardiovascular complications that can be manifest since adolescence.

2.3.2. Community Targeted

School teaching and learning was implemented in primary schools in the three districts, in suburbs with a high density of migrants and/or low-SES families. Primary school in Italy includes children and adolescents aged 6–14. In order to participate, primary schools did not have to be involved in a major health education diet/nutrition-related project. School administrators, school leaders and local health officials worked together to develop and implement the program. Thirty-two school classes were contacted, and 30 accepted to participate in the program. Students as well as families were invited to participate. All the students of the 30 classes were enrolled because the activity was proposed as part of the school program on the decision of the class council.

Participants in the nutritional laboratories were asked anonymously to complete a pre- and a post-intervention questionnaire (after one month) in order to evaluate the learning development. Questionnaires, which differed according to age (6–10 and 11–14 years old), were developed by the Cà Foscari University of Venice, with the collaboration of professionals and academics working in the field of nutrition. Questionnaires included: 3 questions to check the children's knowledge; 2 questions related to their eating habits and the level of physical activity practiced; 3 questions regarding the satisfaction about the activities proposed during the workshop, the cookbook, and the app (Table 2).

Table 2. Lists of questions used to check students' knowledge and habits.

Students Aged 6–10 Years
How often should we eat fruit and/or vegetables?
How much movement should we do?
Check the images of the foods that fall into the vegetable group
How often do you eat fruit and/or vegetables?
How much movement do you do?
Students aged 11–14 years
Which of these represents a complete and balanced meal?
How often should we exercise to stay healthy?
How many servings of fruit and vegetables should be consumed each day?
How many times a week do you usually eat fruit and/or vegetables?
In the last 7 days, how many days have you been exercising (sports, games or activities that make your heart beat faster and can leave you breathless) for a total of at least 60 min a day?

In the questionnaires, students self-reported the number of times they achieved each goal, which referred to a modified behavior. A value of 1 indicated a change of behavior which became equal or greater than the recommended level; a value of 0 indicated a behavior which was lower than the recommended level; missing responses were excluded from the analysis. The number of behaviors that met the recommended level was calculated by adding the values with a maximum score of 6. The primary outcome of this intervention was to increase patients'/students' awareness of the importance of eating five portions of fruit and vegetables daily and of implementing daily physical activity. Parents of the children enrolled in the laboratory received a brochure on the project and on healthy lifestyles, as well as a copy of the cooking book, and were encouraged to download the app.

2.3.3. Individual Approach

- Trained family pediatricians and general practitioners implemented transcultural counselling in their routine activities and during their scheduled periodical free-of-charge physical examinations in both normal weight and overweight children (of 6, 9, and 13 years of age). Moreover, they gave information regarding the possibility of participating in no-cost physical activity in urban spaces organized by UISP, and to download the app and the transcultural recipe book.

- At the secondary and tertiary level Pediatric Obesity Clinic, subjects with the following inclusion criteria were enrolled:

(i) Age from 6 to 16 years;
(ii) BMI-for-age above 1 (overweight) or 2 (obese) standard deviation of the WHO growth reference median, respectively [24];
(iii) Migrant background and/or socioeconomic disadvantage. The family's socioeconomic status (SES) was evaluated by ascertaining the mother's educational level. Educational level was previously identified as an important indicator for SES [25] and was dichotomized into low- (≤14 years of education) and high- (>14 years of education) SES, which differentiates between families with a mother who has completed medium or higher education, college or university training, from other families [26].

Informed and privacy consent were obtained before participation in the study. All subjects were trained on transcultural nutrition by an expert dietician, invited to participate in no-cost physical activity in urban spaces organized by UISP and download the app, as well as received a printed copy of the recipe book.

Weight and height were measured during the recruitment and pre- and post-intervention questionnaires (at 0, 6 and 12 months) were administered to measure any change in nutritional habits and level of physical activity. These questionnaires were developed on the basis of the validated ones used by *Okkio alla Salute* (for 6–10-year-old children and their parents) [8] and in HBSC surveys (for 11–14-year-old youths) [9]. The main result of the intervention at this level was to evaluate the improvement in the daily consumption of vegetables and fruit (at least 3–5 daily portions) and in the increase in physical activity (at least 1 h per day for 5 days a week), as well as to highlight a possible BMI z-score variation.

Statistical analysis was performed using SPSS Software version 25.0 (SPSS Inc., Chicago, IL, USA) and data were expressed in descriptive statistics. The independent *t*-test was used to compare the means of two independent groups. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Phase 1

Trained operators included:

- 125 family pediatricians and general practitioners;
- 24 cultural mediators, nursing sciences students from different countries and volunteers from foreign associations;
- 42 cultural facilitators;
- 59 sport operators.

3.2. Phase 2

Healthy lifestyle tools, developed during the project, were disseminated as subsequently described.

3.2.1. Transcultural Healthy Eating App

The app was downloaded by 392 families (66% Italians, 34% migrants), and the data from the "satisfaction questionnaire" taken by 67 participants (60% Italians, 40% migrants) reported good scores: 4.15/5 on average.

3.2.2. Cookbook with Healthy Multicultural Recipes

Twenty live book presentations were organized, 2396 paper copies were distributed and 1.481 people visualized the book online as a unique view.

3.2.3. UISP Activities

A total of 11 activities in the three districts were organized by UISP and they were offered once a week; each activity lasted approximately 3 months. A total of 172 different children and adolescents participated in the activity. Each child participated in only one activity.

3.2.4. Policy Environment

The project's brochures were distributed to 884 families/community people, 398 pediatricians, 79 general practitioners, 173 other health professionals, 331 students, 225 members of associations, and 75 teachers.

3.3. Phase 3

The target population was enrolled as subsequently described.

3.3.1. Meetings with Communities on Healthy Lifestyles

Twelve meetings were organized by the scientific panel in collaboration with the trained operators. Meetings were addressed to the general population and were organized after press release, and after dissemination through community chat and brochures. Seventy persons attended the meetings.

Other five meetings were organized together with the religious communities, after preliminary meetings with their leaders; 23 persons attended these meetings.

3.3.2. Outpatient Clinic

From November 2018 to April 2020, in the three Obesity Clinics, 97 patients accepted to be enrolled in the project: 83.5% were Italian of low SES, 4.1% were from eastern Europe, 5.1% were Urdu (Pakistan, India, Bangladesh), 2.1% were from South America, 2.1% were from Morocco, and 3.1% from other countries. Migrants of low SES represented 14.4%, and those of high SES represented 2.1%. Twenty-nine children (30%) attended the physical activity in urban spaces organized by UISP. Among these, 55% were Italian of low SES and 34% were from migrant and low-SES families. Only 30 among all the patients enrolled attended the clinic for a second appointment (+6–12 months) and completed the post-intervention questionnaire. Six out of thirty children enrolled by the obesity clinic also attended the physical activity in urban spaces organized by UISP.

Pre–post-intervention variation in the "self-reported" consumption of vegetables and fruit was +14% ($p < 0.0001$) and no variation in physical activity occurred in the cohort. BMI changed from 29.78 $\pm$ 6.56 to 27.38 $\pm$ 4.23 kg/m^2, and the BMI z-score from 2.46 $\pm$ 0.43 to 2.28 $\pm$ 0.69 ($p = 0.15$). Complete data are reported in Tables 3 and 4.

Table 3. Anthropometric and lifestyle data of the overweight and obese subjects enrolled in the outpatient clinic.

Patients Enrolled at the Obesity Clinic	***n* = 97**
Age (years)	10.7 ± 2.27
M/F (*n*)	56/41
M/F (%)	58/42
Italian of low SES	81 (83.5%)
Migrants of low SES	14 (14.4%)
Migrants of high SES	2 (2.1%)
Pre-pubertal/pubertal (*n*)	47/11
Pre-pubertal/pubertal (%)	48/52
BMI at baseline (kg/m^2)	30.86 ± 4.16
BMI z-score at baseline	2.35 ± 0.69
Follow-up data	*n* = 30
Italian of low SES	21 (70%)
Migrants of low SES	9 (30%)
Follow-up duration (months)	8.40 ± 1.73
BMI at baseline (kg/m^2) (*n* = 30)	29.78 ± 6.56
BMI z-score at baseline (*n* = 30)	2.46 ± 0.43
BMI at follow up (kg/m^2) (*n* = 30)	27.38 ± 4.23
BMI z-score at follow up (*n* = 30)	2.28 ± 0.69

Data shown as the mean and standard deviation in brackets. Abbreviations: M, male; F, female; BMI, body mass index.

Table 4. Anthropometric and lifestyle variation from baseline to follow up of overweight and obese subjects enrolled in the outpatient clinic (*n* = 30).

Variation in BMI z-Score	**−0.17 ± 0.63**	***p*= 0.15**
Variation in % of patients with physical activity at least 1 h per day for 5 days a week	0% (from 12.5 to 12.9%)	$p = 0.34$
Variation in % of patients with consumption of 3–5 daily portions of vegetables and fruit	14% (from 25.7 to 29.4%)	$p < 0.0001$

Data shown as the mean and standard deviation in brackets or as percentages. Abbreviations: BMI, body mass index.

3.3.3. UISP Activities

One hundred and seventy-two subjects were enrolled in the UISP physical activities. Only 15 children attended the activities after 3–12 months and completed the post-intervention questionnaire.

3.3.4. School Program

Six-hundred and ninety-five primary school students from 30 classes were recruited and completed the pre-intervention questionnaire; only 402 completed the post-questionnaires due to the closure of schools caused by the SARS-COV2 pandemic.

Among the participants, 52% were female and 48% were male. The mean age was 9 ± 1.2 years. There was a 35% increase (from 69.3 to 93.5%) (p-value < 0.001) in nutritional knowledge on the correct daily frequency of vegetables and fruit consumption, and physical activity knowledge improved by 63% after the project (from 54.5 to 88.9%) (p-value < 0.001);

the reported behavioral change regarding the daily frequency of vegetables and/or fruit consumption and physical activity improved from 36.6 to 51.7% (p-value < 0.001) and from 47.2 to 62.6% (p-value < 0.001), respectively.

4. Discussion

In this study, we investigated the effects of the integrated and comprehensive project "*Smuovi la Salute*", aiming to prevent and manage overweight and obesity among migrant children and/or children of low-SES. According to our knowledge, this was the first systematic approach targeted at this population reported in the literature.

Phases 1 and 2 were preparatory to develop the tools that could help reduce cultural and socioeconomic inequalities in the target population. In Phase 3, we tried to promote transcultural nutrition and healthy lifestyle at different levels.

(i) At the societal level, we adopted a universal approach with the aim of not discriminating against children living in families experiencing socioeconomic deprivation and/or of migrant background. Telematic tools such as apps, online recipe books, and video conferences have been widely used and appreciated, as demonstrated by the number of download and views and by the satisfaction questionnaires. We developed the tools only in Italian language, to encourage integration, and data from the app download (66% Italians, 34% migrant family) reflect the percentage of population in our suburbs with a high density of migrants, confirming that language was not a barrier. In the literature, a few universal approaches aiming to reduce inequalities were reported, and studies have produced poor results [18]. The use of technology-based interventions, although not effective sustaining a reduction in weight, has been shown to be beneficial in short-term weight loss interventions [27,28], and according to our knowledge, this is the first project that produced interactive digital material on transcultural healthy lifestyles.

(ii) Regarding targeted community approaches, working with schools has been a promising strategy. In primary schools, we achieved an increased knowledge and reported change in behavior regarding healthy eating and physical activity. An "ad hoc" study with longitudinal follow up is needed to determine whether this improved knowledge can lead to long-term behavioral improvement. Our finding was also confirmed by a study set in Germany, of third and fourth grade classes with a high proportion of migrant schoolchildren [22]. Three months after the three-day practical nutrition lessons, the group had improved their knowledge, but without changes in nutrition-related behavior and attitudes. Two additional exercise lessons weekly positively affected parameters of fitness and motor skills after 6 months [29]. In boys of Mexican origin, curriculum-based interventions in the community and schools were effective in preventing BMI growth after 1 year [23].

On the other hand, a targeted community approach, culturally tailored towards ethnic communities, has not led to good results in our project. When we understood that in our territory the foreign communities do not represent structured realities with a referent to contact as a moderator to promote the events and the tools developed, we turned to religious leaders. However, the importance of promoting healthy transcultural nutrition was not a priority for religious groups. The involvement of cultural mediators could be another promising strategy, as reported by studies conducted with community-based groups conducting health education and counselling intervention among low-income women participants or with female majority [30,31]. These interventions have been shown to be effective in promoting weight loss at 4 months post-intervention, although this was not consistent over time. In our study, the cultural mediators we trained reported feeling more like translators than peers with a mission to improve lifestyle. Therefore, their effect was lower than expected.

(iii). With regard to the individual approach, the tools and activities offered by the program were meant to be used in primary care settings for overweight prevention, and on second and third level, as a support for obesity treatment. The majority of pediatricians were not effective in promoting and introducing young patients to UISP physical

activities or in encouraging them to download the book's PDF. Having a self-employed role, they probably had to be motivated with some form of incentive to optimize their project collaboration. In the literature, there is evidence of the efficacy in the short-term period (3 to 6 months) of primary care-delivered tailored weight loss programs among deprived groups [32]. Jeffery R.W. et al. demonstrated that after 1 year, their intervention prevented weight gain with age among high-income women; even if after 3 years, there was no significant effect on weight [33,34]. In our second- and third-level centers, the drop out at 6 months of follow up was 62%, and was different from the one of the Caucasian or non-disadvantaged obese population (28–37% of patients attending the three obesity clinic). A greater proportion of migrant children (9 out of 14) did the follow up compared to Italian children (21 of 81), probably because they perceived the project as inclusive. Due to the low number of patients enrolled for each ethnic group, we were unable to build peer groups to be supported by trained cultural mediators as reported for chronic diseases in a previous study [19]. Patients enrolled by the clinics, for 6–12 months, were able to increase their vegetable and fruit consumption (+14%) but not to improve their level of physical activity. In fact, we observed a high rate of drop out during the UISP activities. We identified several possible explanations of this behavior: providing no-cost activities helped in removing barriers and to facilitate the participation of low-SES families; however, on the other hand, participants did not feel the commitment and were often deciding not to come without any advice. In addition, participants were living in different parts of the city or different villages, and it was difficult to organize an activity in a place which was easily accessible for everyone. However, a greater proportion of low-SES migrant children attended the activity sessions compared with low-SES Italian children, confirming that activities organized for free, by previously trained sport operators, facilitated the participation of immigrants.

Overweight children and adolescents from our cohort decreased their BMI z-score by -0.17 ± 0.63; however, this reduction was not significant, probably due to the low sample size of subjects that had at least two visits to the Pediatric Obesity Care (30 subjects), and the consequent impossibility to analyze larger data due to the drop out. However, the BMI z-score change we observed in our group was of the same magnitude as that of structured and intensive multidisciplinary group interventions [35]. In addition, it is slightly better than the mean change of -0.10 kg/m^2 (95% CI -0.14 to -0.05) and -1.53 kg/m^2 (95% CI -2.67 to -0.39) reported, respectively, in overweight or obese children aged 6–12 and 13–18 years of the last Cochrane review [36].

In addition to the concrete results of this project, we understand that our study has some limits: i. low-SES families were identified based on their educational level and not based on family income, which we could not evaluate; ii. the lack of randomization, due to the number of patients that accepted to be enrolled, did not allow us to obtain an adequate sample size to demonstrate a significant variation in lifestyle habits; iii. the follow up for the participants enrolled in schools was relatively short, mainly because of the interruptions of all the activities due to the SARS-COV2 pandemic; iv. due to the action–research design of the study, different populations and outcomes were analyzed, without a priori power size calculation; v. lifestyle behaviors were self-reported without other means to verify. However, we also believe that our study has big potential, supported by strong preliminary results, and can inspire future interventions: i. according to our knowledge, this is the first study reported in the literature regarding obesity clinic which has offered specific tools for obese patients with a migrant background or at a socioeconomic disadvantage. Migrant families appeared more engaged in physical activities and clinical follow up proposed by this project, than Italians of low SES. As reported in the literature, the treatment of childhood obesity remains largely ineffective. Most obesity programs are only successful in highly motivated families that are aware and willing to change their lifestyle and without severe psychosocial problems [37]; ii. the multidimensional approach of the project is, with the involvement of professionals in the health, telematic, social and sport fields, in our experience, a winning approach; iii the large pediatric population involved in

the study; iv. the transferability of the tools developed from the clinical groups to the non-clinical population.

5. Conclusions

In conclusion, for the first time in the literature to the best of our knowledge, we reported the results of a comprehensive and integrated nutritional education project (*Smuovi la Salute*) for overweight and obese children of migrant background or at socioeconomic disadvantage. This study supports the importance of the use of telematics tools (an app, online transcultural recipe book, video-lessons and video-laboratory were developed and implemented) for promoting healthy lifestyles among the general population. Targeted community approaches in schools in suburbs with a high density of migrants, using a brief, low-intensity, nutrition education course as a method to improve students' knowledge, and offering students after-school sport or physical activities, is feasible and effective. The video-laboratory has the advantage of being easily transferable to other schools. It would be interesting to also evaluate, in addition to the students' nutritional education, the potential changes in their lifestyle and family habits. At the individual level, the comprehensive and integrated management of obese patients remains mostly ineffective; therefore, preventive measures are crucial and by using all the tools we developed, designing new tailored strategies of primary care engagement might help enhance the intervention.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13103634/s1, Figure S1: the "transcultural" food pyramid; Figure S2: The cookbook: healthy cooking within everyone's reach; Figure S3: The "Smuovi la Salute" brochure with the project details.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was designed and conducted to ensure compliance with good-clinical practice principles and procedures and adhere to Italian laws. Ethical review and approval were waived for this study, as this study was considered as an effort to organize the existing clinical investigations and did not involve new procedures or tests for the patient.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are not publicly available because they contain information that could compromise the privacy of research participants, but are available from the corresponding author upon reasonable request.

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Is High Milk Intake Good for Children's Health? A National Population-Based Observational Cohort Study

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Abstract: Milk is widely considered as a beneficial product for growing children. This study was designed to describe the milk consumption status of Korean children aged 30–36 months and to investigate its association with the risk of obesity and iron deficiency anemia (IDA). This nationwide administrative study used data from the Korean national health insurance system and child health screening examinations for children born in 2008 and 2009. In total, 425,583 children were included, and they were divided into three groups based on daily milk consumption: low milk group (do not drink or drink <200 mL milk per day, n = 139,659), reference group (drink 200–499 mL milk per day, n = 255,670), and high milk group (drink ≥500 mL milk per day, n = 30,254). After adjusting variable confounding factors, the consumption of a large amount of milk of ≥500 mL per day at the age of 30–36 months was associated with an increased risk of obesity at the age of 42–72 months and IDA after the age of 30 months. These results may provide partial evidence for dietary guidelines for milk consumption in children that are conducive to health.

Keywords: milk; body weight; nutritional state; iron deficiency anemia; children

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1. Introduction

Appropriate linear growth and weight gain are the most crucial issues among healthy children and are related to several factors, such as nutritional status, physical development, calorie intake from diverse types and amounts of food, and level of physical activity [1]. In particular, milk is widely considered as a beneficial product for growing children because it is a complete source of energy and is the richest and the most inexpensive source of high nutritional quality protein, calcium, phosphorus, and vitamin A [2,3]. Consequently, nutritional guidelines in most countries recommend daily milk consumption as a component of a healthy diet [4]. For instance, in the United States (US), the national dietary guidelines recommend amounts of dairy in 2, 2-1/2, and 3 cup equivalents per day for children aged 2–3, 4–8, and 9–18 years, respectively [5]. In China, the Chinese Dietary Guidelines 2016 suggest that school-age children should drink 300 mL of milk per day [1,6]. In Korea, the 2020 Dietary Reference Intakes for Koreans recommends two cups (400 mL) of milk for adolescents and one cup (200 mL) of milk for adults per day [7].

As growth issues such as obesity and stunting have become more of a concern, extensive research has been conducted on milk intake and height or body weight [1], and the majority of these studies have agreed that milk contributes positive benefits to growth in height for children [8–14]. However, the association between milk and body weight or body mass index (BMI) remains under debate due to inconsistent findings [15–23].

In fact, the basis for the dietary guidelines for milk is not sufficient, and data on Korean children's actual daily milk consumption have remained scarce. In Korea, after the period

of bottle-feeding, the amount of milk consumed significantly decreases, or conversely, if the time to stop bottle-feeding has elapsed and the child still depends on the bottle, the majority of calories per day tend to be consumed through milk. Several observational studies have suggested associations between prolonged bottle-feeding and excessive milk intake [24–26]. In this case, an imbalance of nutrients, especially insufficient intake of nutrients that milk does not contain or lacks, may occur. For instance, the finding that consumption of cow's milk by infants and toddlers has adverse effects on iron stores has been documented in many studies [27]. The prevalence of iron deficiency anemia (IDA), which can significantly affect neurodevelopment, has been reported to increase in children aged 1 to 3 years, who have high iron requirements due to rapid growth [28,29]. When a direct relationship exists between milk intake and the growth or nutritional status of children, more specific recommendations on milk intake are essential.

This study was designed to describe the milk consumption status of Korean children aged 30–36 months (generally, after the cessation of bottle-feeding in Korea) and to investigate its association with the BMI z-scores of children aged 42–72 months. Moreover, by analyzing the relationship between milk intake and the risk of IDA, we attempt to obtain more information with regard to milk intake and nutritional status. We anticipate that the findings of this study would help set an appropriate range for daily milk consumption conducive to the health of Korean children.

2. Materials and Methods

2.1. Database: National Investigation of Birth Cohort in Korea Study 2008 (NICKs-2008)

The NICKs-2008, which integrates data from the Korean National Health Insurance System (NHIS) and the National Health Screening Program for Infants and Children (NHSPIC), consists of children born in 2008 (n = 469,248) and 2009 (n = 448,459) in the Republic of Korea.

The NHIS accumulates nationwide healthcare data that contain health records, including sociodemographic variables (age, sex, residential area, and income), healthcare utilization (International Classification of Diseases, 10th revision (ICD-10 codes)), procedure codes, drug classification codes, length of stay, and treatment costs for the entire nation. The NHSPIC conducts seven surveys among children, from the age of 4 to 72 months, comprising a general health questionnaire, developmental screening, anthropometric examination, physical examination, assessment of oral health, and age-specific anticipatory guidance. Detailed information on this study has been provided elsewhere [30].

2.2. Study Setting

This study was designed using the data from the NICKs-2008 database, which consisted of 917,707 subjects—all children born in 2008 and 2009 in Korea. Among the children who underwent the 4th round of NHSPIC, from the age of 30 to 36 months, children whose weight and height were properly recorded were included in the analysis (n = 425,583), excluding those who met the exclusion criteria. Exposure was defined as the consumption of milk per day, which was recorded from the results of the 4th round of the NHSPIC program. The primary outcome was the association between milk consumption and obesity at the age from 42 to 72 months. The association between milk consumption and IDA was also analyzed. The study protocol was reviewed and approved by the Institutional Review Board of the Korea National Institute for Bioethics Policy (P01-201603-21-005).

2.3. Study Population

All children who were born during 2008–2009 and who participated in the health screening program were identified (n = 917,707). We included children (1) who had details available regarding birth weight information in any round of the NHSPIC, (2) who underwent the 4th round of NHSPIC, and (3) whose weight and height were properly recorded in the 4th round of NHSPIC (n = 533,533). Children that were excluded included those (1) who had been admitted to an intensive care unit for more than 5 days within 3 months

after birth, (2) who died, (3) who were born in multiple births, (4) who were diagnosed with at least one of the ICD-10 codes (CXX (malignant neoplasms), K50.X (Crohn's disease), K51.X (ulcerative colitis), and QXX (congenital malformations, deformations, and chromosomal abnormalities)), or (5) who underwent the 4th round of NHSPIC but had no details available regarding the questions related to milk consumption. In total, 425,583 children were included in this study, and they were divided into three groups based on daily milk consumption (Figure 1).

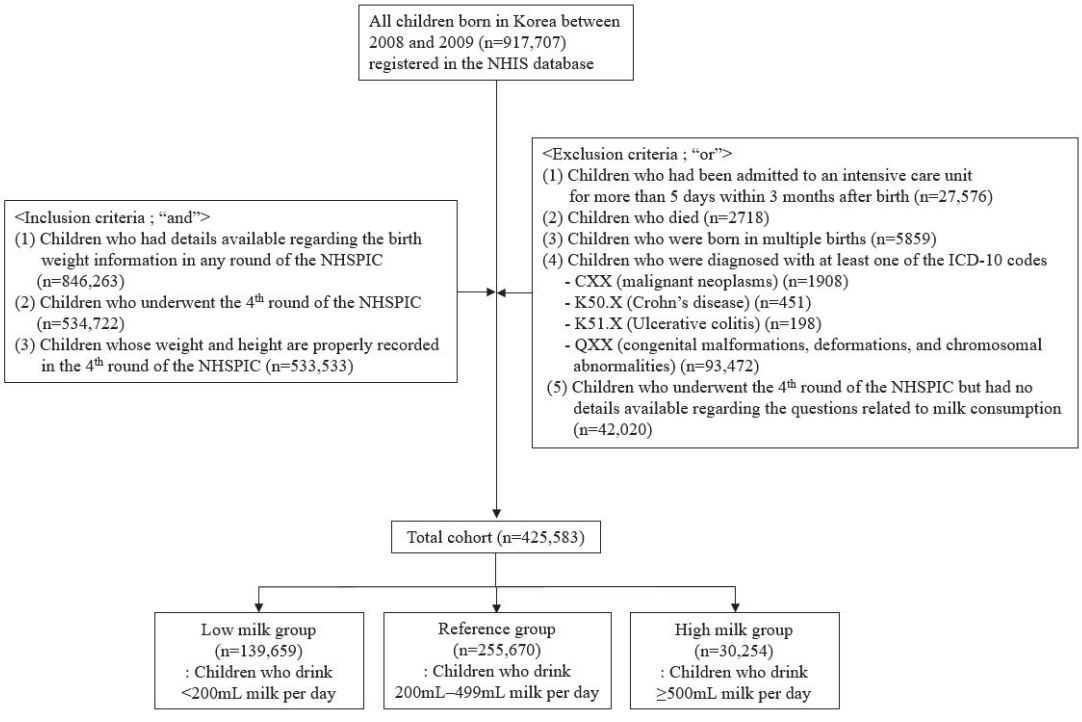

Figure 1. Enrollment diagram and grouping of the study population.

2.4. Exposure: Daily Milk Consumption

The exposure of interest was consumption of milk per day in children aged 30–36 months, which was recorded from the results of the 4th round of the NHSPIC program. Parents answered the following question: "How much milk does your child drink per day?" with the following possible answers: (1) Do not drink, (2) <200 mL, (3) 200–499 mL, (4) 500–999 mL, and (5) >1000 mL. Based on the answers, the children were divided into the following three groups: low milk group—children who do not drink milk or drink <200 mL milk per day (n = 139,659), reference group—children who drink 200–499 mL milk per day (n = 255,670), and high milk group—children who drink ≥500 mL milk per day (n = 30,254). In this study, milk refers to any plain whole, low-fat, and skim cow's milk, not other types of milk such as goat milk or soy milk.

2.5. Outcomes: Obesity and IDA

The primary outcome was the association between milk consumption and obesity at the age of 42 to 72 months. The early adiposity rebound (AR) period (the age of 4–7 years), known as the risk period for obesity, has been determined to have a particularly important relationship with the prevalence of childhood obesity [31,32]. Obesity was defined as BMI

z-score of ≥1.64 (95th percentile) [33,34] based on the BMI recorded at the last round of the 5th–7th NHSPIC [35–37]. BMI was calculated as weight in kilograms divided by height in meters squared.

The additional outcome was the association between milk consumption and IDA. We defined IDA using at least one diagnosis of the ICD-10 code D50.8 (other IDAs) or D50.9 (IDA, unspecified), along with prescription of iron supplements using drug classification codes 322 (Supplementary Table S1). Prescription at least once from the 4th round of NHSPIC to December 2017 was included.

2.6. Statistical Analysis

Data were analyzed using descriptive statistics and presented as counts (percentages) or mean (SD) values. For the analysis of the risk of obesity and IDA based on the milk consumption status in children aged 30–36 months, the relative risk (RR) and 95% confidence intervals (CIs) were estimated using generalized estimated equations, with the GENMOD procedure with log link function and all interactions of these variables. All RRs were presented with 95% CIs, and adjusted RRs (aRRs) in the multivariable analysis considered sex, birth residence, income quintile, birth year, prematurity, birth weight (continuous variable), type of milk feeding from age 4 to 6 months, and amounts of sugar-containing beverages (SCBs) from age 18 to 24 months. Probability (p) values of ≤0.05 were considered to be statistically significant. All statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Daily Milk Consumption during Childhood in Korea and Comparison of Baseline Characteristics between Study Groups

We identified 425,583 children who were eligible for this study (Figure 1). There were 139,659 (32.8%), 255,670 (60.1%), and 30,254 (7.1%) children in the low milk group, reference group, and high milk group, respectively. More than half of Korean children were reported to drink 200–499 mL milk per day. Even in the low milk group, 35,405 (8.3%) children did not drink milk at all.

Table 1 summarizes the baseline sociodemographic characteristics of the three groups. The distributions of sex, birth residence, income quintile, birth year, prematurity, birth weight, type of milk feeding from the age of 4–6 months, and amounts of SCB from the age of 18–24 months were similar across the three groups.

Table 1. Baseline sociodemographic characteristics of children in the low milk, reference, and high milk group.

Parameters	*n* (%)			
	Total Cohort (*n* = 425,583)	**Low Milk Group [a] (*n* = 139,659)**	**Reference Group [b] (*n* = 255,670)**	**High Milk Group [c] (*n* = 30,254)**
Sex				
Male	214,894 (50.5)	67,396 (48.3)	130,930 (51.2)	16,568 (54.8)
Female	210,689 (49.5)	72,263 (51.7)	124,740 (48.8)	13,686 (45.2)
Birth residence [d]				
Seoul	106,558 (25.3)	36,840 (26.6)	62,124 (24.5)	7594 (25.4)
Metropolitan	96,609 (22.9)	29,145 (21.1)	60,481 (23.9)	6983 (23.4)
City	167,318 (39.7)	55,173 (39.9)	100,470 (39.7)	11,675 (39.1)
Rural	51,124 (12.1)	17,231 (12.5)	30,263 (11.9)	3630 (12.1)

Table 1. *Cont.*

Parameters	*n* (%)			
	Total Cohort (*n* = 425,583)	**Low Milk Group [a] (*n* = 139,659)**	**Reference Group [b] (*n* = 255,670)**	**High Milk Group [c] (*n* = 30,254)**
Income quintile [e]				
1 (Lowest)	33,429 (8.2)	10,294 (7.7)	20,421 (8.3)	2714 (9.3)
2	61,501 (15.0)	19,274 (14.3)	37,363 (15.2)	4864 (16.7)
3	112,116 (27.3)	35,996 (26.8)	68,201 (27.7)	7919 (27.2)
4	133,834 (32.6)	44,993 (33.5)	79,946 (32.4)	8895 (30.6)
5 (Highest)	69,206 (16.9)	23,935 (17.8)	40,570 (16.5)	4701 (16.2)
Birth year of child				
2008	203,375 (47.8)	65,945 (47.2)	122,655 (48.0)	14,775 (48.8)
2009	222,208 (52.2)	73,714 (52.8)	133,015 (52.0)	15,479 (51.2)
Prematurity [f]				
No	408,847 (96.1)	134,384 (96.3)	245,508 (96.1)	28,955 (95.8)
Yes	16,445 (3.9)	5201 (3.7)	9972 (3.9)	1272 (4.2)
Birth weight, mean(SD), kg	3.22 (0.41)	3.22 (0.41)	3.22 (0.41)	3.21 (0.42)
Type of milk feeding from age 4 to 6 months [g]				
Breast	115,785 (45.5)	39,945 (47.3)	69,906 (45.6)	5934 (35.3)
Bottle	87,885 (34.5)	27,858 (33.0)	52,681 (34.3)	7346 (43.7)
Mixed [h]	50,132 (19.7)	16,285 (19.3)	30,383 (19.8)	3464 (20.6)
Special formula	889 (0.3)	312 (0.4)	494 (0.3)	83 (0.5)
Amounts of SCB from age 18 to 24 months [i]				
<200 mL/d	263,760 (93.3)	87,068 (93.7)	159,808 (93.3)	16,884 (91.8)
200 to 499 mL/d	17,315 (6.1)	5423 (5.8)	10,607 (6.2)	1285 (7.0)
≥500 mL/d	1531 (0.5)	416 (0.5)	884 (0.5)	231 (1.3)

Data are presented as number (percent) or mean (SD). Abbreviation: SCB, sugar-containing beverages. [a] The group of children who drink <200 mL milk per day. [b] The group of children who drink 200–499 mL milk per day. [c] The group of children who drink ≥500 mL milk per day. [d] Residential status was classified as Seoul, metropolitan, urban, or rural. Metropolitan areas were defined as five metropolitan cities (Busan, Incheon, Gwangju, Daejeon, and Ulsan), urban areas as cities, and rural areas as non-city areas. Of all the participants, information was missing for 1270 in low milk group, 2332 in reference group, and 372 in high milk group. [e] Income was categorized into quintiles of average neighborhood income on the index date. Of all the participants, information was missing for 5167 in low milk group, 9169 in reference group, and 1161 in high milk group. [f] Of all the participants, information was missing for 74 in low milk group, 190 in reference group, and 27 in high milk group. [g] Question in the first round of the NHSPIC program: "What do you usually feed your child with?". Of all the participants, information was missing for 55,259 in low milk group, 102,206 in reference group, and 13,427 in high milk group. [h] Feeding with breast milk, animal milk, and/or formula milk. [i] Parents answered the following question in the third round of the NHSPIC program: "How much fruit juices or sugary beverages does your child drink per day?" with the following possible answers: (1) <200 mL, (2) 200–499 mL, and (3) ≥500 mL. Of all the participants, information was missing for 46,752 in low milk group, 84,371 in reference group, and 11,854 in high milk group.

3.2. Primary Outcome: The Association between Milk Consumption and Obesity in Children

We examined the association between milk consumption and obesity using modified Poisson regression analysis. In the total cohort, we included subjects whose BMI was recorded at least once among the 5th (42–48 months), 6th (54–60 months), and 7th (66–71 months) rounds of NHSPIC (*n* = 377,592). Among the recorded BMI of the three rounds, the BMI of the last round of NHSPIC was analyzed. Obesity was defined as BMI z-score of ≥1.64. In the low milk group, 10,606 (8.54%) children were found to be obese; in

the reference group, 23,138 (10.19%) children were obese; and in the high milk group, 3270 (12.39%) children were determined to be obese ($p < 0.001$).

Table 2 shows the adiposity outcomes at the age of 42–72 months based on the quantity of milk intake at the age of 30–36 months. After adjusting for sex, birth residence, income quintile, birth year, prematurity, birth weight, type of milk feeding from the age of 4 to 6 months, amounts of SCB from the age of 18 to 24 months (Table 1), and obesity at the 4th round of NHSPIC, a positive correlation was observed between milk consumption and obesity. In the adjusted analysis, the aRRs for obesity occurrence were 0.856 (95% CI, 0.835–0.878) for the low milk group and 1.120 (95% CI, 1.077–1.165) for the high milk group. Compared with the reference group, the risk of obesity was significantly greater for children who drank ≥500 mL milk per day.

Table 2. The association between the milk consumption and obesity in children.

Milk Consumption (mL/d)	*n* = 377,592 [a]		RR (95% CI)	
	Subjects, *n*	Obesity [b], *n* (%)	Unadjusted	Adjusted [c]
Low Milk Group [d]	124,236	10,606 (8.54)	**0.820 (0.805 to 0.835)**	**0.856 (0.835 to 0.878)**
Reference Group [e]	226,964	23,138 (10.19)	Ref	Ref
High Milk Group [f]	26,392	3270 (12.39)	**1.249 (1.214 to 1.284)**	**1.120 (1.077 to 1.165)**

Abbreviation: RR, relative risk; CI, confidence interval; BMI, body mass index. [a] Subjects who recorded BMI at least once between the 5th to 7th rounds of NHSPIC were included. [b] Obesity was defined as BMI z-score ≥ 1.64, based on the BMI recorded at the last round of the 5th to 7th NHSPIC. [c] Adjusted for sociodemographic characteristics (Table 1), and obesity at the 4th round of NHSPIC, as recorded in the database. [d] The group of children who drink <200 mL milk per day. [e] The group of children who drink 200–499 mL milk per day. [f] The group of children who drink ≥500 mL milk per day. Bold values indicate $p < 0.05$.

3.3. Secondary Outcome: The Association between Milk Consumption and IDA in Children

We next analyzed the association between milk consumption and IDA in the total cohort. As per our findings, we observed that 1.86% of children in the reference and low milk groups and 2.06% of children in the high milk group were diagnosed with IDA and prescribed iron, respectively ($p = 0.047$). After adjusting for sociodemographic characteristics (Table 1), the risk of IDA was significantly increased in children who drank ≥500 mL milk per day (aRR, 1.079; 95% CI, 1.000–1.176) compared with the reference group (Table 3). No statistical significance was detected between the low milk group and the reference group.

Table 3. The association between the milk consumption and IDA[a] in children.

Milk Consumption (mL/d)	*n* = 455,160		RR (95% CI)	
	Subjects, *n*	IDA, *n* (%)	Unadjusted	Adjusted [b]
Low Milk Group [c]	139,659	2593 (1.86)	1.001 (0.954 to 1.050)	1.016 (0.967 to 1.067)
Reference Group [d]	255,670	4744 (1.86)	Ref	Ref
High Milk Group [e]	30,254	622 (2.06)	**1.108 (1.019 to 1.205)**	**1.079 (1.000 to 1.176)**

Abbreviations: RR, relative risk; CI, confidence interval; BMI, body mass index. [a] IDA was defined with at least one diagnosis of ICD-10 code D50.8 or, D50.9 along with prescription of iron using drug classification codes 322, at least once from the 4th round of NHSPIC to December 2017. [b] Adjusted for sociodemographic characteristics (Table 1), as recorded in the database. [c] The group of children who drink <200 mL milk per day. [d] The group of children who drink 200–499 mL milk per day. [e] The group of children who drink ≥500 mL milk per day. Bold values indicate $p < 0.05$.

4. Discussion

This study was conducted to demonstrate the current status of daily milk consumption in children aged 30–36 months in Korea and to identify its association with health conditions such as obesity and IDA in preschool-aged children. We observed that 60.1% of Korean children drank 200–499 mL milk per day, similar to the recommendations, whereas 7.1% drank >500 mL milk per day. Our results confirmed that children aged between 30 and 36 months who consumed >500 mL milk per day were at an increased risk of obesity at the age of 42–72 months, controlling for various confounding variables. The analysis with regard to overweight (BMI z-score $\geq$ 1.03) also revealed the same significant trend as that of obesity (Supplementary Table S2).

Our finding was consistent with that of previous studies. A US study of the 1999–2004 National Health and Nutrition Examination Surveys suggested consistent positive associations between milk intake and BMI among children aged 2–4 years old [19]. Another longitudinal cohort study of 12,829 US children aged 9–14 years reported that children who drank more than three servings of milk daily had an increased BMI [23]. Mark et al. [20] demonstrated that the volume of milk consumed was related to higher weight status and taller stature. Several potential mechanisms have been speculated in previous studies to explain the relationship between milk intake and obesity. Two studies suggested that the surplus energy provided by milk was stored as fat, thus leading to weight gain by increasing the total calorie consumption [21,23]. Another study speculated that milk has a unique biological effect of weight gain, considering that cow's milk induces rapid growth in calves in terms of skeletal size and body weight [19]. There is also the 'early protein hypothesis' that high protein intake in infancy increases the risk of obesity [38,39]. A systematic literature review study of 34 articles concluded that high protein intake during infancy and early childhood was associated with an increased risk of obesity later in life [40]. However, there is no clear information about which components of milk have the potential to influence BMI; thus, further research is needed on this issue. However, it is true that milk is the only food that is actually produced by mammals for the purpose of childhood consumption [19].

This study was significant, considering that the analyzed age of obesity was 42–72 months, which is the AR period. BMI increases rapidly during the first year of life and then gradually decreases, reaching a minimum at 4–7 years of age, before increasing again during adolescence [31]. That point of minimal BMI has been termed the AR, and early AR has been identified as an indicator predicting later obesity [32]. Similarly, it is conceivable that becoming obese at the age of 4–7 years increases the risk of obesity in adolescence or early adulthood. Therefore, if a large amount of milk consumption is associated with a risk of obesity during this period, this also indicates the possibility of continuing obesity in the future.

We also analyzed additional outcomes about the relationship between milk intake and the risk of IDA by obtaining more information about milk and nutritional status in children. As per our results, it was determined that the risk of IDA was significantly increased in children who drank $\geq$500 mL milk per day compared with the reference group, and this finding was consistent with previous studies. Iron deficiency and IDA are important health issues in young children, given that they are associated with a negative impact on neurodevelopment [41]. Although the importance and prevention of IDA have focused on the first 12 months of life, it is also necessary to focus on toddlers, as the prevalence of IDA between 1 and 3 years is 15%, compared to 3% among those aged 1–2 years [28,29,42]. Some of the previously suggested mechanisms regarding milk intake and the occurrence of IDA include the low iron content of cow's milk, low iron bioavailability for absorption, inhibition of iron absorption due to high amounts of the calcium and casein provided by milk, and occult intestinal blood loss [3]. Especially at the age of toddlers, the preference for food and beverages increases, often resulting in the refusal of healthy iron-rich foods and replacement with significant amounts of milk and fruit juices [41]. For this reason, the Centers for Disease Control and Prevention in the USA limited milk consumption to

<24 oz per day in the second year of life [43], with some clinicians suggesting a stricter limit of 16 oz (approximately 473 mL) per day [29]. Furthermore, our experience in clinic shows that the majority of children who consume a large amount of milk at the age of 30–36 months are still dependent on bottle-feeding. There are several studies supporting associations between bottle-feeding beyond 15 to 18 months of age, excessive milk intake, and iron deficiency [24–26].

The strength of our study is the large sample size that makes our results reliable and generalizable. The study sample included all children born in Korea from 2008 to 2009, making it a nationally representative sample. However, we recognize some limitations of this study. First, as misreporting is common in diet surveys [1], recall bias may exist, as dietary information was self-reported, and it can be difficult for parents to accurately estimate children's milk intake. Second, the questionnaire of the NHSPIC program did not include the consumption of other dairy products and did not distinguish the type of milk, such as whole, low-fat, or skim milk. However, previous studies have shown no relationship between the type of milk consumed and body weight status [18,44], or the association between BMI and dairy products was less consistent than that of milk [19]. Third, we could not adjust for all confounding factors, including physical activity time and energy intake from other foods. Instead, we attempted to adjust for as many confounding factors that could affect obesity as possible, such as prematurity, birth weight, socioeconomic status, feeding type from the age of 4 to 6 months, daily SCB consumption between the age of 18 and 24 months, and body weight status at the age of 30–36 months. In particular, one study demonstrated that children aged 24–47.9 months were more likely to consume SCB with each meal than children of other age groups [2], and much convincing evidence already exists indicating that SCB consumption increases the risk of overweight and obesity [45–48]. In our study, a meaningful result could be that the statistical significance was maintained even after adjusting for daily SCB consumption. Moreover, a significant outcome after adjustment for obesity at the 4th round of NHSPIC could be considered as an important factor. Fourth, the definition of IDA was based on the diagnosis of ICD-10 code along with the prescription of iron supplements. Because the diagnosis or prescription was not made by one person based on the same criteria, this value seems questionable. Further research is required to address these factors.

5. Conclusions

This national study demonstrated the current status of daily milk consumption in children aged 30–36 months in Korea as follows: 24.5% of children drank <200 mL milk per day, 60.1% of children drank 200–499 mL milk per day, 7.1% of children drank >500 mL milk per day, and 8.3% of children did not drink milk at all. Consumption of a large amount of milk of $\geq$500 mL per day at the age of 30–36 months was associated with an increased risk of obesity at the age of 42–72 months and IDA after the age of 30 months. These results indicate that the parents or guardians of children who consume >500 mL milk per day need education with regard to obesity prevention and consuming not only dairy products but also foods rich in iron or other essential nutrients that have low levels in milk. We believe that this study is significant, as it may provide partial evidence for dietary guidelines for milk consumption in children that are conducive to health.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13103494/s1. Table S1: Drug classification codes 322 in NHIS database. Table S2: The association between the milk consumption and overweight in children.

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Protein Quality in Infant Formulas Marketed in Brazil: Assessments on Biodigestibility, Essential Amino Acid Content and Proteins of Biological Importance

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Abstract: Infant formulas, designed to provide similar nutritional composition and performance to human milk, are recommended when breastfeeding is not enough to provide for the nutritional needs of children under 12 months of age. In this context, the present study aimed to assess the protein quality and essential amino acid content of both starting (phase 1) and follow-up (phase 2) formulas from different manufacturers. The chemical amino acid score and protein digestibility corrected by the amino acid score were calculated. The determined protein contents in most formulas were above the maximum limit recommended by FAO and WHO guidelines and at odds with the protein contents declared in the label. All infant formulas contained lactoferrin (0.06 to 0.44 g·100 g^{-1}) and α-lactalbumin (0.02 to 1.34 g·100 g^{-1}) below recommended concentrations, whereas k-casein (8.28 to 12.91 g·100 g^{-1}), α-casein (0.70 to 2.28 g·100 g^{-1}) and β-lactoglobulin (1.32 to 4.19 g·100 g^{-1}) were detected above recommended concentrations. Essential amino acid quantification indicated that threonine, leucine and phenylalanine were the most abundant amino acids found in the investigated infant formulas. In conclusion, infant formulas are still unconforming to nutritional breast milk quality and must be improved in order to follow current global health authority guidelines.

Keywords: breastfeeding; infant formulas; protein quality; whey proteins; caseins; HPLC; amino acid score; amino acid score corrected protein digestibility

1. Introduction

Infant nutrition during the first two years of life is essential for adequate development and crucial for survival and long-term health and well-being. Nourishment deficiencies caused by inadequate nutrition during this development stage may cause immediate damage, increasing infant morbidity and mortality, while also potentiating growth delays and low school achievements and increasing the risks for chronic and degenerative adulthood diseases [1,2]. In this regard, breast milk is the complete and ideal infant nutritional source,

endorsed by health authorities and recommended as the exclusive food source during the first six months of life, aiming at optimal child growth, development, and health. After sixth months, adequate and safe complementary feeding should be given to infants in order to meet their evolving requirements, while breastfeeding should be maintained at least until two years of age [3–6]. Breast-milk substitutes are, however, required when breastfeeding is not possible or recommended or if it does not meet nutritional needs. In this regard, infant formulas (IFs) are the only acceptable milk derivative for infants under one year old, as they can be manipulated to fulfill physicochemical and nutritional characteristics while still maintaining a composition as similar as possible to breast milk [5,6].

IFs are available in both liquid or powdered form and contain proteins isolated from cow milk or other the milk of animal species, as well as from vegetables, in both intact or hydrolyzed forms, although other nutrients may be added in the amounts and ratios recommended for each child age to ensure adequate growth, optimal development and immune and metabolic system maturation [7–9]. Researchers in the infant nutrition field and IFs manufacturers have developed a variety of IFs throughout the last decades, currently available worldwide and developed based on scientific infant nutrition data. IFs are formulated for the first months of life until the introduction of complementary feeding, and from the sixth month onwards, until age one. In addition to traditional formulas, specials IFs can also be formulated to provide specific dietary needs required in special health conditions [3,6].

IFs formulated from cow milk are produced at low-costs and widely marketed, and processed to adjust macro and micronutrient contents to obtain nutritional characteristics as similar as possible to human breast milk, which is safer for neonates or infants under one year old [10]. Cow milk processing aims to reduce protein content to avoid overloading the newborn immature renal tubular systems, mainly through the reduction of casein content and consequent improvement of whey protein: casein ratios in order to enrich IFs with high biological-activity proteins aiming to supply essential amino acids and facilitate milk digestion [1]. Furthermore, the quality and three-dimensional conformation of these proteins are essential to sustain infant safe growth and development, aiming at long-term health, as adequate protein intake during the first two years of life leads to important muscle protein syntheses and linear growth effects, as well as healthy immune and digestive system development and optimal brain development support, including better cognitive evolution [11,12].

Despite the attempts of manufacturers to mimic the composition and/or performance of human milk, it is not yet possible to state that IFs nutrients display the same bioavailability as human milk components since differences between the development and long-term health of infants fed IFs compared to exclusively breastfed individuals are still significant [13]. Furthermore, considering that IFs are the only food source for several neonates and infants, and that protein requirements during initial child developmental stages are the highest, the quality of proteins in marketed IFs should be monitored. In this context, the aim of the present study was to evaluate several IFs brands marketed in Brazil recommended for children from 0 to 6 months of age (starting formulas, phase 1) and for children aged 6 to 12 months (follow-up, phase 2) formulas with regard to protein quality through a combination of different analytical protein determination methods. To this end, total protein content, distribution and profiles and essential amino acid contents were determined and the results were then used to calculate the chemical amino acid score (AAS) and protein digestibility corrected by the amino acid score (PDCAAS).

2. Material and Methods

2.1. Sample Selection

IFs were purchased in commercial establishments located in the metropolitan region of the municipality of Rio de Janeiro, Brazil. All selected IFs are marketed in several commercial establishments, conveniently available to be purchased by consumers in the most populated part of the city. Although no formal inquiry was performed, as the selected

formulas are commonly supplied in the largest supermarkets in town, they are considered a high demand by consumers and were, thus, evaluated in the present study.

All products are registered by the Brazilian regulatory agency ANVISA, marketed as powdered formulas packed in aluminum cans and labeled by each manufacturer. Inclusion criteria consisted of the following: formulas should be prepared exclusively from non-hydrolyzed cow milk proteins, for children aged 0 to 6 months (phase 1 starting IFs) and for children aged 6 to 12 months (phase 2 follow-up IFs). IFs produced using protein sources other than cow milk, such as soy or wheat protein, as well those designed for specific needs, such as lactose-free or hydrolyzed protein conditions, were not selected. A total of ten formulas marketed in Brazil produced by three different manufacturers filled the inclusion criteria. Thus, five phase 1 and five phase 2 formulas, and three distinct batches of each brand were thus selected, totaling thirty samples (N = 30). The three different batches of each brand were identified using the same two capital letters and a number, where the number indicates different batches, from 1 to 3. Manufacturers and brands were not disclosed for ethical reasons, and samples were identified by codes (Supplementary file Table S1)

2.2. Total Protein Contents

Total protein contents were assessed according to the Kjeldahl method proposed by the Association of Official Analytical Chemists [14]. Nitrogenous materials were converted into protein content by employing a 6.25-factor multiplication.

2.3. Protein Fraction Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A protein electrophoretic analysis was carried out in six stages, namely extraction, quantification, preparation and SDS-PAGE resolving followed by gel staining and destaining, and, finally, photo documentation [15]. Protein extraction was performed according to Almeida et al. [16] with modifications. First, proteins were quantified by serial dilution (1:1, 1:10 and 1:100) using Coomassie Blue G-250® at 595 nm and 450 nm using a UV-1800 spectrophotometer (Shimadzu®, KYO, JPN). After establishing the appropriate dilution factor, the samples were diluted in the staining solution (4% SDS, 0.5 M Tris-HCl pH 6.8, 50 mM DTT, 20% glycerol and a pinch of bromophenol blue) and a total of 20 µL of each sample (5 mg·mL^{-1} of protein) were loaded in each well of 4% and 12% stacking and resolution acrylamide gels, respectively, and the runs were performed at 200 V and 25 mA for about 2 h. Apparent molecular weights were estimated using electrophoresis molecular marker weights (Precision Plus ProteinTM Standards, Bio-Rad) applied to the central well. After the runs, the gels were stained with Coomassie Blue G-250 for 24 h, destained with 20% acetic acid, 20% methanol, and 60% distilled water to visualize the protein bands and photo documented using a Gel Doc XR + Gel Documentation System. The relative abundance of the gel bands was estimated by TotalLab Quant® software (TotalLab Ltd., Newcastle-Upon-Tyne, UK), through staining intensity and thickness.

2.4. High-Performance Liquid Chromatography (HPLC)

The identification and quantification of the main target proteins in the investigated IFs, α-lactalbumin (α-LA), β-lactoglobulin (β-LG), K-casein (K-CN), α-casein (α-CN), β-casein (β-CN) and lactoferrin (Lf), were performed by HPLC, following protein extraction according to Bobe et al. [17]. Briefly, about 10 g of each IFs were diluted in 100 mL of Milli-Q® water (Merck Millipore, MA, USA) and 500 µL were mixed to 500 µL of a solution containing 0.1 M bi-tris-HCL/buffer (pH 6.8), 6 M guanidine hydrochloride, 5.37 mM sodium citrate and 19.5 mM d-dithiothreitol (pH 7.0). The resulting suspensions were incubated for 1 h at room temperature and then centrifuged for 20 min at 20,000× *g* at 4 °C. The small lipid layer formed during this step was removed, and the remaining solubilized solution was diluted 1: 3 (v:v) with 4.5 M guanidine hydrochloride and solvent A, and maintained at −20 °C until analysis.

The HPLC system comprised a quaternary pump LC-20AD (Shimadzu®, KYO, JPN), coupled to an analytical column C18 (250 mm × 4.6 mm, I.D., Kromasil®), and a photodiode array detector model SPD-20A (Shimadzu®) managed by the LabSolutions System software (Shimadzu®), employed in two distinct chromatographic conditions: (*i*) K-CN, α-CN, α-La, β-CN and β-Lg separation and identification according to Bonfatti et al. [18] at 214 nm employing an elution at a flow rate set at 0.5 mL·min^{-1} using a 0.1% trifluoroacetic acid (TFA) in water, and 0.1% TFA in acetonitrile mixture. An injection volume of 20 µL was applied, the total analysis time for each sample was 45 min and the column temperature was maintained at 45 °C; and (*ii*) lactoferrin separation and identification as described by Duchén et al. [19] at 205 nm employing a linear gradient and flow rate of 1 mL·min^{-1} and the previously described mobile phase. An injection volume of 10 µL was applied, the total analysis time for each sample was 25 min and the column temperature was set at 45 °C.

Major bovine milk protein standards were prepared in acetonitrile, water, and TFA at a 100:900:1 (v:v:v) ratio. Calibration curves were constructed by plotting increasing protein standard concentrations, and all samples and standards were run in triplicate.

2.5. Essential Amino Acid Identification

Nine essential amino acids threonine, lysine, histidine, valine, methionine, isoleucine, leucine, phenylalanine and tryptophan were identified and quantified in the IFs samples according to Furota et al. [20], with modifications. Phase 1 and phase 2 IFs samples were suspended in Milli-Q® water (Merck Millipore) and 10 mL of each sample were mixed with 10 mL of 6N HCl and heated in an oven at 110 °C for 24 h. Hydrolyzed samples were then transferred to 50 mL volumetric flasks, made up with Milli-Q® water and triplicate aliquots were filtered through non-sterile 0.22 µm MF-Millipore® hydrophilic membranes (Millipore, MA, USA). The filtered solutions were maintained at −20 °C until analysis.

A Thermo Scientific™ UltiMate™ 3000 RSLC nano System (Thermo Fisher Scientific®, CA, USA) liquid chromatograph equipped with a Corona Ultra Charged Aerosol Detector (CAD) (Corona Veo, Thermo Scientific®) was used. Orthogonal chromatography was performed using cyan columns (150 mm × 4.6 mm, I.D., Phenomenex) connected to a C8 column (150 mm × 4.6 mm, I.D., Phenomenex). Data were acquired with the Chromeleon 7.2 software (Thermo Fisher Scientific®).

Essential amino acids were eluted at a 0.5 mL·min^{-1} flow rate using A 0.1% formic acid (FOA) in water and solvent B 100% acetonitrile (ACN) mixture. The gradient elution (solvent B) was increased from 0% to 25% from 0 to 30 min; 25% to 70% from 31 to 46 min and 70% to 0% from 47 to 60 min. A 20 µL injection volume was applied, the column temperature was maintained at 30 °C and the total analysis time for each sample was 60 min. Amino acid standards were purchased from Sigma (Sigma-Aldrich Co, MO, USA) ranging from 50 to 63 mg·100 mL^{-1}.

2.6. In Vitro Gastrointestinal Digestion Simulation

An in vitro gastrointestinal digestion mimicking physiological conditions during the gastric and intestinal phases was performed according to Oomen et al. [21] and Sagratini et al. [22], with modifications.

2.7. Gastric Phase Digestion (GPD)

In a glass vial, 5 g of each sample were mixed with 2 mL of Milli-Q® water and 2.5 mL of artificial gastric fluid, containing 2.75 g NaCl; 0.27 g NaH_2PO_4; 0.82 g KCl; 0.42 g $CaCl_2$; 0.31 g NH_4Cl; 0.65 g glucose; 0.085 g urea; 3 g mucine; 2.64 g swine gastric pepsin; 1 g bovine albumin and 8.3 mL HCl. After adjusting the volume to 500 mL, the pH was adjusted to 2.0 with 5 M HCl, the vials were sealed with a rubber septum and the atmosphere was charged with N_2. The vials were then transferred to an orbital shaker at 260 rpm and 37 °C for 2 h to complete the gastric phase digestion (GPD), followed by the removal of 5 mL aliquots for further analysis.

2.8. Intestinal Phase Digestion (IPD)

In this stage, 0.9 M $NaHCO_3$ were added to the GPD mixture to adjust the pH to 6.0, followed by 2 mL of an artificial intestinal fluid containing 6.75 g NaCl; 0.517 g KCl; 0.205 g $CaCl_2$; 3.99 g $NaHCO_3$; 0.06 g KH_2PO_4; 0.0375 g $MgCl_2$; 0.1375 g urea; 25 g swine bile; 4 g swine pancreatin; 1.2 g albumin bovine, 0.185 mL HCl and volume adjustment to 500 mL. The glass vials were then resealed under an N_2 atmosphere and maintained in the same conditions as the GDP followed by the collection of 5 mL aliquots for further analysis.

2.9. In Vitro Protein Digestibility (IVPD) Assay

The supernatants collected at the end of representative in vitro digestions (gastric and intestinal) were analyzed regarding nitrogen content employing the Kjeldahl AOAC 930.29 method [14]. The IVPD values were calculated according to the following equation:

$$\% \text{ Digestibility} = \frac{\text{Ns} - \text{Nb}}{\text{Ns} \times 100}$$

where Ns and Nb are the nitrogen content of the samples and blanks, respectively.

2.10. Amino Acid Score (AAS) and Protein Digestibility Corrected Amino Acid Score (PDCAAS)

To calculate the AAS, the essential amino acid concentrations in the IFs were estimated and compared with daily amino acid infant requirements using the amino acid content in breast milk proteins as reference to define amino acid scores for infant foods [23]. The PDCAAS was calculated by multiplying the AAS value of each essential amino acid by the protein digestibility score.

2.11. Statistical Analyses

Significant differences in IFs protein contents were assessed through a One-way analysis of variance (ANOVA) with repeated measures. Differences in protein fractions and amino acids between IFs and their respective batches were evaluated by a two-way analysis of variance (ANOVA) with repeated measures. Differences in phase 1 and phase 2 IFs IVPD, AAS and PDCAAS were estimated by a one-way analysis of variance (ANOVA) with repeated measures. An additional post hoc analysis (Bonferroni correction) was performed when a significant F was found. Results were considered significant when $p < 0.05$. Data were expressed as the means ± standard deviations (SD). All statistical analyses were carried out using the Graphpad Prism software version 5 for Windows® (GraphPad Software, CA, USA). All measures were acquired in triplicate.

3. Results

3.1. Crude Protein Contents

Figure 1 displays the comparison between the total protein content of whole cow milk (CM) and phase 1 and phase 2 IFs. Whole CM total protein content was 32.47 ± 0.33 g·100 g^{-1}, higher than all phase 1 and phase 2 IFs. Phase 2 formulas, termed ME2, DM2 and DA2, contained the highest total protein levels, 22.64 ± 0.56 g·100 g^{-1}, 19.95 ± 0.71 g·100 g^{-1}, 19.33 ± 1.20 g·100 g^{-1}, respectively, when compared to the remaining phase 2 and all phase 1 formulas. NC1, NN1 and ME1 contained the lowest total protein contents, of 9.67 ± 0.03 g·100 g^{-1}, 11.94 ± 0.61 g·100 g^{-1} and 11.81 ± 0.35 g·100 g^{-1}, respectively, compared to the other IFs ($p < 0.05$).

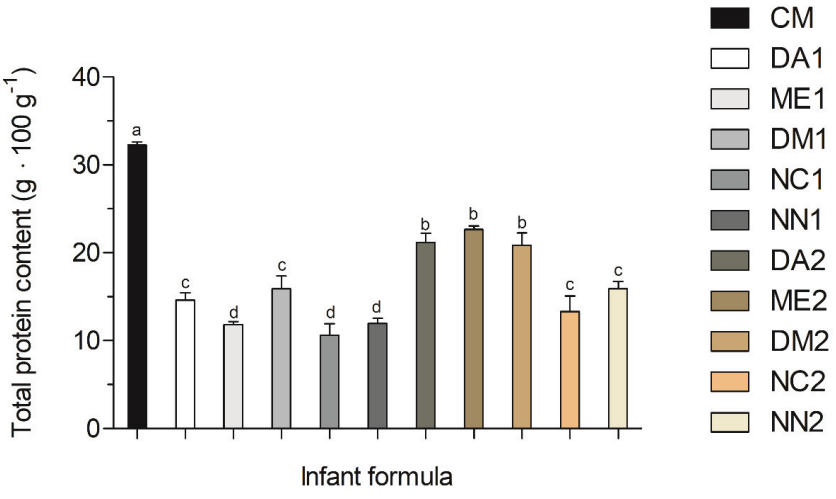

Figure 1. Protein content (g·100 g^{-1}) of cow milk (CM) and phase 1 and phase 2 infant formulas. Formulas from the same brand were coded with the same two capital letters and numbers 1 or 2 to indicate the phase of infant growth. Three samples of each batch were analyzed and the values are expressed as means ± SD (n = 3). Different lowercase letters over the bars indicate differences between brands at a significance level of $p < 0.05$.

3.2. Protein Fractions Analysis

Protein Profiles and Relative Protein Abundance

The SDS-PAGE profiles presented in Figure 2 displayed intense bands with apparent molecular masses similar to lactoferrin (80 kDa), α-casein (23 kDa), β-casein (27 kDa), κ-casein (19 kDa), β-lactoglobulin (18 kDa) and α-lactalbumin (14.2 kDa) in all analyzed IFs. Other bands that could correspond to whey proteins, such as bovine serum albumin (68 kDa), were also identified in the NN, NC and ME formulas.

According to the apparent volume determined by the TotalLab Quant® software, the relative percentage of the protein fractions identified in the analyzed gels, they exhibited different patterns among IFs brands and between batches (Figure 3). Bands identified as caseins ranged from 22.8% to 47%, with 40.6% quantified in DA1; 47.0% in DA2; 29.8% in ME1; 40.7% in ME2; 22.8% in NC1; 27.2% in NC2; 34.4% in NN1 and 35.1% in NN2, while bands identified as whey proteins displayed high variations, ranging from 53.3% to 77.2%, totaling 59.4% in DA1; 53.0% in DA2; 70.2% in ME1; 59.3% in ME2; 77.2% in NC1; 72.8% in NC2; 65.6% in NN1 and 64.9% in NN2. Regarding whey proteins, β-Lg was the most abundant fraction, although numerous other minor proteins were also observed. In addition to proteins in different IFs that were markedly identified in the gels, the TotalLab Quant® software also indirectly revealed other minor proteins.

Major protein fractions were identified and quantified in phase 1 and phase 2 formulas.

The identified CM proteins eluted in the following order: κ-CN, α-La, α-CN, β-CN and β-Lg. The expected separation of major caseins and whey proteins was achieved and their similarity to previously established retention times for major peaks allowed for the identification of protein fractions in every sample, as displayed in a representative chromatogram exhibited in Supplementary file Figure S3. Calibration curves derived from the calculated regression parameters for increasing concentrations of standard individual CM proteins were used to quantify caseins and β-Lactoglobulin (Supplementary file Figure S4). The six fractions were well separated, displaying good peak resolution, i.e., sharpness, and symmetry. Repeatability was observed for multiple measurements for each sample, with a relative standard deviation (RSD) ranging from 0.16 to 0.92% for retention times and from 1.01 to 5.02% for peak areas. Regarding the limit of detection (LOD) and limit of

quantification (LOQ), proteins ranged from 0.01 to 0.15 mg·L^{-1} and 0.28 to 1.02 mg·L^{-1}, respectively, and protein recovery ranged from 88% to 103.4%.

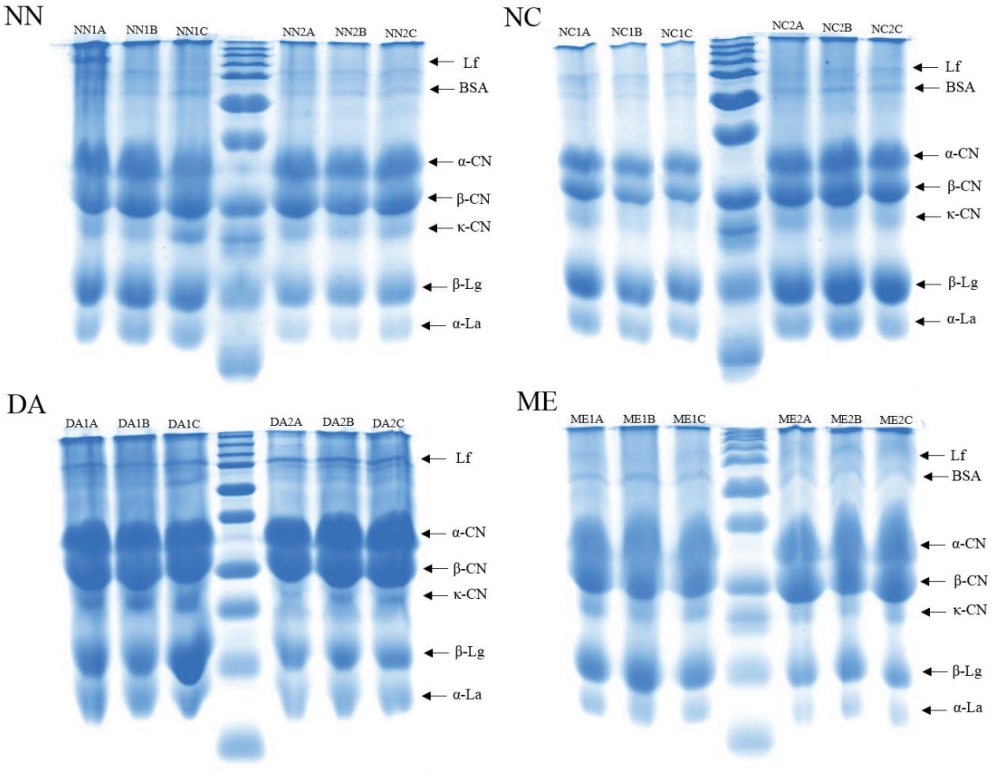

Figure 2. Protein profile of representative infant formulas resolved by SDS-PAGE 12%. Lanes 1, 2 and 3 of each gel represent phase 1 infant formulas (batches A, B and C), the central lanes correspond to Mw markers and lanes 5, 6 and 7 of each gel represent phase 2 infant formulas (batches A, B and C). Lf, lactoferrin; Mw, molecular weight; BSA, bovine serum albumin; α-CN, α-casein; β-CN, β-casein; κ-CN, κ-casein; β-Lg, β-lactoglobulin; α-La, α-lactalbumin. Electrophoresis was run at 200 V and 25 mA for 2 h. Gels were stained with a Coomassie Blue G-250 for 24 h and destained in acetic acid, methanol and distilled water (20:20:60) until the protein bands were visible for photo-documentation.

Figure 3. Approximate percentage of protein fractions identified by SDS-PAGE 12% for each infant formula. Lf, lactoferrin; BSA, bovine serum albumin; α-CN, α-casein; β-CN, β-casein; κ-CN, κ-casein; β-Lg, β-lactoglobulin; α-La, α-lactalbumin. Gels were analyzed by TotalLab Quant® software, in which the apparent volume for each protein band was determined by densitometry. Ratios between whey proteins and caseins were expressed as a percentage using the apparent volume of protein fractions. Unidentified proteins were classified as other proteins.

Average values for major phase 1 and phase 2 IFs protein fractions are presented in Table 1. The detected protein fractions from phase 1 were different from those detected in phase 2 IFs, and protein fractions from distinct formulas from the same phase were also significantly different. IFs with the highest Lf contents comprised DA2, with 3.80 ± 3.01 mg·g^{-1}; ME1, 1.37 ± 0.15 mg·g^{-1}; DM1, 1.37 ± 0.10 mg·g^{-1} and DA1, with 1.13 ± 0.15 mg·g^{-1}. The highest α-CN contents were found in NC1, of 21.17 ± 2.74 mg·g^{-1}, while ME2 contained

18.50 ± 0.81 mg·g^{-1}; ME1, 14.77 ± 2.02 mg·g^{-1} and DA2, 14.71 ± 2.55 mg·g^{-1}. The highest β-CN contents were detected in DM1, at 25.16 ± 3.37 mg·g^{-1}; DM2, at 28.7 ± 3.96 mg·g^{-1} and in NN2, at 22.23 ± 3.62 mg·g^{-1}. The highest κ-CN contents were NN1, 124.50 ± 3.37 mg·g^{-1}; DA1, 106.53 ± 2.72 mg·g^{-1}; ME2, 127.1 ± 3.90 mg·g^{-1}; NN2, 115.53 ± 2.21 mg·g^{-1} and DA2, 107.17 ± 1.14 mg·g^{-1}, and the highest β-Lg contents were observed in NN1, 39.83 ± 1.79 mg·g^{-1}; in NN2, 32.61 ± 3.91 mg·g^{-1} and in ME2, 26.27 ± 3.17 mg·g^{-1}. The highest α-La contents were observed in NC1, 12.10 ± 1.21 mg·g^{-1}; in NN1, 13.23 ± 0.41 mg·g^{-1} and in NN2, 11.97 ± 0.87 mg·g^{-1}.

Table 1. Major protein contents in phase 1 and phase 2 infant formulas.

Infant Formulas	Major Proteins (mg·g^{-1})					
	Lf	**α-CN**	**β-CN**	**κ-CN**	**β-Lg**	**α-La**
CM	0.31 ± 0.02 ^{e}	43.26 ± 0.30 ^{a}	57.95 ± 0.50 ^{a}	125.14 ± 0.39 ^{a}	56.62 ± 0.41 ^{a}	3.53 ± 0.13 ^{c}
Phase 1						
ME1	1.37 ± 0.15 ^{b}	14.77 ± 2.02 ^{d,e}	7.96 ± 0.55 ^{f}	100.50 ± 0.37 ^{d}	22.20 ± 1.35 ^{d,e}	0.33 ± 0.05 ^{f}
NC1	0.57 ± 0.27 ^{d}	21.17 ± 2.74 ^{b}	11.13 ± 0.90 ^{d}	89.90 ± 4.08 ^{e}	19.37 ± 1.76 ^{e}	12.10 ± 1.21 ^{a}
NN1	0.85 ± 0.04 ^{c}	7.60 ± 0.17 ^{g}	11.61 ± 0.01 ^{d}	124.50 ± 3.37 ^{a}	39.83 ± 1.79 ^{b}	13.23 ± 0.41 ^{a}
DM1	1.37 ± 0.10 ^{b}	11.17 ± 0.57 ^{f}	25.16 ± 3.37 ^{b,c}	100.23 ± 0.37 ^{d}	15.77 ± 1.66 ^{f}	3.93 ± 0.89 ^{c,d}
DA1	1.13 ± 0.15 ^{b}	12.57 ± 1.53 ^{e,f}	7.76 ± 0.31 ^{f}	106.53 ± 2.72 ^{c}	14.10 ± 2.30 ^{f}	3.40 ± 1.82 ^{c,d}
Phase 2						
ME2	0.87 ± 0.07 ^{c}	18.50 ± 0.81 ^{c}	5.63 ± 1.06 ^{g}	127.1 ± 3.90 ^{a}	26.27 ± 3.17 ^{c,d}	0.57 ± 0.11 ^{f}
NC2	0.40 ± 0.11 ^{e}	13.37 ± 1.12 ^{d,e}	10.6 ± 0.11 ^{e}	88.17 ± 7.85 ^{e}	17.7 ± 1.75 ^{e,f}	7.57 ± 0.45 ^{b}
NN2	0.67 ± 0.15 ^{c,d}	7.23 ± 0.21 ^{g}	22.23 ± 3.62 ^{c}	115.53 ± 2.21 ^{b}	32.61 ± 3.91 ^{c}	11.97 ± 0.87 ^{a}
DM2	0.71 ± 0.20 ^{c,d}	13.51 ± 0.88 ^{d,e}	28.7 ± 3.96 ^{b}	103.47 ± 3.03 ^{c,d}	18.70 ± 2.66 ^{e,f}	2.10 ± 1.12 ^{d}
DA2	3.80 ± 3.01 ^{a}	14.71 ± 2.55 ^{d,e}	9.03 ± 1.10 ^{f}	107.17 ± 1.14 ^{c}	17.90 ± 2.08 ^{e,f}	1.07 ± 0.11 ^{e}

Cow milk and ten infant formulas marketed in Brazil were evaluated by HPLC analyses. Phase 1 formulas ME1, NC1, NN1, DM1 and DA1 and phase 2 formulas: ME2, NC2, NN2, DM2 and DA2 were analyzed in triplicate to evaluate major protein fractions expressed as means ± SD. Different letters superscript within the same column indicate significant differences between formulas at a significance level $p < 0.01$. CM, cow milk; Lf, lactoferrin; BSA, bovine serum albumin; α-CN, α-casein; β-CN, β-casein; κ-CN, κ-casein; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

Whole powdered bovine milk samples were analyzed to compare phase 1 and phase 2 IFs CM protein fractions. CM contained the highest α-CN (43.26 ± 0.30 mg·g^{-1}), β-CN (57.95 ± 0.50 mg·g^{-1}), κ-CN (125.14 ± 0.39 mg·g^{-1}) and β-Lg (56.62 ± 0.41 mg·g^{-1}) contents compared to all IFs, but the CM the lowest Lf (0.31 ± 0.02 mg·g^{-1}) compared to phase 1 and phase 2 IFs.

Variation of major protein fractions between the three batches of the same phase 1 and phase 2 IFs brands. Table 2 displays the average major protein fractions values from three phase 1 and phase 2 IFs batches. In general, a significant difference in protein fractions was observed among different batches from the same manufacturer, except for Lf in NC1, NN1, DM1, DA1, ME2, NC2 and NN2 and β-CN in NN1, DA1 and NC2. The same was noted for α-La in ME1, ME2 and DA2, revealing protein fraction homogeneity among the different batches of these IFs. A significant difference ($p < 0.001$) between phase 1 and phase 2 formula brands was noted concerning the average value of each protein fraction. Phase 1 mean values ranged from 86.07 to 129.85 mg·g^{-1} for κ-CN, from 7.51 to 22.83 mg·g^{-1} for α-CN, from 0.31 to 13.76 mg·g^{-1} for α-La, from 7.54 to 29.19 mg·g^{-1} for β-CN, from 13.25 to 41.94 mg·g^{-1} for β-Lg and from 0.22 to 1.51 mg·g^{-1} for Lf, while, the means of phase 2 IFs ranged from 82.97 to 129.59 mg·g^{-1} for κ-CN, from 7.09 to 19.22 mg·g^{-1} for α-CN, from 0.51 to 12.57 mg·g^{-1} for α-La, from 4.72 to 33.10 mg·g^{-1} for β-CN, from 15.59 to 36.32 mg·g^{-1} for β-Lg and from 0.28 to 8.55 mg·g^{-1} for Lf. Thus, κ-CN was the most abundant protein, and α-La and Lf, the less abundant ones.

Table 2. Major proteins in phase 1 and phase 2 infant formulas evaluated in three distinct batches from the same manufacturer.

Infant Formulas	Major Proteins (mg·g^{-1})					
	Lf	**α-CN**	**β-CN**	**κ-CN**	**β-Lg**	**α-La**
			Phase 1			
ME1A	1.51 ± 0.15 ^{a}	16.62 ± 0.11 ^{a}	7.62 ± 0.11 ^{b}	100.21 ± 0.27 ^{b}	20.61 ± 0.15 ^{b}	0.31 ± 0.21 ^{a}
ME1B	1.45 ± 0.11 ^{a,b}	12.62 ± 0.22 ^{c}	8.63 ± 0.21 ^{a}	100.36 ± 0.27 ^{b}	22.92 ± 0.15 ^{a}	0.41 ± 0.11 ^{a}
ME1C	1.21 ± 0.21 ^{b}	15.11 ± 0.13 ^{b}	7.75 ± 0.17 ^{b}	100.95 ± 0.17 ^{a}	23.02 ± 0.34 ^{a}	0.39 ± 0.11 ^{a}
NC1A	0.19 ± 0.17 ^{a}	22.83 ± 0.15 ^{a}	10.14 ± 0.27 ^{b}	94.87 ± 0.29 ^{a}	21.43 ± 0.11 ^{a}	13.41 ± 0.23 ^{a}
NC1B	0.22 ± 0.15 ^{a}	18.02 ± 0.45 ^{b}	11.57 ± 0.65 ^{a}	86.07 ± 0.19 ^{c}	18.45 ± 0.31 ^{b}	11.42 ± 0.43 ^{b}
NC1C	0.41 ± 0.26 ^{a}	22.72 ± 0.54 ^{a}	11.84 ± 0.46 ^{a}	88.94 ± 0.48 ^{b}	18.35 ± 0.41 ^{b}	11.29 ± 0.22 ^{b}
NN1A	0.73 ± 0.21 ^{a}	7.82 ± 0.10 ^{a}	11.65 ± 0.43 ^{a}	121.25 ± 0.72 ^{c}	41.94 ± 0.42 ^{a}	13.18 ± 0.12 ^{b}
NN1B	0.88 ± 0.32 ^{a}	7.51 ± 0.12 ^{b}	11.56 ± 0.22 ^{a}	129.85 ± 0.42 ^{a}	38.76 ± 0.21 ^{b}	13.76 ± 0.32 ^{a}
NN1C	0.99 ± 0.12 ^{a}	7.54 ± 0.12 ^{b}	11.76 ± 0.49 ^{a}	122.55 ± 0.83 ^{b}	38.97 ± 0.12 ^{b}	12.95 ± 0.11 ^{b}
DM1A	1.42 ± 0.15 ^{a}	10.57 ± 0.53 ^{b}	27.38 ± 0.34 ^{b}	99.87 ± 0.11 ^{b}	14.08 ± 0.42 ^{c}	4.41 ± 0.19 ^{a}
DM1B	1.43 ± 0.11 ^{a}	11.56 ± 0.14 ^{a}	29.19 ± 0.55 ^{a}	100.57 ± 0.11 ^{a}	16.09 ± 0.12 ^{b}	4.51 ± 0.39 ^{a}
DM1C	1.39 ± 0.13 ^{a}	11.53 ± 0.23 ^{a}	19.19 ± 0.91 ^{c}	100.44 ± 0.31 ^{a}	17.39 ± 0.83 ^{a}	2.93 ± 0.26 ^{b}
DA1A	1.33 ± 0.21 ^{a}	13.55 ± 0.11 ^{a}	7.54 ± 0.43 ^{a}	108.33 ± 0.21 ^{a}	13.25 ± 0.12 ^{b}	2.21 ± 0.25 ^{b}
DA1B	1.15 ± 0.12 ^{a}	13.41 ± 0.21 ^{a}	8.15 ± 0.25 ^{a}	103.42 ± 0.21 ^{b}	15.66 ± 0.13 ^{a}	2.54 ± 0.15 ^{b}
DA1C	1.01 ± 0.28 ^{a}	10.81 ± 0.11 ^{b}	7.74 ± 0.21 ^{a}	107.98 ± 0.13 ^{a}	13.52 ± 0.33 ^{b}	5.54 ± 0.14 ^{a}
			Phase 2			
ME2A	1.06 ± 0.11 ^{a}	19.22 ± 0.14 ^{a}	6.81 ± 0.21 ^{a}	129.09 ± 0.14 ^{b}	22.63 ± 0.27 ^{b}	0.58 ± 0.15 ^{a}
ME2B	0.88 ± 0.22 ^{a}	17.63 ± 0.12 ^{c}	4.72 ± 0.11 ^{c}	129.59 ± 0.14 ^{a}	28.03 ± 0.18 ^{a}	0.51 ± 0.15 ^{a}
ME2C	0.87 ± 0.28 ^{a}	18.77 ± 0.28 ^{b}	5.42 ± 0.18 ^{b}	122.56 ± 0.25 ^{c}	28.24 ± 0.18 ^{a}	0.76 ± 0.13 ^{a}
NC2A	0.57 ± 0.19 ^{a}	13.36 ± 0.26 ^{b}	10.76 ± 0.14 ^{a}	82.97 ± 0.41 ^{c}	17.74 ± 0.29 ^{b}	8.06 ± 0.24 ^{a}
NC2B	0.39 ± 0.37 ^{a}	12.45 ± 0.17 ^{c}	10.54 ± 0.45 ^{a}	97.25 ± 0.22 ^{a}	19.35 ± 0.15 ^{a}	7.66 ± 0.23 ^{a}
NC2C	0.42 ± 0.14 ^{a}	14.46 ± 0.63 ^{a}	10.71 ± 0.15 ^{a}	84.47 ± 0.23 ^{b}	15.86 ± 0.16 ^{c}	7.15 ± 0.24 ^{b}
NN2A	0.61 ± 0.16 ^{a}	7.09 ± 0.24 ^{b}	25.61 ± 0.17 ^{a}	113.17 ± 0.21 ^{c}	28.54 ± 0.54 ^{c}	12.57 ± 0.11 ^{a}
NN2B	0.71 ± 0.15 ^{a}	7.39 ± 0.25 ^{a,b}	22.71 ± 0.28 ^{b}	116.12 ± 0.43 ^{b}	33.01 ± 0.11 ^{b}	11.68 ± 0.21 ^{b}
NN2C	0.73 ± 0.19 ^{a}	7.47 ± 0.14 ^{a}	18.41 ± 0.63 ^{c}	117.43 ± 0.33 ^{a}	36.32 ± 0.72 ^{a}	11.83 ± 0.11 ^{b}
DM2A	0.92 ± 0.17 ^{a}	14.24 ± 0.26 ^{a}	25.40 ± 0.23 ^{c}	109.33 ± 0.15 ^{a}	21.53 ± 0.51 ^{a}	2.82 ± 0.22 ^{a}
DM2B	0.94 ± 0.77 ^{a}	13.81 ± 0.56 ^{b}	27.62 ± 0.36 ^{b}	100.96 ± 0.35 ^{b}	18.46 ± 0.14 ^{b}	2.71 ± 0.33 ^{a}
DM2C	0.28 ± 0.21 ^{b}	12.53 ± 0.14 ^{c}	33.10 ± 0.39 ^{a}	100.25 ± 0.14 ^{c}	16.26 ± 0.24 ^{c}	0.82 ± 0.52 ^{b}
DA2A	8.55 ± 0.41 ^{a}	12.51 ± 0.18 ^{b}	7.93 ± 0.11 ^{c}	108.54 ± 0.24 ^{a}	19.37 ± 0.13 ^{a}	1.02 ± 0.21 ^{a}
DA2B	1.54 ± 0.21 ^{b}	17.52 ± 0.25 ^{a}	9.15 ± 0.12 ^{b}	107.34 ± 0.11 ^{b}	18.98 ± 0.15 ^{b}	1.29 ± 0.11 ^{a}
DA2C	1.44 ± 0.21 ^{b}	14.11 ± 0.22 ^{c}	10.13 ± 0.22 ^{a}	105.74 ± 0.32 ^{c}	15.59 ± 0.36 ^{c}	1.09 ± 0.11 ^{a}

Thirty different batches, three batches for each infant formula, marketed in Brazil were evaluated by HPLC analyses. Analyzes for each sample were performed in triplicate (n = 90) and data are reported as means ± SD. Different letters superscript within the same column indicate significant differences between infant formulas at a significance level $p < 0.01$. Lf, lactoferrin; BSA, bovine serum albumin; α-CN, α-casein; β-CN, β-casein; κ-CN, κ-casein; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

3.3. Amino Acid Quantification in Phase 1 and Phase 2 IFs

The average amino acid content in phase 1 and phase 2 IFs are presented in Table 3. Histidine ranged from 0.11 to 0.16 mg·g^{-1} and tryptophan, from 0.10 to 0.14 mg·g^{-1}, and similar contents for both amino acids were observed in both phase 1 and phase 2 samples. DM1, however, presented the highest valine contents, of 1.34 ± 0.02 mg·g^{-1}, as well as me threonine, 1.99 ± 0.01 mg·g^{-1}; isoleucine, 1.69 ± 0.01 mg·g^{-1} and phenylalanine, 3.24 ± 0.01 mg·g^{-1}, when compared to other phase 1 IFs. DA1 and DM1 presented the highest threonine and leucine contents, of 4.34 ± 0.01 and 4.19 ± 0.02 mg·g^{-1} and 4.14 ± 0.02 and 3.96 ± 0.02 mg·g^{-1}, respectively, when compared to other phase 1 IFs. Furthermore, the highest lysine contents were found in NN1, NC1 and ME1, of 0.26 ± 0.03, 0.20 ± 0.03 and 0.20 ± 0.04 mg·g^{-1}, respectively. Concerning phase 2 formulas, DM2 contained the highest threonine content, 4.70 ± 0.02 mg·g^{-1}, as well as methionine,

2.13 ± 0.02 mg·g^{-1}; isoleucine, 1.99 ± 0.01 mg·g^{-1}; leucine, 4.56 ± 0.01 mg·g^{-1} and phenylalanine 3.28 ± 0.02 mg·g^{-1} compared to other phase 2 IFs. The highest lysine contents were observed in DM2 and NC2, 0.19 ± 0.01 and 0.19 ± 0.01 mg·g^{-1}, respectively, and the highest valine values, of 1.29 ± 0.03, 1.27 ± 0.01 and 1.25 ± 0.01 mg·g^{-1} were detected in NC2, NN2 and DM2, respectively.

Table 3. Average of essential amino acids contents from phase 1 and phase 2 infant formulas.

	Reference	Infant Formulas									
		Phase 1 (mg·g^{-1})					Phase 2 (mg·g^{-1})				
Amino Acids	**FAO/WHO (mg·g of Protein^{-1}) ***	**ME1**	**NC1**	**NN1**	**DM1**	**DA1**	**ME2**	**NC2**	**NN2**	**DM2**	**DA2**
Threonine	31	3.93 ± 0.02 ^{c,E}	3.08 ± 0.03 ^{e,I}	3.18 ± 0.01 ^{d,H}	4.19 ± 0.02 ^{b,C}	4.34 ± 0.01 ^{a,B}	4.07 ± 0.02 ^{b,D}	3.24 ± 0.01 ^{d,G}	3.04 ± 0.02 ^{e,I}	4.70 ± 0.02 ^{a,A}	3.72 ± 0.02 ^{c,F}
Lysine	57	0.20 ± 0.04 ^{a,A,B}	0.20 ± 0.03 ^{a,A,B}	0.26 ± 0.03 ^{a,A}	0.15 ± 0.01 ^{b,C}	0.14 ± 0.01 ^{b,C}	0.16 ± 0.01 ^{b,C}	0.19 ± 0.01 ^{a,B}	0.10 ± 0.01 ^{c,D}	0.19 ± 0.01 ^{a,B}	0.15 ± 0.01 ^{b,C}
Histidine	20	0.15 ± 0.01 ^{a,A}	0.14 ± 0.01 ^{a,A}	0.13 ± 0.01 ^{a,A}	0.14 ± 0.03 ^{a,A}	0.14 ± 0.04 ^{a,A}	0.12 ± 0.02 ^{a,A}	0.14 ± 0.01 ^{a,A}	0.16 ± 0.02 ^{a,A}	0.16 ± 0.02 ^{a,A}	0.11 ± 0.03 ^{a,A}
Valine	43	0.33 ± 0.01 ^{c,C}	1.23 ± 0.03 ^{b,B}	1.27 ± 0.01 ^{b,B}	1.34 ± 0.02 ^{a,A}	0.27 ± 0.05 ^{c,D}	0.35 ± 0.01 ^{b,C}	1.29 ± 0.03 ^{a,B}	1.27 ± 0.01 ^{a,B}	1.25 ± 0.01 ^{a,B}	0.26 ± 0.02 ^{c,D}
Methionine	28	0.95 ± 0.01 ^{e,F}	1.34 ± 0.01 ^{c,D}	1.40 ± 0.02 ^{b,C}	1.99 ± 0.01 ^{a,B}	1.19 ± 0.01 ^{d,E}	0.97 ± 0.01 ^{d,F}	1.41 ± 0.01 ^{b,C}	1.36 ± 0.01 ^{c,D}	2.13 ± 0.02 ^{a,A}	0.87 ± 0.02 ^{e,G}
Isoleucine	32	0.94 ± 0.02 ^{c,D}	0.90 ± 0.03 ^{c,D}	0.90 ± 0.04 ^{c,D}	1.69 ± 0.01 ^{a,B}	1.32 ± 0.02 ^{b,C}	0.96 ± 0.03 ^{b,D}	0.90 ± 0.03 ^{b,D}	0.81 ± 0.01 ^{c,E}	1.99 ± 0.01 ^{a,A}	0.91 ± 0.02 ^{b,D}
Leucine	66	0.75 ± 0.04 ^{d,G}	2.66 ± 0.04 ^{c,E}	2.69 ± 0.01 ^{c,E}	3.96 ± 0.02 ^{b,C}	4.14 ± 0.02 ^{a,B}	3.29 ± 0.02 ^{b,D}	2.72 ± 0.02 ^{c,E}	2.56 ± 0.01 ^{d,F}	4.56 ± 0.01 ^{a,A}	3.24 ± 0.03 ^{b,D}
Phenylalanine	52	2.61 ± 0.01 ^{b,C}	2.39 ± 0.01 ^{c,E}	2.60 ± 0.03 ^{b,C}	3.24 ± 0.04 ^{a,A}	2.60 ± 0.03 ^{b,C}	2.92 ± 0.01 ^{b,B}	2.52 ± 0.01 ^{d,D}	2.63 ± 0.02 ^{c,C}	3.28 ± 0.02 ^{a,A}	2.50 ± 0.02 ^{d,D}
Tryptophan	8.5	0.14 ± 0.09 ^{a,A}	0.13 ± 0.07 ^{a,A}	0.12 ± 0.07 ^{a,A}	0.13 ± 0.09 ^{a,A}	0.11 ± 0.08 ^{a,A}	0.11 ± 0.09 ^{a,A}	0.10 ± 0.07 ^{a,A}	0.14 ± 0.01 ^{a,A}	0.12 ± 0.06 ^{a,A}	0.14 ± 0.06 ^{a,A}

Ten different infant formulas marketed in Brazil were analyzed by HPLC. Analyzes were performed in triplicate and data are reported as means ± SD. Different superscript lowercase letters within the same line indicate significant differences between infant formulas of the same phase at a significance level $p < 0.001$. Different superscript uppercase letters within the same line indicate significant differences between phase 1 and phase 2 infant formulas at a significance level $p < 0.01$. * Recommended amino acids daily intake for children aged 0 to 12 months (Joint WHO/FAO/UNU Expert Consultation, 2007).

When comparing phase 1 vs. phase 2 IFs amino acid contents, a significant difference in phase 1 lysine, valine and isoleucine contents was observed, higher in phase 1 compared to phase 2. On the other hand, higher threonine, methionine, leucine and phenylalanine were detected in phase 2 IFs. Finally, histidine and tryptophan contents were similar in both types of formula (Table 3).

3.4. Phase 1 and Phase 2 IFs %IVPD

Phase 1 and phase 2 IFs %IVPD are displayed in Figure 4. After gastric digestion, ME1, NC1, NN1 and DM1 presented higher %IVPD values than DA1. Concerning intestinal digestion, ME1, NN1 and DM1 exhibited higher %IVPD than NC1 and DA1. Among phase 2 IFs, ME2 and NN2 presented the highest %IVPD when compared to NC2, DM2 and DA2 after gastric digestion. However, the %IVPDs after intestinal digestion were not significantly different when comparing all phase 2 IFs.

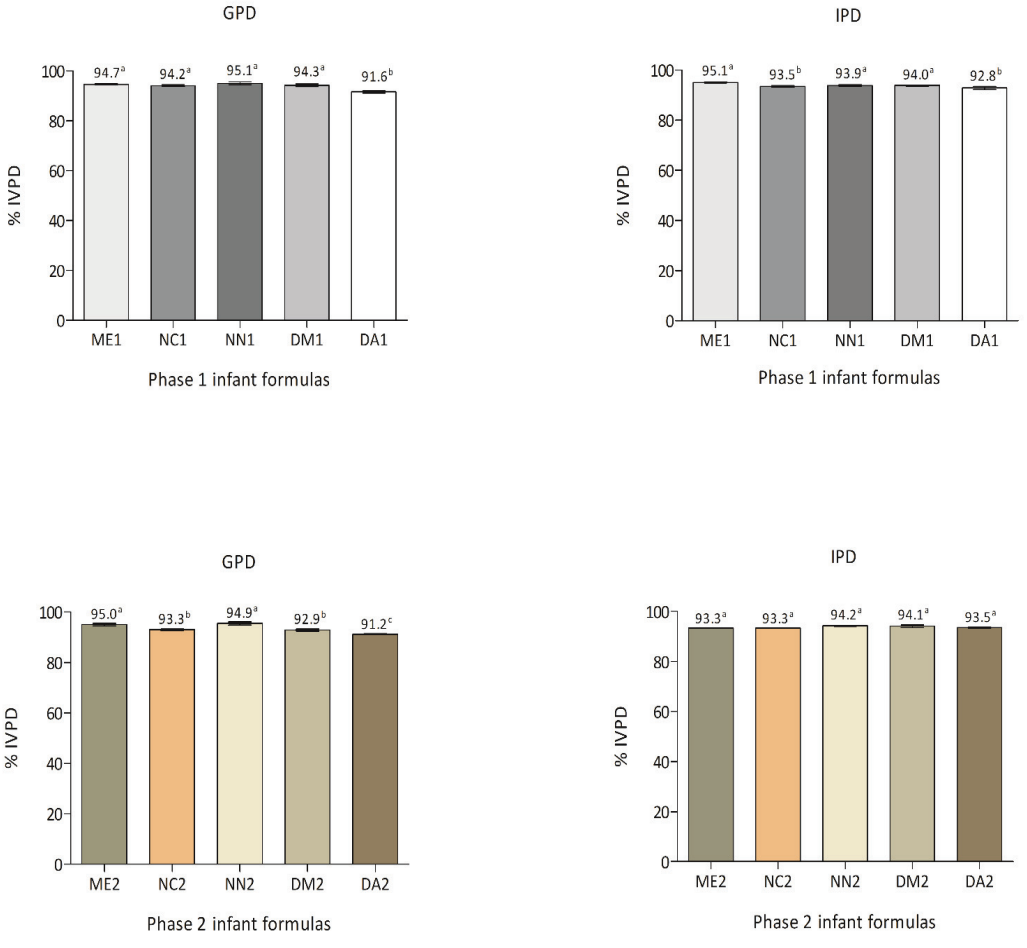

Figure 4. In vitro protein digestibility (%IVPD) of phase 1 and phase 2 infant formulas. Values are expressed as means ± SD (n = 3). Different letters denote difference at a significance level of $p < 0.05$. The percentage of IVPD was estimated based on the nitrogen content by the micro-Kjeldahl method.

3.5. Phase 1 and Phase 2 IFs AAS and PDCAAS

Chemical amino acid scores (AAS) were calculated as recommended by FAO/WHO (2007), considering the ratio between essential amino acid contents and those recommended for children aged 0 to 12 months old, used to identify infant formulas that could limit essential infant amino acid supplies (Table 4). The AAS calculation indicated that all essential amino acids in phase 2 IFs presented a chemical score below 1.0. Similar results were also observed for phase 1 IFs, except for threonine in ME1, NC1, DM1 and DA1, where the AAS was slightly higher than 1.0, as follows: 1.011 ± 0.005 mg·g^{-1}, 1.019 ± 0.010 mg·g^{-1}, 1.008 ± 0.007 mg·g^{-1} and 1.169 ± 0.002 mg·g^{-1}, respectively. Almost all amino acids presented higher AAS values in phase 1 compared to phase 2 IFs ($p < 0.01$).

Table 4. Amino acid scores (AAS) for phase 1 and phase 2 infant formulas calculated for children aged 0–6 to 7–12 months.

Amino Acids	Phase 1 (mg·g^{-1})					Phase 2 (mg·g^{-1})				
	ME1	NC1	NN1	DM1	DA1	ME2	NC2	NN2	DM2	DA2
Threonine	1.011 ± 0.005 ^{b,B}	1.019 ± 0.010 ^{b,B}	0.970 ± 0.014 ^{c,C}	1.008 ± 0.007 ^{b,B}	1.169 ± 0.002 ^{a,A}	0.655 ± 0.007 ^{c,D}	0.595 ± 0.021 ^{d,E}	0.644 ± 0.012 ^{c,D}	0.890 ± 0.014 ^{a,C}	0.665 ± 0.017 ^{b,D}
Lysine	0.025 ± 0.003 ^{a,A}	0.040 ± 0.003 ^{a,A}	0.035 ± 0.007 ^{a,A}	0.030 ± 0.014 ^{a,b,A}	0.029 ± 0.002 ^{b,B}	0.016 ± 0.003 ^{b,C}	0.026 ± 0.001 ^{a,B}	0.017 ± 0.002 ^{b,C}	0.032 ± 0.008 ^{a,B}	0.025 ± 0.005 ^{a,B}
Histidine	0.058 ± 0.005 ^{a,b,A}	0.065 ± 0.002 ^{a,A}	0.045 ± 0.007 ^{c,C}	0.050 ± 0.009 ^{b,B}	0.059 ± 0.005 ^{a,b,A}	0.030 ± 0.004 ^{b,D}	0.035 ± 0.003 ^{b,D}	0.020 ± 0.015 ^{b,D}	0.050 ± 0.001 ^{a,B}	0.032 ± 0.004 ^{b,D}
Valine	0.065 ± 0.002 ^{c,E}	0.261 ± 0.001 ^{a,A}	0.215 ± 0.007 ^{b,B}	0.205 ± 0.008 ^{b,B}	0.053 ± 0.007 ^{c,E}	0.040 ± 0.008 ^{c,E}	0.145 ± 0.007 ^{b,D}	0.185 ± 0.010 ^{a,C}	0.170 ± 0.008 ^{a,C}	0.039 ± 0.006 ^{c,E}
Methionine	0.263 ± 0.001 ^{c,D}	0.436 ± 0.013 ^{a,A}	0.365 ± 0.007 ^{b,B}	0.445 ± 0.008 ^{a,A}	0.357 ± 0.003 ^{b,B}	0.170 ± 0.001 ^{d,F}	0.240 ± 0.007 ^{c,E}	0.306 ± 0.019 ^{b,C}	0.450 ± 0.001 ^{a,A}	0.170 ± 0.001 ^{d,F}
Isoleucine	0.228 ± 0.001 ^{c,D}	0.255 ± 0.001 ^{b,C}	0.210 ± 0.011 ^{d,E}	0.340 ± 0.021 ^{a,B}	0.345 ± 0.001 ^{a,B}	0.140 ± 0.017 ^{b,F}	0.135 ± 0.015 ^{b,F}	0.159 ± 0.015 ^{b,F}	0.370 ± 0.003 ^{a,A}	0.160 ± 0.013 ^{b,F}
Leucine	0.088 ± 0.001 ^{d,G}	0.368 ± 0.001 ^{b,C}	0.285 ± 0.008 ^{c,D}	0.375 ± 0.020 ^{b,C}	0.523 ± 0.003 ^{a,A}	0.235 ± 0.010 ^{d,E}	0.200 ± 0.007 ^{e,F}	0.243 ± 0.001 ^{c,E}	0.410 ± 0.003 ^{a,B}	0.270 ± 0.008 ^{b,D}
Phenylalanine	0.387 ± 0.007 ^{b,B}	0.419 ± 0.023 ^{a,A}	0.370 ± 0.010 ^{b,B}	0.380 ± 0.007 ^{b,B}	0.418 ± 0.001 ^{a,A}	0.265 ± 0.006 ^{c,D}	0.230 ± 0.001 ^{d,E}	0.317 ± 0.029 ^{b,C}	0.375 ± 0.011 ^{a,B}	0.265 ± 0.008 ^{c,D}
Tryptophan	0.133 ± 0.009 ^{a,A}	0.145 ± 0.001 ^{a,A}	0.105 ± 0.009 ^{b,B}	0.115 ± 0.003 ^{b,B}	0.112 ± 0.06 ^{b,B}	0.070 ± 0.002 ^{c,D}	0.067 ± 0.011 ^{c,D}	0.111 ± 0.011 ^{a,B}	0.082 ± 0.008 ^{b,C}	0.091 ± 0.001 ^{b,C}

Analyses were performed in triplicate and data are reported as means ± SD. Different superscript lowercase letters within the same line indicate significant differences between infant formulas of the same phase at a significance level $p < 0.001$. Different superscript uppercase letters within the same column indicate significant differences between phase 1 and phase 2 infant formulas at a significance level $p < 0.01$.

Protein digestibility values are displayed in Table 5. The AAS in this case was corrected for protein digestibility (PDCAAS) based on the amino acid requirements for children aged 0 to 12 months old. Even after correction, values remained lower than 1.0 for all phase 1 and phase 2 IFs, indicating that essential amino acid concentrations in the investigated IFs were under the recommended limits. Furthermore, almost all PDCAAS were higher in phase 1 compared to phase 2 IFs ($p < 0.01$).

Table 5. Protein digestibility-corrected by amino acid scores (PDCAAS) from phase 1 and phase 2 infant formulas calculated for children age 0–6 and 7–12 months.

Amino Acids	Phase 1 (mg·g^{-1})					Phase 2 (mg·g^{-1})				
	ME1	NC1	NN1	DM1	DA1	ME2	NC2	NN2	DM2	DA2
Threonine	1.010 ± 0.007 ^{b,B}	0.965 ± 0.007 ^{c,C}	0.967 ± 0.010 ^{c,C}	0.995 ± 0.009 ^{c,C}	1.115 ± 0.007 ^{a,A}	0.595 ± 0.005 ^{c,F}	0.575 ± 0.020 ^{d,F}	0.580 ± 0.014 ^{c,F}	0.850 ± 0.014 ^{a,D}	0.635 ± 0.009 ^{b,E}
Lysine	0.024 ± 0.007 ^{b,B}	0.030 ± 0.005 ^{a,A}	0.031 ± 0.002 ^{a,A}	0.025 ± 0.004 ^{b,A}	0.025 ± 0.003 ^{b,B}	0.016 ± 0.004 ^{b,B}	0.024 ± 0.003 ^{b,B}	0.015 ± 0.005 ^{b,B}	0.030 ± 0.003 ^{a,A}	0.018 ± 0.004 ^{b,B}
Histidine	0.055 ± 0.013 ^{a,A}	0.063 ± 0.010 ^{a,A}	0.044 ± 0.010 ^{a,A}	0.045 ± 0.010 ^{a,A}	0.055 ± 0.012 ^{a,A}	0.027 ± 0.005 ^{b,B}	0.030 ± 0.001 ^{b,B}	0.015 ± 0.007 ^{c,C}	0.045 ± 0.008 ^{a,A}	0.030 ± 0.001 ^{b,B}
Valine	0.061 ± 0.018 ^{d,E}	0.255 ± 0.013 ^{a,A}	0.210 ± 0.07 ^{b,B}	0.180 ± 0.005 ^{c,C}	0.051 ± 0.012 ^{d,E}	0.038 ± 0.008 ^{c,E}	0.135 ± 0.001 ^{b,D}	0.175 ± 0.007 ^{a,C}	0.165 ± 0.011 ^{a,C}	0.035 ± 0.015 ^{c,E}
Methionine	0.255 ± 0.011 ^{c,D}	0.415 ± 0.010 ^{a,B}	0.350 ± 0.07 ^{b,C}	0.425 ± 0.007 ^{a,A}	0.345 ± 0.001 ^{b,C}	0.160 ± 0.002 ^{d,E}	0.230 ± 0.015 ^{c,D}	0.290 ± 0.010 ^{b,D}	0.430 ± 0.004 ^{a,A}	0.165 ± 0.005 ^{d,E}
Isoleucine	0.225 ± 0.012 ^{b,B}	0.245 ± 0.010 ^{b,B}	0.180 ± 0.012 ^{c,C}	0.319 ± 0.015 ^{a,A}	0.325 ± 0.011 ^{a,A}	0.137 ± 0.011 ^{b,D}	0.133 ± 0.011 ^{c,D}	0.150 ± 0.011 ^{b,D}	0.350 ± 0.018 ^{a,A}	0.150 ± 0.009 ^{b,D}
Leucine	0.085 ± 0.001 ^{d,G}	0.340 ± 0.008 ^{b,C}	0.275 ± 0.011 ^{c,D}	0.355 ± 0.011 ^{b,C}	0.505 ± 0.005 ^{a,A}	0.225 ± 0.008 ^{b,E}	0.190 ± 0.14 ^{c,F}	0.230 ± 0.001 ^{b,E}	0.390 ± 0.005 ^{a,B}	0.255 ± 0.015 ^{b,D}
Phenylalanine	0.369 ± 0.014 ^{c,B}	0.395 ± 0.013 ^{b,B}	0.335 ± 0.011 ^{d,C}	0.370 ± 0.009 ^{b,B}	0.415 ± 0.003 ^{a,A}	0.255 ± 0.013 ^{c,D}	0.227 ± 0.017 ^{d,D}	0.315 ± 0.018 ^{b,C}	0.373 ± 0.011 ^{a,B}	0.250 ± 0.007 ^{c,D}
Tryptophan	0.125 ± 0.004 ^{b,B}	0.142 ± 0.008 ^{a,A}	0.101 ± 0.010 ^{c,C}	0.080 ± 0.010 ^{d,D}	0.105 ± 0.003 ^{c,C}	0.069 ± 0.09 ^{c,D}	0.065 ± 0.011 ^{c,D}	0.105 ± 0.015 ^{a,C}	0.080 ± 0.006 ^{b,D}	0.085 ± 0.012 ^{b,D}

Analyzes were performed in triplicate and data are reported as means ± SD. Different superscript lowercase letters within the same row indicate significant differences between infant formulas of the same phase at a significance level $p < 0.001$. Different superscript uppercase letters within the same column indicate significant differences between phase 1 and phase 2 infant formulas at a significance level $p < 0.01$.

4. Discussion

IFs aimed for children under the age of one are formulated from cow milk or from the milk of other animal species or their mixture, and/or eventually, other ingredients suitable for infant feeding [23]. Herein, several IFs formulated from cow milk were evaluated. Human and cow milk differ not only in the amount of proteins, but also their quality. Human milk protein contents range from 0.8 to 1.3 g·100 mL^{-1}, varying throughout the lactation stage, while whole cow milk contains about 3.33 g·100 mL^{-1}, about three-fold higher than human milk [24–26]. The current Brazilian legislation, which agrees with European Commission Directive 2006/141/EC recommendations, establishes a minimum protein content for phase 1 cow milk-derivative formulas of 1.8 g·100 kcal^{-1} and a maximum of 3.0 g·100 kcal^{-1}, while protein concentrations in phase 2 formulas should range between 1.8 g·100 kcal^{-1} to 3.5 g·100 kcal^{-1} [27,28]. On the other hand, the FAO/WHO

Codex Alimentarius, comprising 189 member countries and one European Union member, which establishes international food and nutrition standards and guidelines, recommends that the protein content of cow-milk derivative formulas should range between 1.8 to 3.0 g·100 kcal^{-1}, not exceeding the maximum limit [7,23].

In the present study, whole cow milk contained higher total protein contents than both phase 1 and phase 2 IFs, demonstrating that IFs manufacturers are making an effort to reduce protein contents from raw material seeking similar protein compositions to human milk. Additionally, protein contents in samples ME1B, NC1A, NC1C, NN1A, NN1C, DM2B and DA1B were all in accordance with their labels. Considering the protein content limits established by Brazilian legislation and Directive 2006/141/EC [27,28] for IFs labels (g·100 kcal^{-1}), our findings indicate that ME1C, DM1A and DM1B phase 1 IFs contained protein contents above recommendations, but not below the minimum limit. In addition, all phase 2 IFs contained protein concentrations within the limits established by Brazilian current legislation. However, when considering the limits established by the Codex Alimentarius, more than one batch of each brand of some phase 1 and phase 2 IFs exceeded the maximum protein recommendation of 3.0 g·100 kcal^{-1}, namely ME1C, DM1A, DM1B phase 1 IFs and ME2B, ME2C, NC2C, NN2B, DM2A, DM2C, DA2A and DA2C phase 2 IFs (Supplementary File Table S2).

Disagreements concerning the minimum and maximum protein content values bring attention to the lack of consensus of worldwide guidelines. According to Food and Drug Administration (FDA) guidelines, the maximum protein content in IFs is established as 3.5 g·100 kcal^{-1}, similar to the Brazilian limit, while the European Commission has updated infant food legislation and now established that minimum and maximum protein contents should be reduced to 1.6 to 2.5 g·100 kcal^{-1}, below the former levels of 1.8 to 3.0 g·100 kcal^{-1} [29].

Nutritional information on food labels aids consumers and professionals to select a balanced diet, contributing to reducing the incidence of unhealthy conditions associated with inadequate eating habits [30]. Non-compliance to nutritional content label statements is foreseen in the Brazilian RDC nº 360 December 2003, which establishes a 20% tolerance considering label and experimental evaluation divergences [31]. At least one batch of the ME1, NC1, NN1, DM1, DA1, ME2, NC2, NN2 and DA2 IFs did not meet the current legislation, as protein discrepancies between protein concentrations and labeled values were higher than 20%. Non-compliance with regulatory agency statements concerning IFs components can lead to infant health risks, as excessive protein intake can overload the metabolic capacity of young children, impairing liver and kidney function and increasing dehydration risks [32,33].

Breast milk or IFs are the recommended exclusive protein sources for newborns and infants up to six months of age. Thus, the quality, amounts, and conformation of IFs proteins should be strictly controlled in order to sustain adequate and safe infant growth, ensuring short and long-term health and proper development. The amount and quality of protein intake during the first two years of life have important effects on muscle protein development and linear growth, supporting optimal brain development and, consequently, better cognitive evolution, and ensuring the development of healthy and mature immune and digestive systems [11,12]. Protein restrictions lead to low levels of insulin-like growth factor 1 (IGF-1), compromising not only growth but also adipogenesis control. On the other hand, excess protein, such as CM protein concentrations, is associated with greater weight gain, higher body mass index (BMI), and risk of overweight or later obesity [34].

A broad multi-center randomized and controlled clinical trial conducted in several countries investigated the effects of protein intake on infant growth and adiposity, where healthy infants fed cow milk-formulas containing high protein concentrations ranging from 2.9 to 4.4 g·100 kcal^{-1} or low protein concentrations varying from 1.77 to 2.2 g·100 kcal^{-1} before and after the 5th month of life, respectively, were compared to a group of exclusively breastfed babies. The ingestion of high protein formulas resulted in greater weight in the first two years of life, although no growth effect was observed [35]. Weight gain

during childhood promoted by excess protein intake is the result of increased insulin-hyper aminoacidemia levels which, in turn, stimulates IGF-1 secretion, increasing later risks concerning obesity and associated diseases, reinforcing the requirement for strict IFs protein adjustments [35–37]. Furthermore, a significant effect of protein type and quality on gene expression is also observed, especially those encoding IGF-1 and insulin-like growth factor-binding proteins 1 (IGFBP-1), both involved in whole-body protein synthesis, thus affecting growth and body composition [12].

Considering protein IFs content and the Koletzko trials [35], the consumption of high protein formulas over the recommended amounts can result in significant weight gains in the first two years of life. Thus, one can assume that the regular intake of the ME1C (3.2 g·100 $kcal^{-1}$), DM1A (3.1 g·100 $kcal^{-1}$) and DM1B (3.3 g·100 $kcal^{-1}$) IFs investigated herein, containing the maximum recommended protein content by children under the age of one may increase the risk for obesity. Protein IFs content should preferentially be kept closer to the minimum limit, in order to manage childhood risk for overweight and obesity.

Poor nutrition during pregnancy is related to low nephron endowment, which may comprise a potential driver for hypertension and renal disease later in life [38]. Prematurity and low birth weight have been associated with smaller kidneys and lower nephron endowment, predisposing individuals to kidney disease and hypertension in adulthood [39,40]. These findings support the hypothesis that kidneys may be programmed while nephrogenesis takes place. As kidneys quickly increase in size and functional capacity during the first months of life, an appropriate nutritional intervention could exert long-term cardiovascular and kidney health effects [33]. Nevertheless, excessive protein intake is harmful to infants due to increased protein metabolite filtration, mainly urea, which leads to glomeruli hyperplasia and immature renal tubules, increasing kidney growth due to enhanced renal workload, evidenced by the urea/creatinine serum ratio in infants fed high protein formulas [38]. Additionally, abnormal kidney sizes seem to be a compensatory mechanism to allow for the excretion of high protein loads. Furthermore, increased body weight following high protein intake stimulates the secretion of IGF-1, leading to a transient increase in body organ size, such as kidneys. Fortunately, kidneys can return to their normal volume by simply ceasing excess protein intake [33].

Ensuring nutrient consistency and standardization in distinct batches of the same brand of IFs should be a major goal concerning the nutritional quality of these formulas, as these formulas may comprise the exclusive food source offered to some infants for over six months, and marketing IFs with unrecommended nutrient composition/concentration can lead to inadequate child development. In the present study, consistency among batches from the same manufacturer was observed for DM1/DM2, DA1/DA2, ME2, NC2 and NN2, while brands ME1, NC1 and NN1, exhibited a non-uniform composition with statistical protein content differences in at least one of the three tested lots (Supplementary file Table S2). This may be due to differential milk composition, as even milk samples from the same species do not contain exactly the same macro and micronutrient contents, and milk composition can be influenced by animal breed, lactation period or diet [41,42], as well as the manufacturing process, due to different purification methods (membrane filtration vs. ion exchange) and/or thermal processing [42,43].

In general, mammal milk contains insoluble proteins and soluble whey proteins, which include smaller floating proteins associated with milk fat globule membranes (MFGM). Caseins comprise the majority of insoluble milk proteins and are found in micellar form as α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN, while whey proteins, i.e., β-Lg, α-La, Lf, lysozyme, bovine serum albumin, immunoglobulins (Igs) and other smaller soluble proteins, present in colloidal form and in their glycosylated forms, compose the MFGM [44–47]. While the whey: casein proteins ratio in breast milk is about 90:10, in colostrum, and 60:40 in mature milk, this ratio reaches 20:80 in CM, indicating that CM is richer in casein than human milk [48]. Caseins are hard to digest, as they coagulate in the stomach under acidic pH and attack intestinal cells, hampering nutrient absorption and resulting in intestinal bleeding, diarrhea, anemia, cramps, allergies and weight gain impairments [49]. In addition, caseins

delay amino acid release due to slow digestion. On the other hand, whey proteins are quickly digested and highly bioavailable [48,50]. Herein, the IFs evaluation by SDS-PAGE indicated α-CN, β-CN, κ-CN, α-La, β-Lg, Lf as the main proteins, with other, less abundant ones, also detected. The amount of whey proteins α-La, β-Lg and Lf was higher than caseins in almost all IFs brands, except for DA1, DA2 and ME2 (Figure 3).

Whey or casein CM protein profiles differ from human milk. Human milk contains most protein subunits, such as α_{s1}-CN, β-CN κ-CN and γ-CN, except for the α_{s2}-CN variant, and main caseins correspond to β-CN, a highly phosphorylated protein, which aids in calcium absorption and contributes to zinc absorption. CM, however, contains main casein subunits α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN and γ-CN, with higher concentrations of α-CN and κ-CN [51–54]. Among CM whey proteins, β-Lg corresponds to about 50%, while α-La comprises about 20%, and Lf contributes to ≈ 4%. In contrast, β-Lg is not present in human milk, and is, therefore, considered a potential allergen present in CM. α-La is the major protein in human milk, reaching approximately ≈ 40% among whey proteins, and rich in essential amino acids, mainly tryptophan and cysteine, involved in lactose synthesis and playing a role as a mineral absorption facilitator [55,56]. Lf contributes to ≈ 25% of whey proteins in human milk and exhibits strong antimicrobial activity against a broad spectrum of bacteria, viruses, yeast, fungi, and parasites [56–58]. IFs should, therefore, mimic the overall proportion of casein and whey proteins and contain whey protein profiles similar to mature human milk.

The HPLC analyses carried out herein, an analytical method more sensitive and accurate than the SDS-PAGE technique, demonstrated that casein IFs contribution to total protein contents was higher compared to whey proteins. κ-CN was detected by HPLC in higher concentrations compared to SDS-PAGE, where it was apparently the least abundant protein. κ-CN may undergo thiol disulfide linkages with proteins containing disulfide bonds, such as α-La, β-Lg, BSA, IGs and Lf, when subjected to high temperatures, resulting in κ-CN aggregates that move slowly or remain stacked in the stacking gel during the electrophoresis run, altering correct concentration estimations [59]. Consequently, the caseins and whey protein contents detected in the investigated IFs are not in agreement with public health authority recommendations and are dissimilar to human milk composition, where whey proteins should be present in higher amounts compared to caseins, as they are better digested due to coagulation in the acidic stomach environment, and consequently, delay the release of free amino acids [60]. The ideal formula to fulfill infant needs should contain α-CN, β-CN and β-Lg in lower amounts to those detected in the investigated IFs, as the most common type of food allergen for infants and young children, β-casein A1, has been associated with a range of allergic diseases, including type 1 diabetes [49,60–62]. When β-casein A1, commonly found in CM in Europe, the United States, Australia, and New Zealand [63], is digested, it releases the β-casomorphin-7 peptide, which presents opioid and inflammatory properties and is considered an allergen. However, although β-casein A1 comprises the main allergen agent, other factors should also be considered.

SDS-PAGE can simultaneously identify caseins and whey proteins, although information obtained following IFs sample SDS-PAGE seems to be less accurate compared to urea-PAGE or native-PAGE for caseins and whey proteins, respectively. Caseins display similar molecular weights, differing only slightly from each other, resulting in inadequate SDS-PAG discrimination [64]. Reverse phase-HPLC, on the other hand, can improve protein detection sensitivity and accuracy and may be useful in establishing a standard approach to evaluate milk proteins in complex samples such as infant formulas [18,19].

The protein concentrations described herein indicate significant differences between IFs brands and batches. Cow milk differences can be explained by protein milk content and the relative concentrations of individual proteins present during the lactation period, cattle feeding or formula manufacturing, which can spoil heat-sensitive nutrients [17,65]. Whey proteins, including β-CN, are more susceptible to heat treatment, as this affects their 3D-conformations, leading to loss of functional and nutritional characteristics, reducing the biovailability of calcium and zinc and, thus, affecting child and adulthood health [66,67].

Similarly, α-La may lose its ability to bind to calcium and zinc ions, and thus, decrease the bioavailability of these nutrients. On the other hand, Lf tends to lose its antimicrobial activity following heat exposure [68].

The supply of essential amino acids in the first month of a child's life is provided by the set of proteins found in breast milk and/or IFs that must meet physiological newborn needs [69]. Whey protein fractions are richer in essential amino acids compared to caseins and, therefore, the general amino acid profile found in cow milk differs from breast milk [70], corroborating the results reported herein. Babies fed IFs exhibit greater differences in plasma amino acids compared to breastfed infants, with tryptophan levels, in particular, lower in the former [25].

Recommendation of essential and semi-essential amino acid contents per 100 kcal of IFs have been established considering breast milk proteins as reference [27,28]. The degree of compliance between the concentrations described in the present study observed herein and the content established by Brazilian RDCs (in mg·kcal^{-1}) reveal that most of the investigated IFs contain amino acid concentrations under established guidelines, except for threonine in ME1, ME2, DM1, DM2, DA1 and DA2, which were above 77 mg·kcal^{-1} (reference value), and methionine in NC1, NC2, NN1, NN2, DM1, DM2 and DA1, detected at 24 mg·kcal^{-1}, higher than the reference limit. It is important to note that a lower supply of essential amino acids and non-compliance to recommendations in the starting IFs (phase 1) can be more harmful when compared to the follow-up IFs (phase 2), as IF phase 1 is the only feeding recommended in the first six months of age when breast milk is not available. Unlike the starting IFs, follow-up IFs are offered to complement the introduction of new proteins from vegetable and animal origins. Therefore, even if the follow-up IFs are below the recommended intake, other protein sources can compensate and fulfill recommended essential amino acids concentrations.

Most essential amino acids were detected in all IFs, although at lower concentrations compared to established guidelines, probably due to reduced concentration of α-La and Lf whey proteins, the best source of essential amino acids, as indicated by the HPLC analysis (Tables 1 and 2). Essential IFs amino acid contents were lower than the reference values and exhibited tryptophan as the first limiting amino acid, corroborating other studies [10,69]. Essential amino acids were significantly different among IFs brands and batches, reflecting protein fraction variability (Table 3), with the relative concentration of each amino acid also depending on CM composition [26,71].

Essential and semi-essential amino acids supplies, digestibility, absorption and transport through the gastrointestinal tract influence infant growth, and are important for cell and tissue maintenance [23]. The absorption of free amino acids, dipeptides or tripeptides in the small intestine depends on the bioaccessibility of IFs proteins, that release these nitrogenous compounds following gastrointestinal digestion [72]. Digestion and absorption processes are highly integrated, dynamic and complex, regulated by both neural and hormonal controls and responsive to various stimuli in order to efficiently release nutrients required for body growth, maintenance and reproduction. In vitro digestibility bioassays are reproducible and can provide reliable digestibility estimates for a wide variety of food matrices [73]. In vitro bioaccessibility assays simulate the composition and physicochemical features of the gastric and small intestinal fluids including mineral salts, different compounds and enzymes, mimicking the physiological conditions found in the human body [72]. Good bioaccessibility was verified following simulating independent gastric and intestinal IFs digestions, with IVPD over 90%, in both phases, with only a discrete difference between them. Regarding the intestinal phase, no differences were observed in the bioaccessibility of phase 2 IFs. The digestibility of breast milk, due to its composition and the physical structure of proteins and fat globules, differs from CM digestibility [74]. Casein digestibility is around 85% in CM and 94% in breast milk, while whey proteins reach 97–98% [75]. Furthermore, the IVPDs% reported herein are comparable to previously reported values, ranging from 85–95%, higher than CM and near breast milk values [76]. Taken together, the results regarding gastric the simulated gastric and intestinal diges-

tions confirmed that the proteins present in phase 1 and 2 IFs are adequately digested, supporting suitable infant absorption.

Assessing the protein quality of foodstuffs is important when considering nutritional infant benefits. In this regard, the AAS and PDCAAS are commonly employed to assess the protein quality of food matrices [23]. The AAS compares the content of each essential amino acid present in a protein source with a protein reference, while the PDCAAS is defined as the ratio between the content of the first limiting amino acid in the protein and the content of that amino acid in a reference protein, multiplied by digestibility. Concerning infant requirement estimates, it is assumed that the human milk of a healthy and well-nourished mother during the first six months of life is an ideal intake source. Thus, the quality of the protein assessed by the chemical score is based on the limiting essential amino acid, where values greater than 1.0 and 100% for AAS and PCDAAS, respectively, indicate that good quality protein, containing essential amino acids capable of meeting human needs. Furthermore, according to the FAO/WHO [23] and IOM [43], IFs proteins should comprise at least 80% of breast milk protein. Herein, AAS and PDCAAS values concerning essential amino acid concentrations in IFs were lower than in breast milk, indicating that the most essential amino acids are limiting and do not reach the minimum established thresholds. When the quality of IFs proteins formulated from unmodified CM was evaluated through the chemical amino acid score (AAS), and corrected by the PDCAAS, threonine was the only amino acid that achieved a good chemical score of 80% in all phase 1 IFs and in the DM2 phase 2 formula.

According to IFs composition protocols, essential and semi-essential amino acids can be added to improve formula protein quality, but in amounts just enough to fill this purpose [27,28]. The findings obtained herein indicate that the protein quality from the investigated IFs was much lower than recommended. Therefore, alternatives to improve the protein quality should be carried out to supplement IFs with free essential amino acids or proteins of high biological value, such as α-La [77]. IFs enrichment with bovine α-La has been conducted in many clinical studies, improving the plasmatic levels of essential amino acids, resulting in infant development closer to that of breastfed ones [78,79]. Bovine α-La is noteworthy as a high biological value protein, as its essential amino acids, especially tryptophan, cysteine and lysine, reach similar concentrations found in human breast milk [26,49,68].

It is important to note that studies concerning the amino acid scores of CM-based IFs are scarce. In the present study, protein composition and essential amino acid ratios were variable, and total protein contents reported on the investigated IFs labels are not adequate and do not reflect IFs quality, making it difficult for pediatricians and nutritionists to accurately compare between available commercial products.

5. Conclusions

Despite attempts to mimic the composition or performance of breast milk by IFs manufacturers, proteins present in IFs cannot be proven as exhibiting the same bioavailability as those found in human milk. All formulas displayed adequate protein bioaccessibility, with proteins almost entirely digested in the intestine, generating free amino acid, dipeptides and tripeptides available for absorption. Only some IFs brands exhibited total protein content in accordance with product labels. Total protein content varied between batches from the same manufacturer, which may result in negative effects in children that consume the same brand regularly over six months. In addition, most IFs displayed protein contents above the maximum limits established by Brazilian regulation, as well as the European Commission Directive 2006/141/EC and Codex Alimentarius (FAO and WHO).

Casein contents were higher than whey proteins in all IFs, presenting three different subunits and with K-CN as the most abundant. Concerning whey protein contents, Lf amounts were lower than in human milk, jeopardizing newborn defenses, as this protein exhibits strong antimicrobial activity. High β-Lg concentrations comprised a non-desirable

component, as this protein, which is not present in human breast milk, can induce allergy in susceptible individuals.

Therefore, despite the advances noted in technological IFs processing in recent years leading to safer and more adequate IFs for children under the age of one, the investigated brands still displayed marked nutritional quality differences when compared to human breast milk, requiring continuous improvements to follow current international guidelines due to the major health effects associated to poor nutrition or obesity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13113933/s1, Table S1: Coded infant formulas. Table S2: Protein label content vs. determined crude protein in different infant formulas (IFs). Figure S3: Representative chromatogram of a mixture of bovine milk proteins and lactoferrin standards (A) and a protein fraction chromatogram from infant formulas (B). Figure S4: Standard curves of bovine milk protein fractions identified by HPLC.

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Prevalence and Correlates of Overweight, Obesity and Physical Activity in Italian Children and Adolescents from Lombardy, Italy

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Abstract: Investigating pediatric overweight and physical activity correlates is essential to design effective preventive programs. We used regional data (Lombardy, northern Italy) from the 2019 survey "OKKio alla Salute" (3093 children aged 8–9 years with measured anthropometric data), and from the 2018 wave of the "Health Behaviour in School-aged Children" survey (2916 adolescents aged 11–15 years with self-reported anthropometric data). In both the surveys, a cluster sampling methodology was used. Unconditional multiple logistic regression models were applied to estimate the odds ratios (OR) and corresponding 95% confidence intervals (CI) of overweight, obesity and poor physical activity. The prevalence of overweight (including obesity) was 22.4% for children aged 8–9 years and 14.4% for adolescents aged 11–15 years. A higher prevalence of overweight was observed among males, children with greater birth weight and those with obese parents. Scant physical activity was higher among females and older adolescents. There was a direct relationship between obesity and increased psychological distress (OR = 2.44; 95% CI: 1.12–5.27) or being victims of bullying (OR = 2.25; 95% CI: 1.17–4.34). Increasing physical activity significantly decreased the frequency of mental health outcomes. Prevention campaigns should be promoted to safeguard childhood physical and psychological wellbeing.

Keywords: childhood obesity; childhood overweight; physical activity; cross sectional study; adolescents; screen time; Italy; HBSC

1. Introduction

Over the past three decades Europe has undergone a substantial socio-economic transition that has changed its inhabitants' dietary habits, leading to a more obesogenic environment [1]. The increased availability, access and affordability of energy-high-caloric foods, along with their intense marketing, are recognized examples of the environmental factors that help to explain excess energy intake and subsequent weight gain [2]. As a consequence, in most European countries the prevalence of overweight in children and adolescents has steadily risen since 1980, reaching its peak in 2007–2010 [3,4].

In Italy, childhood overweight and obesity show a special pattern. Italy ranks as the fourth country for overweight (including obesity) among children aged 7–9 years, out of 36 European countries [5], but its position in the ranking drops sharply with age. Out of 43 European countries, Italy ranks 4th among children aged 11 years, 11th among children aged 13 years, and 20th among children aged 15 years [6]. This huge reduction is even more marked in adulthood, with Italy ranking as the country with the lowest prevalence of overweight (including obesity) among 30 countries included in the Eurobarometer data [7]. Estimates from the Italian National Institute of Statistics show similar patterns [8].

There is evidence that the prevalence of childhood overweight and obesity is substantially higher in southern than northern Italy [9,10]. This is also true for the adult general population [10].

The increasing tendency to a sedentary lifestyle and insufficient physical activity, even in childhood, in most high-income countries contributes to raise this issue [11]. In the last few decades, several studies have already documented that children and adolescents from most high-income countries have become less physically active and more sedentary [12,13]. Screen time behaviors, such as watching TV, playing videogames or using computers for other purposes, such as social media or surfing the internet, have all contributed to the overall increase in sedentary time. Recent estimates show that less than 20% of European children and adolescents meet the World Health Organization (WHO) recommendations on physical activity [14,15]. A WHO report indicated that the prevalence of adolescents (aged 11–15 years) reaching the recommended level of at least 60 min of physical activity per day in Italy (11%) is lower than in several other European countries [16]. In addition, Italian schools tend to devote less time to physical exercise, compared to those from other European countries [15,17]. This issue is becoming more compelling given the increase in sedentary habits acquired with remote learning during the pandemic [18].

Childhood obesity and scant physical activity are strongly related with poor physical health but less is known about their association with mental health [19]. Some studies, however, have already shown that obese children and those with low levels of physical activity have an impaired psychological wellbeing [20–23]. Moreover, obese children are often victims of bullying and weight stigmatization [24–26]. Therefore, these factors need to be considered in body mass index (BMI) surveillance programs and when planning interventions to improve not only physical but also psychological wellbeing.

Comprehensively investigating which are the characteristics of the overweight and poorly active children and adolescents is essential to design effective prevention programs. The aim of the present study is to provide up-to-date consistent knowledge on the prevalence and correlates of overweight and poor physical activity among Italian children and adolescents, with special focus on familial characteristics, lifestyle habits and psychological wellbeing, taking advantage of two Italian surveillance systems: OKKio alla Salute, on children aged 8–9 years, and Health Behaviour in School-aged Children (HBSC), on adolescents aged 11–15 years. In order to provide estimates on determinants of overweight and poor physical activity not affected by the geographical gradient, we focused our analysis to Lombardy (northern Italy), the most populous region counting one-sixth of the Italian population [27], with a population size comparable to Sweden and Portugal.

2. Materials and Methods

We used Lombardy regional data from the 2019 wave of the Italian National Surveillance System OKKio alla Salute, coordinated by the National Institute of Health (Istituto Superiore di Sanità) and from the 2018 wave of the HBSC survey.

OKKio alla Salute is a national nutritional surveillance system, part of the Childhood Obesity Surveillance Initiative (COSI) of the WHO Regional Office for Europe, established in 2007 with the objective of monitoring the nutritional status and lifestyle behaviors among primary school children. The survey involved children in the third grade of primary schools, aged 8–9 years. A cluster sampling method was used, with classes as the sampling unit. Sampling selection was done at the Local Health Unit level (often corresponding to the Province), with a sampling list of third primary classes provided by the corresponding Provincial School Offices [28]. For each school, the probability of having their classes extracted was proportional to the number of students enrolled. The final sample consisted of 166 classes and 3093 children, with anthropometric measurements provided by trained local health staff.

HBSC is an international multicenter study [29] conducted in more than 40 countries across Europe and North America, in collaboration with the Regional Office for Europe of the WHO with the aim of monitoring the nutritional status and gaining greater insight into determinants of health and wellbeing in adolescents. The HBSC study population comprises school young adolescents aged 11–15 years. The sampling procedure followed international guidelines. Classes were selected according to a systematic sampling method from the complete list of schools provided by the Italian Ministry of Education, University and Research. Cluster sampling was used to select participants, with school classes representing the primary sampling unit. In the Italian HBSC schools from Lombardy were oversampled to ensure sufficient statistical power to obtain robust frequency estimates at a regional level [30]. In all, 2916 students from 188 classes, self-reporting height and weight, were included in the study.

Details on sampling methods and data collection in the two surveys are reported elsewhere [28,31]. The protocol of the OKKio alla Salute survey was approved by the Institutional Ethical Board of the National Institute of Health (General protocol: ISS AOO-ISS 12/12/2018 0037697). The protocol of the HBSC survey was approved by the Institutional Ethical Board of the National Institute of Health (General protocol: PRE-876/17).

2.1. Outcome Measures

Body mass index (BMI) was calculated according to the sex-and-age specific international Cole's cut-offs proposed by the International Obesity Task Force (IOTF) [32,33]. Underweight corresponded to a BMI value equivalent to a BMI lower than 18.5 kg/m^2 extrapolated for the 18 years population, the overweight definition corresponded to a BMI value equivalent to a BMI ranging between 25–29 kg/m^2 extrapolated for the 18 years population and the obesity definition corresponded to a BMI value $\geq$ 30 kg/m^2 extrapolated for the 18 years population, for age and sex. Children and adolescents' nutritional status was classified accordingly. Additionally, for both age groups we performed the BMI z-scores according to the growth charts developed in 2000 from the Centers for Disease Control and Prevention (CDC) for the United States [34]. BMI was classified into four groups, including underweight (<−2 standard deviations, SD), normal weight (−2 to 1 SD), overweight (1 to 2 SD) and obesity (>2 SD) according to sex and age-specific cut-offs of BMI z-scores recommended by WHO in 2007. Chronic malnutrition was further defined as a z-score <−3 SD [35]. In the OKKio alla Salute survey, information on physical activity was derived from a structured questionnaire filled in by children's parents, who provided the average number (from 0–7) of days per week the child practiced sport or played for at least 1 hour. In the HBSC survey, physical activity was provided by adolescents as the average number (from 0–7) of days per week the adolescent practiced physical exercise for at least 1 hour. Children and adolescents' physical activity was then classified according to a tertile

distribution, as low (poor physical activity, equal to fewer than 3 days/week), intermediate (3 days/week) and high (4 days/week or more).

A section of the questionnaire focused on perceived psychological wellbeing and on possible perceived threats to it: adolescents reported how often they felt low, nervous or irritable (providing a score between 1, approximately every day, to 5, rarely or never). Psychological distress was defined as the average value of the three scores. The four variables where then categorized in often, occasionally and rarely, according to the tertiles. Adolescents also reported if they had been victims of bullying in the previous two months.

2.2. Other Measures

In OKKio alla Salute, parents [28,31] provided information on their level of education, self-reported socio-economic status, nationality, their child's birth weight and the average amount of time per day their child spent watching TV and played with the computer or other digital devices. Parents also provided their self-reported height and weight, that were used to derive BMI [36]. In the HBSC study [28,31], adolescents provided information on their nationality (i.e., Italian vs. not Italian), their parents' highest level of education and their family socio-economic status, obtained as a combination of selected variables: adolescents reported if they had a family car, if they had their own bedroom, the number of computers owned by their family, if they had a dishwasher at home, the number of bathrooms at home and if they had been on any family holidays outside of Italy the year before. A total score was derived from the previous questions and adolescents' socio-economic status was classified as low, intermediate and high, accordingly. They also reported the average amount of time per day they spent watching TV and playing with the computer or other digital devices.

2.3. Statistical Analysis

The normality of the distribution was tested for anthropometric measures, and non-normally distributed variables were expressed as medians and interquartile ranges. Chi-squared tests were used to compare non-continuous variables. Assuming a sample size of 3000 subjects and an overall conservative prevalence of overweight of 14% (and alpha = 0.05), the data had sufficient statistical power (>0.80) to observe an odds ratio (OR) of 1.33, for an equally distributed dichotomous exposure variable. We used unconditional multiple logistic regression models to calculate the odds ratios (OR) and corresponding 95% confidence intervals (CI) for overweight (including obesity) and poor physical activity. All the models were adjusted for selected socio-demographic variables, i.e., sex, age (for HBSC data) and parents' highest level of education. The ORs for overweight (including obesity) were further adjusted for physical activity (tertiles) and those for poor physical activity were adjusted for BMI (normal/underweight, overweight and obese). Additional sex-stratified analyses were performed for the ORs for overweight (including obesity), adjusting for the same variables. We also performed separate multiple logistic regression models to investigate if overweight, obesity and scant physical activity were determinants of each outcome of poor mental health: feeling low, nervous, irritable, having general psychological distress and being victims of bullying. All the models were adjusted for the same variables. A statistical weight was applied to OKKio alla Salute analyses to guarantee the representativeness of the sample at regional or LHU levels. All statistical analyses were done with software SAS, version 9.4 (Cary, NC, USA).

3. Results

3.1. BMI and Physical Activity

Table 1 shows the distribution of BMI categories and levels of physical activity among the OKKio alla Salute and HBSC study participants. Of the 3093 children aged 8–9 years, 2.3% were underweight, 75.3% normal weight, 17.6% overweight and 4.8% obese, with no significant differences between males and females ($p = 0.109$). On the total sample of 2916 adolescents aged 11–15 years, 2.9% were underweight, 83.2% normal weight, 12.2%

overweight and 1.8% obese, with a higher proportion of overweight and obese adolescents among males ($p < 0.001$). A significant difference of physical activity levels was observed by gender, with a higher proportion of poorly active children and adolescents among females ($p < 0.001$). The distribution of BMI and levels of physical activity statistically significantly differed by age category ($p = 0.001$ for BMI and $p < 0.001$ for physical activity).

Supplementary Figure S1 shows the distribution of BMI levels by sex according to the z-scores. In children aged 8–9 years, the mean z-score was 0.21 (SD = 1.19) for males and 0.09 (SD = 1.10) for females. In adolescents, the mean z-score was 0.02 (SD = 1.19) for males aged 11 years, 0.12 (SD = 1.03) for those aged 13 years and −0.06 (SD = 1.15) for those aged 15 years. The corresponding values in female adolescents were −0.32 (SD = 1.08), −0.15 (SD = 1.00) and −0.19 (SD = 0.84), respectively. In adolescents, chronic malnutrition was 1.3% in males and 0.9% in females (data not shown in tables).

3.2. Sex, Socio-Demographic and Familial Findings

Table 2 shows the ORs and 95% CIs for childhood overweight (including obesity) and poor physical activity, according to selected socio-demographic and family characteristics, in children aged 8–9 years (OKKio alla Salute survey) and 11–15 years (HBSC survey). Among adolescents aged 11–15 years, males were more frequently overweight or obese (OR = 2.11; 95% CI: 1.69–2.64), and in both age groups they were less frequently inactive, compared to females (OR = 0.58; 95% CI: 0.50–0.68 for children aged 8–9 years; OR = 0.61; 95% CI: 0.52–0.71 for those aged 11–15 years). Poor physical activity was more frequent with increasing age (p for trend < 0.001) and both overweight and poor physical activity were less frequent with increasing levels of parents' education (p for trend from <0.001 to 0.016) in both age groups. Higher socio-economic status was related to a decreased prevalence of overweight or obesity (p for trend <0.001 for children aged 8–9 years; p for trend = 0.010 for those aged 11–15 years) and poor physical activity (p for trend <0.001 for adolescents aged 11–15 years). Children aged 8–9 years with a foreign nationality were less active than Italian children of the same age (OR = 1.61; 95% CI: 1.31–1.98). Overweight status was more reported with increasing child's birth weight (p for trend <0.001) and in children with at least one parent obese (OR = 3.90; 95% CI: 2.98–5.11).

3.3. Overweight, Physical Activity and Time Spent on Screen

In both age groups, overweight was less frequent with increasing time spent for physical activity (p for trend <0.001 for children aged 8–9; p for trend = 0.001 for those aged 11–15 years; Table 3). In adolescents aged 11–15, overweight and poor physical activity were both related to greater time spent watching TV (p for trend <0.001) and, in both age groups, with increasing time spent playing with the computer or other digital devices (p for trend between 0.001 and 0.012). Poor physical activity was more frequently reported with increasing BMI (p for trend 0.004 for children aged 8–9 years; p for trend <0.001 for those aged 11–15 years).

Table 1. Percentages (%) of children from the OKKio alla Salute and HBSC surveys, according to various anthropometric measures. Lombardy, 2018–2019.

Characteristics	OKKIO alla Salute Age 8–9 Years %			HBSC Age 11–15 Years %					
	Total	Gender		Total	Gender		Age (Years)		
		Males	Females		Males	Females	11	13	15
No. of children	*3093*	*1595*	*1498*	*2916*	*1461*	*1455*	*859*	*1003*	*1054*
Weight (kg), median (IQR)	30.0 (26.4–34.8)	30.4 (26.8–35.3)	29.5 (26.0–34.3)	50.0 (43.0–60.0)	54.0 (43.0–64.0)	50.0 (42.0–55.0)	40.0 (36.0–46.0)	51.5 (45.0–60.0)	58.0 (52.0–65.0)
Height (cm), median (IQR)	133.5 (129.0–137.8)	133.8 (129.9–138.4)	132.9 (128.0–137.2)	163.0 (155.0–170.0)	166.0 (155.0–175.0)	161.0 (155.0–167.0)	152.0 (146.0–158.0)	165.0 (159.0–170.0)	170.0 (164.0–175.0)
BMI (kg/m^2), median (IQR)	16.6 (15.1–18.6)	16.6 (15.2–18.8)	16.5 (15.1–18.6)	19.1 (17.3–21.2)	19.4 (17.6–21.6)	18.9 (17.1–20.8)	17.6 (16.0–19.6)	19.1 (17.5–21.3)	20.1 (18.4–21.9)
BMI categories									
Underweight	2.3	1.9	2.8	2.9	2.5	3.2	4.3	1.9	2.6
Normal weight	75.3	75.1	75.5	83.2	79.2	87.2	81.6	81.9	85.7
Overweight	17.6	17.6	17.7	12.2	16.0	8.5	11.8	14.7	10.3
Obese	4.8	5.5	4.0	1.8	2.3	1.2	2.3	1.6	1.5
Physical activity (approximate tertiles) [1]									
Low	32.9	27.2	38.9	32.8	27.6	38.0	25.1	31.8	40.0
Intermediate	29.2	31.0	27.3	19.6	17.7	21.6	19.4	20.0	19.5
High	37.9	41.7	33.8	47.5	54.7	40.4	55.5	48.2	40.5

IQR: Interquartile range. [1] In both surveys: <3 days per week/3 days per week/≥4 days per week. We excluded 45 children from OKKio alla Salute and 24 from HBSC because they did not provide information on physical activity.

Table 2. Odds ratios (OR) and corresponding 95% confidence intervals (CI) for childhood overweight (including obesity) and poor physical activity according to selected socio-demographic and family characteristics. Lombardy, 2018–2019.

Characteristics	Overweight [1]		Poor Physical Activity [2]	
	OR (95% CI)		OR (95% CI)	
	OKKio alla Salute Age 8–9 Years	HBSC Age 11–15 Years	OKKio alla Salute Age 8–9 Years	HBSC Age 11–15 Years
Total, No.	*3021*	*2833*	*3048*	*2892*
Sex				
Female	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Male	1.13 (0.95–1.34)	**2.11 (1.69–2.64)**	**0.58 (0.50–0.68)**	**0.61 (0.52–0.71)**
Age category				
11	-	1.00°	-	1.00°
13	-	1.14 (0.87–1.51)	-	**1.52 (1.22–1.89)**
15	-	0.79 (0.58–1.06)	-	**2.18 (1.75–2.71)**
p for trend	-	0.079	-	**<0.001**
Highest parental education				
Low	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Intermediate	**0.71 (0.57–0.90)**	0.76 (0.49–1.17)	**0.78 (0.63–0.96)**	0.73 (0.52–1.01)
High	**0.55 (0.43–0.70)**	**0.58 (0.37–0.91)**	**0.74 (0.59–0.92)**	**0.58 (0.41–0.82)**
p for trend	**<0.001**	**0.009**	**0.016**	**0.001**
Family socio-economic status				
Low	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Intermediate	**0.71 (0.59–0.86)**	0.87 (0.67–1.14)	**0.79 (0.67–0.95)**	**0.72 (0.59–0.88)**
High	**0.55 (0.41–0.73)**	**0.65 (0.47–0.90)**	1.02 (0.80–1.30)	**0.63 (0.50–0.80)**
p for trend	**<0.001**	**0.010**	0.482	**<0.001**
Nationality				
Italian	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Other	1.23 (0.98–1.54)	1.31 (0.80–2.14)	**1.61 (1.31–1.98)**	0.87 (0.58–1.32)
Birth weight (g) [4]				
<2500	1.00 [3]	-	1.00 [3]	-
2500–3300	1.08 (0.75–1.56)	-	0.83 (0.63–1.11)	-
>3300	**1.72 (1.20–2.47)**	-	0.81 (0.61–1.09)	-
p for trend	**<0.001**	-	0.267	-
Parental BMI [4]				
Both parents normal weight	1.00 [3]	-	1.00 [3]	-
At least one parent overweight (not obese)	**1.95 (1.56–2.44)**	-	1.08 (0.90–1.29)	-
At least one parent obese	**3.90 (2.98–5.11)**	-	1.06 (0.83–1.37)	-
p for trend	**<0.001**	-	0.508	-

[1] ORs for overweight (overweight/obesity vs. normal weight) were calculated in unconditional multiple logistic regression models, after adjustment for sex, parents' highest level of education and physical activity (low, intermediate, high); HBSC data were further adjusted for age. Underweight children and adolescents were excluded from the analyses. Estimates in bold type are significant at 0.05. [2] ORs for poor physical activity (first vs. second and third tertiles of physical activity) were calculated in unconditional multiple logistic regression models, after adjustment for sex, parents' highest level of education and BMI (underweight/normal weight, overweight, obese); HBSC data were further adjusted for age; 45 children from OKKio alla Salute and 24 from HBSC did not provide information on physical activity and were therefore excluded. Estimates in bold type are significant at 0.05. [3] Reference category. [4] Information on the child's birth weight and parents' BMI were not available for the HBSC survey.

Table 3. Odds ratios (OR) and corresponding 95% confidence intervals (CI) for childhood overweight (including obesity) and poor physical activity according to selected lifestyle habits and other characteristics. Lombardy, 2018–2019.

Characteristics	Overweight [1]		Poor Physical Activity [2]	
	OR (95% CI)		OR (95% CI)	
	OKKio alla Salute Age 8–9 Years	HBSC Age 11–15 Years	OKKio alla Salute Age 8–9 Years	HBSC Age 11–15 Years
Physical activity				
Low	1.00 [3]	1.00 [3]	-	-
Moderate	0.82 (0.66–1.02)	0.85 (0.63–1.14)	-	-
High	**0.67 (0.54–0.82)**	**0.66 (0.52–0.85)**	-	-
p for trend	**<0.001**	**0.001**	-	-
Time spent watching TV (approximate tertiles) [4]				
Low	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Intermediate	1.11 (0.87–1.41)	1.09 (0.81–1.46)	0.90 (0.72–1.12)	0.90 (0.73–1.11)
High	1.18 (0.95–1.46)	**1.81 (1.40–2.33)**	1.19 (0.99–1.44)	**1.44 (1.19–1.74)**
p for trend	0.132	**<0.001**	0.091	**<0.001**
Time spent playing with the computer (approximate tertiles) [5]				
Low	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Intermediate	1.14 (0.90–1.45)	1.26 (0.93–1.71)	1.14 (0.93–1.40)	1.18 (0.95–1.45)
High	**1.46 (1.15–1.85)**	**1.65 (1.22–2.22)**	**1.37 (1.10–1.69)**	**1.31 (1.06–1.62)**
p for trend	**0.002**	**0.001**	**0.004**	**0.012**
BMI				
Underweight	-	-	**1.65 (1.01–2.70)**	1.49 (0.94–2.36)
Normal weight	-	-	1.00 [3]	1.00 [3]
Overweight	-	-	**1.28 (1.05–1.57)**	**1.29 (1.02–1.65)**
Obese	-	-	**1.71 (1.21–2.42)**	**2.33 (1.32–4.13)**
p for trend	-	-	**0.004**	**<0.001**

[1] ORs for overweight (overweight/obesity vs. normal weight) were calculated in unconditional multiple logistic regression models, after adjustment for sex, parents' highest level of education and physical activity (low, intermediate, high). HBSC data were further adjusted for age. Underweight children and adolescents were excluded from the analyses. Estimates in bold type are significant at 0.05. [2] ORs for poor physical activity (first vs. second and third tertiles of physical activity) were calculated in unconditional multiple logistic regression models, after adjustment for sex, parents' highest level of education and BMI (underweight/normal weight, overweight, obese); HBSC data were further adjusted for age; 45 children from OKKio alla Salute and 24 from HBSC did not provide information on physical activity and were therefore excluded. Estimates in bold type are significant at 0.05. [3] Reference category. [4] In OKKio alla Salute: <1.17 h per day/1.17–1.54 h/≥1.55 h per day. In HBSC: <1.17 h per day/1.17–2.22 h/≥2.23 h per day. [5] In OKKio alla Salute: <34 min per day /34 min–1.16 h/≥1.17 h per day. In HBSC: <38 min per day/38 min −1.51 h/≥1.52 h per day.

3.4. Perceived Nervousness, Irritability and Experienced Bullying

In adolescents, obese subjects reported more frequently nervousness (OR = 2.37; 95% CI: 1.32–4.26), general psychological distress (OR = 2.44; 95% CI: 1.12–5.27) and bullying episodes in the previous two months (OR = 2.25; 95% CI: 1.17–4.34; Table 4). Feelings of irritability increased with an increasing level of BMI (*p* for trend = 0.038). Increasing physical activity significantly decreased the frequency of all mental health outcomes (*p* for trend $\leq$ 0.002).

Table 4. Odds ratios [1] (OR) and corresponding 95% confidence intervals (CI) for selected mental health outcomes by body mass index (BMI) levels and physical activity. Lombardy, 2018.

Characteristics	HBSC Age 11–15 Years				
	Nervous	Feeling Low	Irritable	Psychological Distress [2]	Being Bullied
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
BMI levels					
Underweight	0.89 (0.56–1.40)	1.16 (0.73–1.83)	0.83 (0.53–1.31)	0.97 (0.60–1.59)	1.42 (0.79–2.53)
Normal weight	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Overweight	1.15 (0.91–1.45)	1.18 (0.93–1.49)	1.20 (0.95–1.52)	1.09 (0.84–1.40)	1.25 (0.91–1.72)
Obese	**2.37 (1.32–4.26)**	1.36 (0.76–2.45)	1.44 (0.79–2.62)	**2.44 (1.12–5.27)**	**2.25 (1.17–4.34)**
p for trend	**0.007**	0.203	**0.038**	0.075	0.069
Physical activity					
Low	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]	1.00 [3]
Moderate	**0.66 (0.53–0.81)**	**0.76 (0.61–0.95)**	**0.77 (0.62–0.96)**	**0.66 (0.52–0.84)**	**0.67 (0.49–0.93)**
High	**0.74 (0.62–0.88)**	**0.75 (0.63–0.89)**	**0.67 (0.56–0.80)**	**0.62 (0.51–0.75)**	**0.72 (0.56–0.92)**
p for trend	**<0.001**	**0.002**	**<0.001**	**<0.001**	**0.001**

[1] ORs for all mental health outcomes were calculated in unconditional multiple logistic regression models, after adjustment for sex, age group, parents' highest level of education and mutually for BMI levels and physical activity. Estimates in bold type are significant at 0.05. [2] Psychological distress was computed as the average score of the three previous measures (nervousness, feeling low, irritability). [3] Reference category.

Distribution and percent prevalence of overweight (including obese) and poor physical activity for each variable are reported in Supplementary Tables S1 and S2. Supplementary Tables S3 and S4 show the ORs and corresponding 95% CIs for childhood overweight (including obesity), stratified by sex. No substantial differences were observed.

4. Discussion

In 2018–2019 in northern Italy the prevalence of overweight (including obesity) was 22% in children aged 8–9 years and 14% in adolescents aged 11–15 years. The prevalence was higher in males, in families with a low socio-economic status and obese parents, and in those reporting longer time at a screen. Poor physical activity increased with age and was higher in females. Overweight and poor physical activity were both related to several psychological wellbeing shortcomings (i.e., feeling nervous, irritable) and having been a victim of bullying.

In all, the prevalence of childhood overweight in northern Italy was substantially lower than in Europe as a whole, estimated at 29% for boys and 27% for girls aged 7–9 years, and 25% for boys and 16% for girls aged 11–15 years [5,6]. Compared to the other Italian regions, Lombardy estimates showed a substantially lower prevalence of overweight [37,38], confirming a national north–south gradient [10] not only for adults [9]. Comparing our estimates with the corresponding Lombardy figures of previous waves (2014–2016), the prevalence of overweight remained substantially stable [39,40].

Adolescence represents a special transition period from childhood to adulthood [41], encompassing elements of psychological, biological and hormonal changes and major social role transitions, that complicate a comparison with other periods of life. However, our data, estimated using two different methods (Cole's cut-offs and z-scores), are in line with a decrease in overweight (including obesity) prevalence from pre-pubertal age to pubertal age [5,6]. Self-reported measures in adolescents may possibly suffer reporting bias [42,43], resulting in slightly lower BMI; nevertheless, our data are consistent with those from the National Institute of Statistics, collecting data with homogeneous methodology for all age groups [8]. The already documented sharp decrease in overweight and obesity prevalence with age in Italy might be partially explained by present policies or natural course. Body dissatisfaction and the "thin-ideal of feminine beauty" are phenomena documented in adolescence, particularly in females [44,45]. These attitudes might contribute to the attenuation in overweight prevalence in pubertal age, leading to a consequent higher prevalence in

malnutrition among girls. However, when comparing adolescent males and females in our data, the prevalence of chronic malnutrition was not higher among females. Among the reasons for the observed decrease in overweight prevalence with age, we can also include the possibly less healthy dietary habits of children compared to adolescents. This has been observed by a previous Italian study [46], showing a lower prevalence of good adherence to Mediterranean diet in students attending primary than secondary school, suggesting that younger children are more subjected to unhealthy choices. This result is in contrast with the majority of data from other countries reporting a negative trend in Mediterranean diet adherence with age [47,48]. In addition, the relatively high prevalence of overweight among Italian children could be related to the restricted time devoted to physical activity in the Italian primary schools compared to other countries.

Our data confirm the higher prevalence of overweight among males, and poor physical activity among females, in line with Italian and European estimates on children [5,6,49–52]. In agreement with recent European estimates, poor physical activity was more frequent with increasing age [13].

We confirm the well-known relationship between low socio-economic status, including parental education, and childhood overweight, obesity and poor physical activity [49]. Moreover, our estimates indicated that, particularly among children aged 8–9 years, those with a non-Italian nationality appeared at increased risk of overweight (or obesity) and poor physical activity. These results agree with other studies [53,54] and are possibly explained by the low socio-economic level of families with a foreign nationality or by the lack of knowledge among immigrant parents about available facilities and opportunities for physical activity for their children [54,55]. Therefore, preventing campaigns should focus particularly on low-socio-economic and foreign families.

Overweight and poor physical activity were more prevalent in children with at least one obese parent. These results suggest that an obese family might be a key target for intervention efforts to prevent overweight and promote adequate physical activity among these children [56,57]. In addition, as already shown [58,59], higher birth weight was a major determinant for overweight, suggesting that prenatal factors including genes and nutrition play major roles in wellness and BMI later in life.

Our findings confirmed that longer time watching TV and playing videogames was related to overweight and poor physical activity [60,61]. Parents should be aware of this direct relationship and regulate the screen time of their children. Sedentary behavior has also been associated with other unhealthy habits such as snacking on junk food [62,63]. This is particularly critical since leisure time is increasingly spent in sedentary pursuits [62,63].

In line with current literature, higher levels of BMI and poorer physical activity were related to lower psychological wellbeing, particularly in the form of feeling discomfort, such as irritability and feeling nervous [21–23]. In addition, adolescents who had been victims of bullying were more frequently obese and with low levels of physical activity, confirming findings from other studies highlighting the risk factors implied in such events for both psychological and physical health [24,25]. In school, weight-based bullying is among the most frequent forms of peer harassment reported by students [24,25], and weight stigmatization can further sharpen unhealthy eating behaviors and reduce physical activity [26]. Media campaigns should stress that preventing obesity has important implications not only for the physical health of children but also for their moods and psychological wellbeing. However, any dietary education and overweight prevention program, at any age group, must focus on the development of a correct relationship with food and pay particular attention to avoid the risk of developing any negative behavior, as, for example, eating disorders.

Our study needs to be interpreted in light of some limitations, mainly inherent to the cross sectional study design, not allowing us to derive any causal inference from the relationships observed. Another limitation is the self-reporting of anthropometric measures by adolescents aged 11–15 years. Moreover, the different methods of data collection used in the two surveys did not allow us to make any comparisons between the two

studies. In addition, differences in sampling methodology may have caused systematic discrepancies in the two samples. Thus, for example, the proportion of foreign families was 17% among children aged 8–9 years and 4% among adolescents aged 11–15 years. In addition, since data for children and adolescents come from two different surveys (designed for different target populations), some questions were formulated differently. This may have further accentuated the observed differences between the two surveys. In addition, being this is a population-based study, we did not have the possibility to investigate any clinical data, particularly important when studying overweight correlates. New data considering clinically relevant measures are therefore required to have a clearer and more complete evaluation of correlates of overweight. Among the strengths, we can include the large sample size that enabled us to detect even the smallest differences, the representativeness of the two samples, collected using two national surveys, and the measured anthropometric data in the OKKio alla Salute survey. Another strength of this study is the comprehensiveness of the two surveys, produced in collaboration with the Regional Office for Europe of the WHO, considering exhaustively several (not frequently studied) aspects related to overweight and poor physical activity, such as psychological wellbeing and bullying, that represent the added value of this study, enabling a clearer definition of the characteristics of children and adolescents affected by overweight or obesity. In addition, limiting our cover to the Lombardy region meant we had no heterogeneous data due to differences across the country. The Lombardy region represents the most populous Italian region, with approximately 10 million inhabitants (one-sixth of Italy). Although having restricted our study to a homogeneous population is a strength of our study, in order to have a broader evaluation of the determinants of overweight, data from different countries should be collected and compared.

In conclusion, in northern Italy three out of four children or adolescents are of normal weight. Obesity intervention programs should particularly target and prioritize low socio-economic families and those with overweight or obese parents.

Special attention needs to be paid to psychological wellbeing in schools, by monitoring and possibly reducing specific threats or risk factors such as bullying or negative moods (i.e., feeling nervous and irritable). Policymakers and other stakeholders, including parents, should also increase the opportunities for young people to participate in daily physical activity and explore solutions to reduce excessive screen time in order to preserve the wellbeing of children and adolescents. Future research should evaluate the efficacy of population prevention programs focused on these identified fragile subgroups.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14112258/s1. Figure S1: Distribution of children aged 8–9 years and adolescents aged 11–15 years according to BMI levels measured with z-scores. Table S1: Frequencies and percent distribution (%) of overweight (including obesity) and poor physical activity according to selected socio-demographic and family characteristics. Lombardy, 2018–2019. Table S2: Frequencies and percent distribution (%) of overweight (including obesity) and poor physical activity according to selected lifestyle habits and other characteristics. Lombardy, 2018–2019. Table S3: Odds ratios (OR) and corresponding 95% confidence intervals (CI) for childhood overweight (including obesity) stratified by sex, according to selected socio-demographic and family characteristics. Lombardy, 2018–2019. Table S4: Odds ratios (OR) and corresponding 95% confidence intervals (CI) for childhood overweight (including obesity) stratified by sex, according to selected lifestyle habits and other characteristics. Lombardy, 2018–2019.

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Impact of Lifestyle Modifications on Alterations in Lipid and Glycemic Profiles and Uric Acid Values in a Pediatric Population

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Abstract: Cardiometabolic risk factors are frequent in children and adolescents with excess weight. The aim of this study was to evaluate the effects of lifestyle modifications on alterations in lipid and glycemic profiles and uric acid values in a pediatric population at increased cardiovascular risk. The study involved 276 subjects with a mean age of 10.6 (2.3) years. Body mass index (BMI) z-score and biochemical parameters (serum low-density lipoprotein (LDL) cholesterol, triglycerides and uric acid and homeostasis model assessment to quantify insulin resistance (HOMA index)) were assessed at baseline and at the end of a median follow-up of 14.7 (12.4, 19.3) months. Throughout follow-up, all children received a non-pharmacological treatment based on increased physical activity, reduced sedentary activity and administration of a personalized, healthy and balanced diet. All children attended periodic quarterly control visits during follow-up. Multivariable statistical analyses showed that each BMI z-score point reduction at follow-up was associated with an 8.9 (95% CI −14.2; −3.6) mg/dL decrease in LDL cholesterol ($p = 0.001$), 20.4 (95% CI −30.0; −10.7) mg/dL in triglycerides ($p < 0.001$), 1.6 (95% CI −2.2; −1.0) in HOMA index ($p < 0.001$), and 0.42 (95% CI −0.66; −0.18) mg/dL in uric acid ($p = 0.001$) values. At each reduction of the BMI z-score by one point, the odds of presenting with insulin resistance and hyperuricemia at follow-up significantly decreased (OR 0.23, 95% CI 0.10–0.50, and OR 0.32, 95% CI 0.10–0.95, $p < 0.001$ and $p < 0.05$, respectively). Improvement of dietary habits and lifestyles may improve lipid and glycemic profiles and serum uric acid values in a pediatric population.

Keywords: children; lifestyle modifications; cardiovascular risk; overweight; obesity; dyslipidemia; insulin resistance; uric acid

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1. Introduction

Healthy lifestyles and diets are recommended for everyone and at all ages, to maintain good health and prevent non-communicable diseases. In the presence of cardiovascular risk factors, all guidelines propose a dietary-behavioral intervention as a first step [1–4]. This approach is especially recommended for children and adolescents, populations in which medication is used only in very select cases [5–7]. Although in children there is no precise definition of metabolic syndrome due to the lack of agreement of scientific societies on the cut off values of individual parameters, it is accepted that, even in childhood and adolescence, obesity is frequently associated with excess visceral adiposity, glucose metabolism disorders, increased triglycerides and decreased HDL cholesterol. In addition, an increase in serum uric acid values has been shown in overweight and/or hypertensive children and adolescents [8–10]. Therefore, cardio-metabolic risk factors, such alterations in lipid and glycemic profile and hyperuricemia, are frequently present in children and

adolescents that are overweight or obese. There is evidence on the effectiveness of non-pharmacological dietary-behavioral treatment in reducing both excess weight and blood pressure values in children and adolescents with excess weight [11,12]. Two meta-analyses showed a positive effect of lifestyle modification and exercise training on LDL cholesterol and triglyceride values and insulin resistance markers in this population [13,14]. However, in the scientific literature, the information on the effectiveness of dietary-behavioral intervention administered as a therapy in children and adolescents with excess weight to correct altered metabolic profiles is still scarce and fragmentary. The aim of this study is to evaluate the effects of dietary-behavioral treatment on alterations in lipid and glycemic profiles and uric acid values in a population of children and adolescents referred to a second-level outpatient clinic for cardiovascular risk assessment in pediatric age.

2. Materials and Methods

2.1. Subjects

We studied a cohort of 276 children and adolescents (4–18 years), consecutively referred from 16 December 2002 to 12 September 2017 to our Unit for Cardiovascular Risk Assessment in Children (at the Istituto Auxologico Italiano, Milan, Italy) by their primary care pediatricians, because of evidence of excess weight and/or alterations in the lipid and/or glycemic profile and/or plasma uric acid values. Exclusion criteria were: impaired glucose tolerance, diabetes, any form of secondary hypertension, and treatment with antihypertensive drugs. The Unit for Cardiovascular Risk Assessment in Children included a pediatrician, a cardiologist, a nephrologist and a nutrition expert. The team interacted to cover all clinical aspects related to the patients.

2.2. Baseline and Follow-Up Assessments

In all children, anthropometric and biochemical parameters were assessed once at baseline and a second time at the end of follow-up. Between the baseline and the final follow-up assessment, periodic visits were performed every three months, during which the children's anthropometric parameters were again recorded. The median follow-up was 14.7 (12.4, 19.3) months. Between the baseline and final assessments, a minimum of three visits and a maximum of six visits were performed in each child. All children consecutively referred to our center who had two complete assessments (at baseline and follow-up) of all outcomes (anthropometric parameters, complete lipid profile, glycemic profile with insulin dosage, and uric acid) were considered for analysis (Supplementary Figure S1). Follow-up ended in September 2017.

2.3. Anthropometric Parameters

Height, weight and waist circumference (WC) were measured. Weight was approximated to the nearest 100 g, and height to the nearest 1 mm. Waist circumference was measured to the nearest 0.5 cm by a non-elastic flexible tape in standing position. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. Waist-to-height-ratio (WtHr) was calculated by dividing WC by height. BMI z-scores were calculated using the Centre for Disease and Control prevention charts available at https://www.cdc.gov/growthcharts/clinical_charts.htm (accessed on 27 January 2022). Weight class was defined according to the International Obesity Task Force classification [15], distinguishing between normal weight (NW), overweight (OW) and obese (OB) classes. Pubertal stage was assessed by a medical examination and children were classified into two categories: pre-pubertal and pubertal according to Tanner [16], considering prepubertal boys with gonadal stage 1 and girls with breast stage 1.

2.4. Biochemical Parameters

Blood samples were taken from all subjects after a 12-h fasting period in order to measure serum concentrations of total cholesterol, high density lipoprotein (HDL), triglycerides, glucose, insulin, uric acid and creatinine. Commercial kits, normally used for routine exam-

inations of patients, were employed for all analyses. In detail: enzymatic colorimetric test Cholesterol Gen.2 Cobas Roche, for total cholesterol assay; colorimetric enzymatic test in homogeneous phase HDL-Cholesterol Gen.4 Cobas Roche, for HDL cholesterol; enzymatic colorimetric test Triglycerides Cobas Roche, for triglycerides assay; enzymatic method with hexokinase Glucose HK Gen.3 Cobas Roche, for glucose assay; immunoassay in Electro-ChemiLuminescence Elecsys Insulin Cobas Roche, for insulin assay; colorimetric enzymatic test Uric Acid 2 Cobas Roche, for uric acid assay; and the colorimetric kinetic test based on the Jaffé method, Creatinine Jaffé Gen.2 Cobas Roche, for creatinine assay. LDL cholesterol was calculated using Friedewald's formula, LDL cholesterol = total cholesterol − [HDL cholesterol + (triglyceridemia/5)]. HOMA index was calculated by dividing the product of serum insulin (μU/mL) and serum glucose (mmol/L) by 22.5 [17]. Glomerular filtration rate was estimated (eGFR) by means of the Schwartz formula using serum creatinine and height measurements and a k constant of 0.55 [18].

2.5. Definition of Metabolic Variables

The definition of dyslipidemia was based on the reference values of the National Cholesterol Education Program (NCEP) Expert Panel on Cholesterol Levels in Children: LDL cholesterol $\geq$ 130 mg/dL, HDL cholesterol < 40 mg/dL, triglycerides $\geq$ 100 mg/dL (children < 10 years), or $\geq$130 mg/dL (children $\geq$ 10 years) [19]. Dyslipidemia was defined as the presence of altered values for at least one of these parameters (LDL cholesterol, HDL cholesterol, triglycerides).

Insulin resistance was defined as the presence of HOMA Index values > 90th percentile according to sex and age in normal-weight children and adolescents [20].

Hyperuricemia was defined as the presence of uric acid values greater than the 90th percentile for sex and age according to [21] 90th percentile cut-off points for boys, 4.5 mg/dL for 6–9 years, 5.7 mg/dL for 10–13 years, and 6.4 for 14–17 years; for girls, 4.8 mg/dL for 6–9 years, 5.2 mg/dL for 10–13 years, and 5.3 for 14–17 years.

2.6. Recommended Lifestyle Modifications

All the children and adolescents were advised to perform at least two/three hours of structured physical activity every week [22], to increase the time dedicated to movement-play and to reduce sedentary activities (videogames or TV watching) to no more than one hour daily, following the recommendations of the Italian Society of Pediatrics (https://sip.it/2017/09/25/la-sip-presenta-la-piramide-dellattivita-motoria/) (accessed on 28 January 2022).

The participants received general advice for a healthy and balanced diet (increasing the consumption of fruit, vegetables, non-fat milk and dairy products, and reducing the intake of simple sugars and eliminate soft drinks) with adequate salt consumption (no more than 5 g/day, i.e., 2 g of sodium) according to the World Health Organization (WHO) guidelines (www.who.int/nutrition/publications/guidelines/sodium_intake/en/) (accessed on 27 January 2022).

During the baseline visit, an expert nutritionist assessed the physical activity and eating habits of the children by interviewing their parents/caregivers. Based on the obtained data, appropriate changes in lifestyle and nutrition were proposed. A nutritional analysis was performed in order to prepare a personalized dietary scheme for each child, by a dedicated software (Dietosystem, DS Medica S.r.l., Milan, Italy). Balanced dietary patterns for what regards the content of macronutrients (carbohydrates 55%, lipids 30%, proteins 15% of energy intake) and correct content of free sugars, saturated fatty acids and fiber were then proposed to all participants.

Depending on the pathological conditions (excess weight, alteration of lipid profile, alteration of glycemic profile, elevated uric acid values), specific recommendations were provided. When a child presented more than one of these conditions, a combination of the different dietary patterns was proposed. For each pathological condition, the ratio-

nale for the dietary intervention was explained to caregivers, to stimulate caregiver and family empowerment.

Excess weight OW or OB subjects were supplied with a weekly dietary scheme, whose caloric content had been calculated on the basis of both the basal metabolic rate by the Schofield equation [23] and the functional metabolism [24], through the analysis of the child's physical activity and the usual energy expenditure during an average day. In younger children, a balanced dietary model was proposed, equal to the calculated energy expenditure (normocaloric regime), whereas in adolescents with severe excess weight, a mildly hypocaloric regime (−10%) was suggested.

Alterations of lipid profile Children with hypercholesterolemia were advised to completely eliminate foods containing trans-fatty acids, to reduce energy intake from saturated fatty acids to less than 5–10%, and to limit consumption of some meats, dairy products, chocolate, baked goods and fried and processed foods. Instead, the consumption of foods containing mono- and polyunsaturated fatty acids was favored. The intake of dietary cholesterol was not allowed to exceed 100 mg per 1000 kcal of the diet; to this end, the intake of foods of animal origin such as meat, egg yolks, shellfish and whole milk dairy products was limited, whereas the consumption of foods containing soluble fiber (whole grains, vegetables and legumes) and phytosterols was favored. In case of increased triglyceride levels at baseline, dietary advice included, besides aiming at a reduction of body weight, a limitation of calories deriving from free sugars to less than 10% of total calories, and in particular a reduction in the intake of fructose through the complete abolition of sugary drinks. In the case of mixed dyslipidemia, all the above indications were recommended and, in all participants, the consumption of fish (salmon, tuna and blue fish) was encouraged to increase the intake of ω3 [4,25].

Insulin resistance The dietary-behavioral treatment implemented to reduce HOMA index values coincided with that to reduce excess weight (see above). From a qualitative point of view, the consumption of non-starchy vegetables and fruits rich in fiber, vitamins, and minerals citrus fruits, legumes and preferably whole grains, lean meats, fresh cheeses, fish and nuts, and unsweetened dairy products was encouraged. On the other hand, the intake of sugary drinks, fruit juices, carbonated and soft drinks, starchy vegetables, such as potatoes, pumpkin and corn, processed snacks and canned foods sugary sweets, ice cream and chocolate was limited.

Elevated uric acid Children with high uric acid levels were instructed to completely eliminate all sugary drinks from their diet (soft and energy drinks, ready-to-drink teas, fruit juices and nectars, etc.) and to reduce consumption of sweet foods. To caregivers, a list of foods with high purine content was provided, with the recommendation to reduce as much as possible the intake of these foods (red meat, lamb and pork, sweetbreads, seafood and especially shellfish such as shrimps, lobsters, mussels, anchovies and sardines). The recommended foods with low purine content comprised non-fat dairy products, fresh fruit and vegetables, cereals and derivatives. Eggs, fish, chicken and white meats were allowed in moderation.

At each periodic visit, information to assess the compliance with diet modifications, increase in physical activity, decrease in time spent watching television or with videogames was requested from family members according to a panel of questions, and the answers were reported in the medical record. Reinforcement of dietary and of lifestyle recommendations was given when necessary.

2.7. Statistical Analysis

The characteristics of the cohort, overall, at baseline and at follow-up, were described as mean and standard deviation (SD) or median and interquartile range (IQR) if the variables were continuous, and as frequencies and percentages if they were categorical. Univariate analyses to compare the characteristics of the children at baseline and at follow-up were conducted through the *t*-test for paired data or the Wilcoxon signed-rank test for continuous variables, and by the McNemar test for categorical variables.

The Z-score value was calculated by the equation $((\text{BMI value}/M)^L - 1)/(L \times S)$ where L, M, and S parameters specific for age are available at this link https://www.cdc.gov/growthcharts/percentile_data_files.htm (accessed on 28 January 2022). The distribution of weight class and WtHr $\leq$ 50% or >50% at baseline and at follow-up was described by bar plots and the proportions were compared by an extension of the McNemar test for the marginal homogeneity of categorical data with more than two levels [26], or by the McNemar test.

The distributions of dyslipidemia, insulin resistance and hyperuricemia at baseline and at follow-up were displayed through alluvial plots.

2.7.1. Different Multiple Linear Regression Models Were Used to Assess

The impact of gender, age, puberty transition, the difference between BMI z-score at baseline and at follow-up, family history of dyslipidemia/diabetes on LDL cholesterol or HDL cholesterol or triglycerides or HOMA index or uric acid plasma values at follow-up. The models were adjusted for values of LDL cholesterol or HDL cholesterol or triglycerides or HOMA index or uric acid plasma at baseline.

2.7.2. Multiple Logistic Regression Models Were Used to Assess

The impact of gender, age, puberty transition, the difference between BMI z-score at baseline and at follow-up, family history of dyslipidemia/diabetes on dyslipidemia or insulin resistance or hyperuricemia at follow-up. The models were adjusted for the presence of dyslipidemia or insulin resistance or hyperuricemia at baseline. The same linear and logistic regression models were performed including the difference between WtHr at baseline and at follow-up as covariate instead of the difference between BMI z-scores.

Statistical analyses were performed with R 4.1.2 (http://www.R-project.org) (accessed on 28 January 2022). All *p*-values were two-sided, with *p*-values < 0.05 considered statistically significant.

3. Results

The study involved 276 children with a mean age of 10.6 (SD = 2.3) years (56% were males). Table 1 shows the clinical characteristics and hematochemical variables of the population at baseline and at the end of follow-up.

At recruitment, 14% of children were NW, 35% were OW, and 50% were OB (Figure 1A), and 70% of subjects had a WtHr > 50% (Figure 1B). Forty-three patients (15.8%) had a family history of diabetes mellitus, and 117 (42.9%) had a family history of dyslipidemia. At least one alteration in plasma lipid values was present in 69 children (25.0%), 157 (56.9%) had HOMA index values > 90th percentile, and 46 (16.7%) had uric acid levels > 90th percentile. At the end of the follow-up period, BMI z-score and WtHr had significantly decreased (from 1.8 to 1.5 and from 53.6% to 50.7%, respectively, $p < 0.001$). The proportion of NW subjects had significantly increased, whereas the proportion of OB subjects was significantly lower than at baseline ($p < 0.001$) (Figure 1A). Only 50% of the study population had WtHr > 50% at the end of follow-up ($p < 0.001$) (Figure 1B). Changes in the prevalence of normal weight, overweight, obesity and WtHr > 50% are shown in Figure 1C,D. In detail, 40 of 237 (16.9%) children with excess weight at baseline were NW at follow-up and 63 (26.6%) had improved their weight class. In contrast, the number of subjects with a worsening of weight class was 8 out of 137 (5.8%). Regarding the prevalence of WtHr > 50%, 55 of 192 (28.6%) subjects had normalized their WtHr values (i.e., WtHr $\leq$ 50%) at follow-up, whereas 3 of 83 (3.6%) children with WtHr $\leq$ 50% at baseline showed elevated WtHr values (i.e., >50%) at follow-up. The percentage of children with HOMA index > 90th percentile at the end of follow-up was significantly lower than that at baseline (56.9% vs. 45.3%, $p = 0.002$), whereas we did not observe any significant change in the number of subjects with dyslipidemia and in those with uric acid values > 90th percentile. However, a significant reduction in LDL cholesterol levels was observed (from 95.0 to 90.4 mg/dL, $p < 0.001$).

Table 1. Anthropometric and clinical characteristics at baseline and at follow-up.

Parameter	Baseline (n = 276)	Follow-Up (n = 276)	p
Age (years), mean (SD)	10.6 (2.3)	12.1 (2.3)	<0.001
Gender (males), n (%)	154 (55.8)	-	
Puberty, n (%)	115 (42.0)	179 (71.6)	<0.001
Weight (kg), median (IQR)	51.4 (40.0, 62.5)	55.9 (44.4, 68.1)	<0.001
Height (cm), mean (SD)	145.3 (14.3)	153.1 (13.5)	<0.001
BMI (kg/m^2), mean (SD)	24.2 (4.5)	23.8 (4.5)	0.004
BMI (z-score), median (IQR)	1.8 (1.3, 2.1)	1.5 (1.0, 1.8)	<0.001
Waist circumference (cm), mean (SD) [§]	77.9 (12.1)	77.6 (11.7)	0.125
WtHr (%), mean (SD)	53.6 (6.9)	50.7 (6.6)	<0.001
Family history of diabetes (mother and/or father), n (%)	43 (15.8)	-	
Family history of dyslipidemia (mother and/or father), n (%)	117 (42.9)	-	
Total cholesterol (mg/dL), mean (SD)	163.9 (28.3)	160.4 (28.4)	0.002
HDL cholesterol (mg/dL), median (IQR)	52.0 (46.0, 60.0)	53.0 (45.0, 62.0)	0.335
HDL cholesterol < 40 mg/dL, n (%)	31 (11.2)	30 (10.9)	0.999
LDL cholesterol (mg/dL), median (IQR)	95.0 (78.8, 111.7)	90.4 (74.9, 106.2)	<0.001
LDL ≥ 130 mg/dL, n (%)	28 (10.1)	16 (5.8)	0.010
Triglycerides (mg/dL), median (IQR)	65.0 (49.0, 88.3)	66.0 (47.0, 88.0)	0.967
Triglycerides ≥ 100 mg/dL or ≥130 mg/dL [#], n (%)	27 (9.8)	24 (8.7)	0.735
Dyslipidemia, n (%)	69 (25.0)	58 (21.0)	0.170
Glucose (mg/dL), mean (SD)	83.1 (7.6)	83.9 (8.4)	0.118
Insulin (μU/mL), median (IQR)	12.1 (7.7, 16.0)	10.8 (7.32, 14.9)	0.925
HOMA Index [$], median (IQR)	2.5 (1.5, 3.4)	2.2 (1.5, 3.1)	0.480
HOMA Index > 90th percentile, n (%)	157 (56.9)	125 (45.3)	0.002
Uric acid (mg/dL), mean (SD)	4.4 (1.1)	4.6 (1.2)	<0.001
Uric acid > 90th percentile, n (%)	46 (16.7)	45 (16.3)	1.000
Creatinine (mg/dL), mean (SD)	0.5 (0.1)	0.6 (0.1)	<0.001
eGFR (mL/min), mean (SD)	155.5 (25.5)	146.3 (24.2)	<0.001
Follow-up time (months), median (IQR)	14.7 (12.4, 19.3)	-	

SD, standard deviation; IQR, interquartile range. Comparisons were conducted through the t-test for paired data or the Wilcoxon signed-rank test for continuous variables, and by the McNemar test for categorical variables. BMI, body mass index; WtHr, waist-to-height-ratio; eGFR, estimated glomerular filtration rate. [#] Triglycerides ≥ 100 if children < 10 years or ≥130 if children ≥ 10 years. [$] Calculated as plasma insulin (μU/mL) × plasma glucose (mmol/L)/22.5. [§] missing at baseline n = 1, missing at follow-up n = 28.

Changes in the prevalence of dyslipidemia and elevated HOMA index and uric acid values are described in Figure 2. In detail, 32 of 69 (46.4%) children with dyslipidemia at baseline had a normal lipid profile at follow-up. In contrast, 21 of 207 (10.1%) subjects who were not dyslipidemic at baseline had dyslipidemia at follow-up. Considering the presence of insulin resistance (HOMA index > 90th percentile), 67 of 157 (42.7%) subjects with elevated HOMA index values at baseline had normal values at follow-up, whereas 35 of 119 (29.4%) children with HOMA index ≤ 90th percentile at baseline showed elevated values at follow-up. Finally, 26 of 46 (56.5%) children with elevated uric acid values (>90th percentile) at baseline had values ≤90th percentile at follow-up. In contrast, 25 of 230 (10.9%) subjects with normal uric acid values presented with hyperuricemia at follow-up (Figure 2).

Multivariate analyses showed that each one-point reduction in BMI z-score was associated with a reduction of 8.9 mg/dL in LDL cholesterol and 20.3 mg/dL in triglycerides (p < 0.001, Table 2), 1.63 points in the HOMA index (p < 0.001, Table 3) and 0.42 mg/dL in uricemia (p = 0.001, Table 4). The loss of one-point BMI z-score was also associated with a 77% decrease in the risk of having a HOMA index value > 90th percentile at the end of follow-up (p < 0.001, Table 3 and a 68% decrease in the risk of having uric acid levels >90th percentile (p < 0.05, Table 4). Higher baseline values of LDL cholesterol, triglycerides, HOMA index and uric acid were strongly associated with the likelihood of no improvement at follow-up (p < 0.001, Tables 2–4). The presence of dyslipidemia, HOMA index values > 90th percentile and uric acid values > 90th percentile was associated with a higher risk of having the same condition at the end of follow-up (p < 0.001, Tables 2–4). Male sex and transition from pre-pubescence to pubescence during follow-up were factors

associated with higher uric acid values at follow-up ($p = 0.01$, Table 4). When the model was adjusted for modifications of eGFR between baseline and follow-up, the result did not change (data not shown).

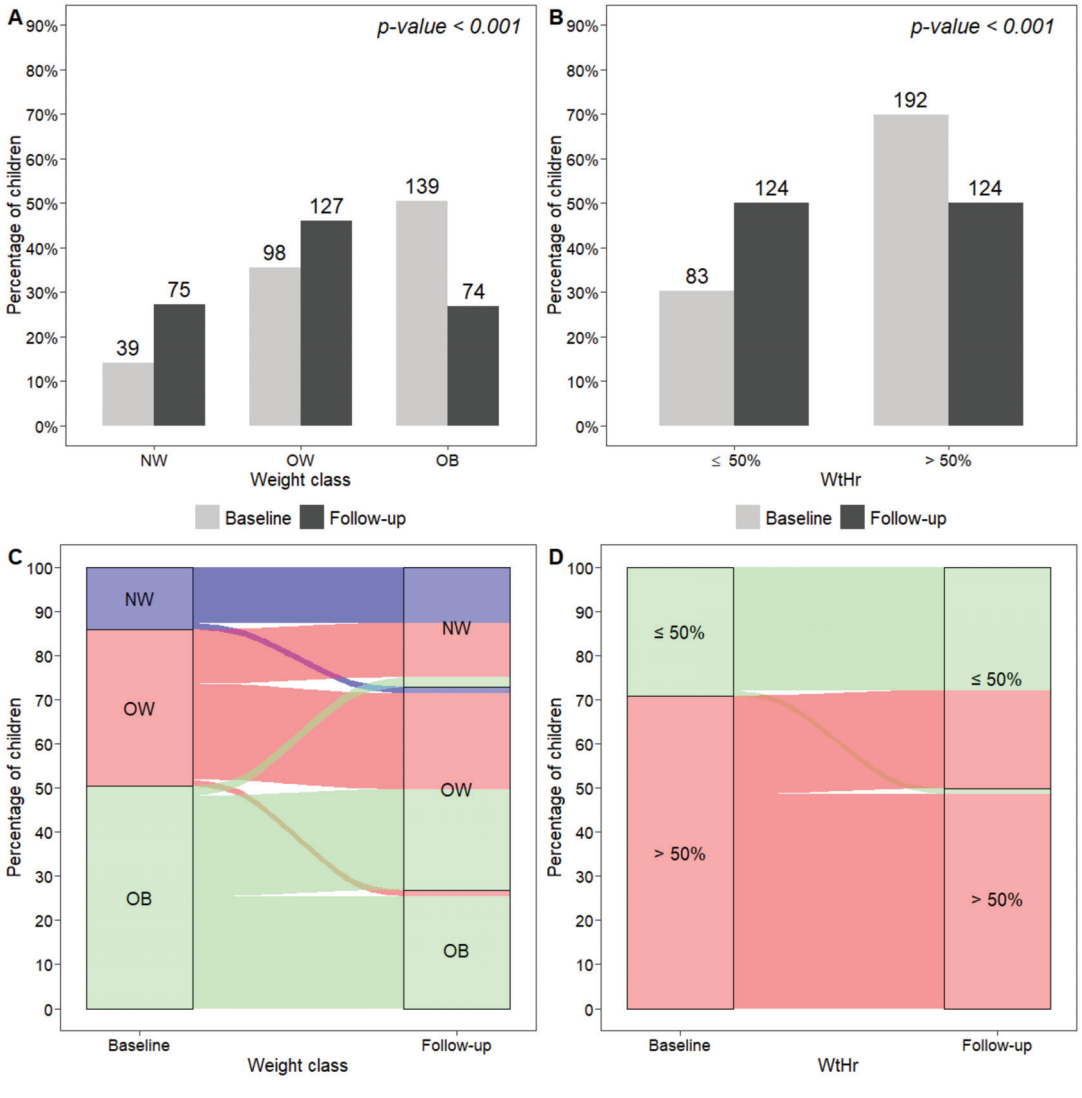

Figure 1. Distribution of weight classes (**A**) and WtHr ≤ 50% or >50% (**B**) at baseline and at follow-up, and graphical representation of subjects moving from one weight class to another (**C**), and from a WtHr value > 50% to a value ≤50% and vice versa (**D**), from baseline to follow-up. NW, normal weight; OW, overweight; OB, obese; WtHr, waist-to-height-ratio.

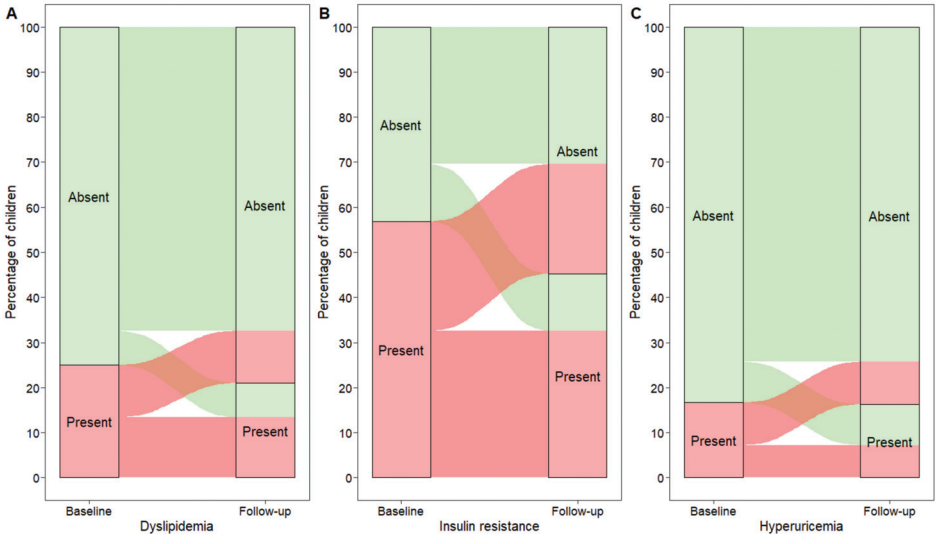

Figure 2. Graphical representation of subjects moving from a pathological to a normal clinical condition and vice versa ((**A**) Dyslipidemia; (**B**) Insulin resistance; (**C**) Hyperuricemia), from baseline to follow-up.

Table 2. Effect of LDL, HDL or triglycerides at baseline, gender, transition from pre-pubescent to pubescent, BMI or WtHr, family history of dyslipidemia on LDL, HDL or triglycerides at follow-up, by multiple linear regression models. Effect of dyslipidemia at baseline, gender, transition from pre-pubescent to pubescent, BMI or WtHr, family history of dyslipidemia on dyslipidemia at follow-up, by a multiple logistic regression model.

LDL Cholesterol at Follow-Up							
Variable	**b**	**(95% CI)**	***p***	**Variable**	**b**	**(95% CI)**	***p***
Intercept	23.167	(15.135; 31.200)	<0.001	Intercept	22.264	(14.359; 30.169)	<0.001
LDL cholesterol at baseline	0.743	(0.668; 0.818)	<0.001	LDL cholesterol at baseline	0.754	(0.679; 0.829)	<0.001
Gender (males)	−3.151	(−7.017; 0.715)	0.110	Gender (males)	−3.695	(−7.618; 0.228)	0.065
Becoming pubescent during follow-up	1.809	(−2.550; 6.168)	0.414	Becoming pubescent during follow-up	2.815	(−1.618; 7.249)	0.212
BMI (Δz-scores)	−8.869	(−14.152; −3.586)	0.001	ΔWtHr	−0.803	(−1.226; −0.379)	<0.001
Family history of dyslipidemia	3.234	(−0.751; 7.219)	0.111	Family history of dyslipidemia	2.335	(−1.677; 6.346)	0.253
HDL Cholesterol at Follow-Up							
Variable	**b**	**(95% CI)**	***p***	**Variable**	**b**	**(95% CI)**	***p***
Intercept	16.049	(10.873; 21.224)	<0.001	Intercept	16.643	(11.573; 21.713)	<0.001
HDL cholesterol at baseline	0.718	(0.628; 0.808)	<0.001	HDL cholesterol at baseline	0.718	(0.628; 0.808)	<0.001
Gender (males)	−1.723	(−3.955; 0.509)	0.130	Gender (males)	−1.581	(−3.871; 0.709)	0.175
Becoming pubescent during follow-up	−0.379	(−2.885; 2.127)	0.766	Becoming pubescent during follow-up	−0.997	(−3.572; 1.578)	0.446
BMI (Δz-scores)	1.965	(−1.090; 5.020)	0.206	ΔWtHr	0.098	(−0.147; 0.343)	0.431
Family history of dyslipidemia	−0.318	(−2.547; 1.912)	0.779	Family history of dyslipidemia	−0.299	(−2.570; 1.973)	0.796

Table 2. *Cont.*

Triglycerides at Follow-Up							
Variable	**b**	**(95% CI)**	***p***	**Variable**	**b**	**(95% CI)**	***p***
Intercept	37.394	(27.145; 47.642)	<0.001	Intercept	34.731	(24.725; 44.737)	<0.001
Triglycerides at baseline	0.570	(0.466; 0.673)	<0.001	Triglycerides at baseline	0.544	(0.442; 0.645)	<0.001
Gender (males)	−0.865	(−7.958; 6.228)	0.810	Gender (males)	−0.390	(−7.457; 6.676)	0.913
Becoming pubescent during follow-up	3.827	(−4.161; 11.815)	0.346	Becoming pubescent during follow-up	6.997	(−0.967; 14.961)	0.085
BMI (Δz-scores)	−20.366	(−30.046; −10.687)	<0.001	ΔWtHr	−1.212	(−1.968; −0.456)	0.002
Family history of dyslipidemia	0.379	(−6.754; 7.513)	0.917	Family history of dyslipidemia	0.379	(−6.680; 7.438)	0.916
Dyslipidemia at Follow-Up							
Variable	**OR**	**(95% CI)**	***p***	**Variable**	**OR**	**(95% CI)**	***p***
Dyslipidemia at baseline	11.187	(5.523; 23.674)	<0.001	Dyslipidemia at baseline	11.418	(5.570; 24.509)	<0.001
Gender (males)	1.343	(0.654; 2.817)	0.426	Gender (males)	1.210	(0.580; 2.571)	0.614
Becoming pubescent during follow-up	1.085	(0.197; 1.497)	0.841	Becoming pubescent during follow-up	1.200	(0.521; 2.686)	0.661
BMI (Δz-scores)	0.557	(0.436; 1.885)	0.255	ΔWtHr	0.939	(0.864; 1.014)	0.120
Family history of dyslipidemia	0.915	(0.436; 1.885)	0.812	Family history of dyslipidemia	0.877	(0.410; 1.837)	0.730

b indicates multivariate coefficient; CI, confidence interval; BMI, body mass index; WtHr, waist-to-height-ratio; OR, odds ratio; Δ indicates the difference between the baseline value and the follow-up value.

Table 3. Effect of HOMA index at baseline, gender, transition from pre-pubescent to pubescent, BMI or WtHr, family history of diabetes on HOMA index at follow-up by a multiple linear regression model. Effect of insulin resistance at baseline, gender, transition from pre-pubescent to pubescent, BMI or WtHr, family history of diabetes on insulin resistance at follow-up by a multiple logistic regression model.

HOMA Index at Follow-Up							
Variable	**b**	**(95% CI)**	***p***	**Variable**	**b**	**(95% CI)**	***p***
Intercept	1.775	(1.255; 2.295)	<0.001	Intercept	1.311	(0.785; 1.836)	<0.001
HOMA index at baseline	0.495	(0.385; 0.605)	<0.001	HOMA index at baseline	0.502	(0.386; 0.618)	<0.001
Gender (males)	−0.128	(−0.557; 0.301)	0.558	Gender (males)	−0.030	(−0.479; 0.418)	0.894
Becoming pubescent during follow-up	0.377	(−0.112; 0.866)	0.130	Becoming pubescent during follow-up	0.562	(0.051; 1.073)	0.031
BMI (Δz-scores)	−1.637	(−2.228; −1.047)	<0.001	ΔWtHr	−0.072	(−0.121; −0.024)	0.004
Family history of diabetes	0.098	(−0.495; 0.690)	0.746	Family history of diabetes	0.151	(−0.474; 0.777)	0.634
Insulin Resistance (HOMA Index > 90th Percentile) at Follow-Up							
Variable	**OR**	**(95% CI)**	***p***	**Variable**	**OR**	**(95% CI)**	***p***
Insulin resistance at baseline	4.055	(2.299; 7.340)	<0.001	Insulin resistance at baseline	3.716	(2.116; 6.683)	<0.001
Gender (males)	1.752	(1.009; 3.073)	0.048	Gender (males)	1.654	(0.952; 2.900)	0.076
Becoming pubescent during follow-up	1.140	(0.608; 2.138)	0.682	Becoming pubescent during follow-up	1.296	(0.689; 2.449)	0.421
BMI (Δz-scores)	0.227	(0.097; 0.503)	<0.001	ΔWtHr	0.951	(0.894; 1.009)	0.102
Family history of diabetes	0.613	(0.282; 1.295)	0.205	Family history of diabetes	0.514	(0.227; 1.116)	0.100

b indicates multivariate coefficient; CI, confidence interval; BMI, body mass index; WtHr, waist-to-height-ratio; OR, odds ratio; Δ indicates the difference between the baseline value and the follow-up value.

Table 4. Effect of uric acid at baseline, gender, transition from pre-pubescent to pubescent, BMI or WtHr on uric acid at follow-up by a multiple linear regression model. Effect of hyperuricemia at baseline, gender, transition from pre-pubescent to pubescent, BMI or WtHr on hyperuricemia at follow-up by a multiple logistic regression model.

Uric Acid at Follow-Up							
Variable	**b**	**(95% CI)**	***p***	**Variable**	**b**	**(95% CI)**	***p***
Intercept	1.087	(0.710; 1.464)	<0.001	Intercept	1.016	(0.627; 1.406)	<0.001
Uric acid at baseline	0.766	(0.688; 0.845)	<0.001	Uric acid at baseline	0.770	(0.688; 0.852)	<0.001
Gender (males)	0.307	(0.130; 0.485)	0.001	Gender (males)	0.293	(0.108; 0.477)	0.002
Becoming pubescent during follow-up	0.259	(0.058; 0.460)	0.012	Becoming pubescent during follow-up	0.293	(0.084; 0.503)	0.006
BMI (Δz-scores)	−0.421	(−0.664; −0.177)	0.001	ΔWtHr	−0.028	(−0.048; −0.008)	0.006
Hyperuricemia (Uric Acid > 90th Percentile) at Follow-Up							
Variable	**OR**	**(95% CI)**	***p***	**Variable**	**OR**	**(95% CI)**	***p***
Hyperuricemia at baseline	6.236	(2.803; 12.101)	<0.001	Hyperuricemia at baseline	6.052	(2.641; 14.117)	<0.001
Gender (males)	0.821	(0.395; 1.715)	0.597	Gender (males)	0.710	(0.332; 1.514)	0.374
Becoming pubescent during follow-up	2.157	(0.970; 4.758)	0.056	Becoming pubescent during follow-up	2.651	(1.165; 6.040)	0.019
BMI (Δz-scores)	0.318	(0.097; 0.952)	0.048	ΔWtHr	0.920	(0.842; 1.000)	0.059

b indicates multivariate coefficient; CI, confidence interval; BMI, body mass index; WtHr, waist-to-height-ratio; OR; odds ratio; Δ indicates the difference between the baseline value and the follow-up value.

When WtHr was included in the models instead of BMI z-score, the results were overlapping (Tables 2–4).

4. Discussion

Our study shows that, without the use of pharmacological therapy, an intervention based only on the modification of dietary habits and lifestyles is able not only to improve the weight status in a population of children and adolescents, but also to correct, in a large number of cases, the metabolic alterations present (alterations in the lipid profile, insulin resistance and hyperuricemia). However, in spite of the intervention, a certain number of subjects still experience an increase in BMI and a worsening of the metabolic picture, even if in a smaller percentage than those who improve.

Overall, we obtained a clear improvement in the weight status of the study population. The percentage of children with obesity was significantly reduced and, in parallel, the number of subjects with normal body weight and those who were in excessive weight but without evidence of obesity increased. The magnitude of BMI reduction was strongly associated with an improvement in the metabolic risk profile. Importantly, only a small minority of children (less than 6%) worsened their weight class, whereas a higher proportion (about 27%) experienced improvement. A Cochrane meta-analysis including 70 randomized clinical trials, based on both diet and physical activity interventions, showed that the intervention led to a significant reduction of the BMI z-score [11].

Numerically few data are available on the effects of nonpharmacological treatment on the metabolic profile of children/adolescents and, as highlighted in the meta-analysis by Ho et al., studies often analyzed only small samples of children [13]. Dyslipidemia has a non-negligible prevalence in pediatric age, especially in children and adolescents with obesity [8,27]. Isolated hypercholesterolemia is still the most frequently observed form, but the current prevalence of excess weight in younger generations is contributing to an increase in mixed forms and of those characterized only by the presence of hypertriglyceridemia [25]. In adults, the effectiveness of dietary-behavioral treatment in improving the hematochemical parameters that characterize dyslipidemia has been widely demonstrated; decreasing saturated fat and increasing fiber in the diet is associated with reductions in total and LDL cholesterol [28,29], whereas increasing physical activity and reducing free sugar

consumption primarily lead to a decrease in triglycerides values [30,31]. The meta-analysis by Ho et al. [13], which included five studies, showed that lifestyle intervention had a significantly greater impact on triglycerides (−0.20 mmol/L in the short-term and −0.09 mmol/L in the long-term studies) and LDL cholesterol values (−0.30 mmol/L) than no treatment. A more recent study designed to evaluate the effect of a community lifestyle intervention (achieving healthy eating patterns and increasing physical activity) on components of the metabolic syndrome in adolescents found an improvement in triglycerides in boys and HDL cholesterol in both genders [32]. These findings are in line with those of our study, in which intervention is associated with a decrease in plasma LDL cholesterol and triglyceride values, which is closely related to the decrease in BMI z-score. Baseline LDL cholesterol and triglyceride values have an important influence on the values found at follow-up; the higher the baseline value, the higher the final value. Although the prevalence of a family history of dyslipidemia is high in our sample (43%), having one or two dyslipidemic parents is not a determining factor for the response to treatment. It is possible to hypothesize that the altered lipid values in this population are largely due to poor dietary habits in the family, which causes an altered lipid picture in both children and their parents, rather than to the actual presence of a familial dyslipidemia.

The meta-analysis of Ho et al. [13] included four studies that reported results on insulin resistance: the difference in HOMA index was −2.32 (95% CI: −3.25 to −1.39) in favor of lifestyle intervention compared to the control group, However, the heterogeneity (among these studies) was high. In addition, a meta-analysis that analyzed the ability of exercise training to lower insulin resistance in children and/or adolescents classified with obesity or as being overweight showed a significant reduction of HOMA index (−0.61, 95% CI: −1.19 to −0.02) [14]. However, a study designed to evaluate the effects of recommendations to follow the DASH diet vs. a usual diet on the characteristics of metabolic syndrome in Iranian adolescents resulted in a decrease in serum insulin levels (101.4 vs. 90 pmol/L, $p = 0.04$) without any significant reduction in HOMA index in the DASH group [33]. A gradual decline in insulin secretion from normal glucose tolerance to impaired glucose tolerance to type 2 diabetes mellitus in adolescents with obesity has been documented [34]. A high HOMA index was the most frequent metabolic alteration in our sample; in fact, more than half of children had a HOMA index > 90th percentile. It is known that in pediatric ages, insulin resistance increases physiologically with age and, in particular, with pubertal development [35]. In our population, the proportion of prepubertal subjects decreased from 58 to 28% from baseline to follow-up, and this was accompanied, as expected, by an increase in mean HOMA index values. However, we observed a significant reduction in HOMA index values in children/adolescents who decreased in body weight: for each lost BMI z-score point, the HOMA index was reduced by 1.6. In addition, the likelihood of insulin resistance (i.e., HOMA index > 90th percentile) decreased by 77%. Although the presence of high HOMA index values at baseline strongly conditions the outcome, it seems clear that this parameter responds favorably to nonpharmacological treatment. We might have expected that the presence of a diabetic parent would have an impact on the presence and/or persistence of insulin resistance in the offspring. However, familiarity of diabetes mellitus did not influence the results regarding HOMA index, neither when analyzed as a continuous variable nor as a categorical variable. This finding is similar to what we observed for a family history of dyslipidemia; having a parent with dyslipidemia had no significant impact either on the presence of dyslipidemia at baseline or at follow-up in children. In a small sample of girls with obesity, Browning et al. evaluated the difference in some metabolic variables after six months of an intervention combining diet, behavioral counseling, and exercise training. In patients who lost weight (slightly more than half of the study population), the authors observed a reduction in LDL cholesterol (but not triglycerides) and basal blood glucose compared with those who gained weight [36].

In our population, the number of children with a WtHr > 50% significantly decreased after the intervention. Approximately 29% of subjects with a WtHr > 50% at baseline had a WtHr < 50% at the end of the follow-up period. Replacing BMI z-score by WtHr

(visceral fat index) in the statistical models had no additional effect on outcomes, although from a theoretical point of view, waist circumference should be better associated with metabolic alterations than BMI z-score. However, our result confirms the validity of WtHr in determining pediatric cardiovascular risk, but also suggests that this parameter does not provide any substantial advantage over BMI. It is possible to assume, however, that more accurate estimates of central adiposity (such as the use of dual-energy x-ray absorptiometry) could have led to different results. In a small sample of Japanese children, dietary intervention combined with exercise treatment led to a reduction in the areas of subcutaneous and visceral fat, quantified in CT images obtained by a body fat scan, in association with a significant decrease in plasma values of total cholesterol, triglycerides and insulin [37]. Abdominal-visceral obesity is associated with unfavorable metabolic activity and increased cardiovascular risk. The metabolic activity of visceral fat is a key factor in the development of obesity-related complications [38]. Visceral obesity results in a chronic inflammatory state in adipose tissue. Visceral adipose tissue accumulation leads to immune cell infiltration, release of vasoconstrictor mediators, dysfunctional remodeling and fibrosis of with increased vascular stiffness [39]. Therefore, fat distribution more than BMI may determine cardiovascular disease risk. Although some individuals may apparently accumulate excess body fat without developing cardiometabolic diseases [40], it has been shown that a non-negligible proportion of "metabolically healthy" children with obesity have elevated values of non-traditional risk factors such as HOMA index and serum uric acid [41].

In children, as well as in adults, an association has been demonstrated between obesity and uric acid levels [10,42,43]. There is no agreement on which of the two factors determines the other, and some evidence suggests that this relationship may be bidirectional [44]. From our data, it appears that hyperuricemia is the variable that is least responsive to nonpharmacologic treatment, as the percentage of children with uric acid > 90th percentile at baseline and at follow-up is overlapping. It should be noted, however, that half of the subjects with hyperuricemia at baseline are different from those with hyperuricemia at follow-up. A recent longitudinal study evaluating the temporal relationship between BMI and uric acid showed that changes in BMI preceded changes in uric acid, providing evidence of a causal relationship between obesity and hyperuricemia [45]. Our data, showing a significant association between reduction of BMI z-score (and WtHr) and uric acid values at follow-up, seem to support this hypothesis and also suggest that, at least in pediatric ages, if an increase in weight leads to an increase in uric acid, its reduction can reverse at least in part the phenomenon. Another recent study performed in a population of children and adolescents with obesity demonstrated a reduction in uric acid levels in children who underwent a multifactorial lifestyle intervention and lost weight during the trial, and an increase in those who gained weight [46]. In our population, the higher the uric acid values at baseline, the lower the response to the intervention, and children with uric acid values > 90th percentile at baseline were six times more likely to be hyperuricemic even at follow-up. It has been shown that children with higher levels of uricemia are those in whom it is more difficult to obtain a benefit from the intervention also in terms of reduction of blood pressure values, a parameter associated with high levels of uric acid also in pediatric age [47]. An association between uric acid values and intake of sugar-sweetened beverages (SSBs) has been robustly demonstrated in children [48,49]. However, studies that have investigated the impact of measures to reduce SSBs intake have not explored the effect of the intervention on plasma uric acid, but only on anthropometric parameters [50–53]. The factors that influence uric acid values are complex and not only depend on weight and diet, but also on the genetic characteristics of the subject. It is therefore possible that uric acid values are not reduced in all children in the same way, even if a similar decrease in BMI z-score is obtained. As expected, eGFR decreased with increasing age of the study sample. However, no child/adolescent showed creatinine/eGFR values outside the normal range. It has been shown that creatinine values increase progressively from the first years of life, in accordance with the increase of muscle mass with consequent reduction of eGFR

values [54]. Since this is a physiological phenomenon that occurs in all subjects and since BMI has been indexed for age (z-score) in all statistical models, we think that, in the absence of renal disease, the physiological reduction of eGFR should not influence our results.

Vegetarian diets in adults are assumed to be healthy; however, it is unclear whether this is also true for children and adolescents. So-called "plant-diets" have been shown to have a protective effect on the risk of type 2 diabetes, cardiovascular disease, and obesity in adults [55]. Existing data do not allow to reach a solid conclusion on the health benefits or risks of vegetarian diets on the nutritional and health status of children and adolescents [56]. A vegan diet can potentially be critical in children because of inadequate intake of protein, long-chain fatty acids, iron, zinc, vitamin D, iodine, calcium, and in particular vitamin B12 [57]. However, some scientific societies allow it, but only if properly integrated and controlled [58]. In our study we chose to propose a Mediterranean diet, as it is closer to the culture of Italy, including specific indications based on metabolic alterations present in individual cases. Moreover, we have always prescribed an increase in the consumption of fruits and vegetables.

A strength of our study is that, whereas most previously published studies on this topic were limited to analyzing the differences between pre- and post-intervention values in children who lose weight compared with those who do not, we also provide a precise estimate of the effect of our intervention. For each point of BMI z-score lost, we described a reduction of about 9 mg/dL of LDL cholesterol, 20 mg/dL of triglycerides, 1.6 of HOMA index and 0.4 mg/dL of uric acid. In the available studies, anthropometric variables were not always indexed, and the influence that age and puberty have on lipid and glucose patterns and uric acid values in developmental age was often not considered. In our study, all variables were analyzed as both continuous and indexed variables, and all models were adjusted for the onset of puberty.

A weakness of our study is that we could detect only one indirect parameter of adiposity (waist circumference). A more correct estimation of visceral fat and its changes in relation to those of hematochemical parameters could give interesting additional information. Moreover, as our society has become increasingly multiethnic, other factors, such as sociocultural influences, may contribute, beyond biological factors, to the success of non-pharmacological treatment directed at a population of children and adolescents with excess weight. The presence of community workers specifically trained to promote healthy behaviors that take into account cultural background, psychosocial stressors, and economic limitations would likely have improved compliance among our population and allowed for better outcomes. Unfortunately, our center is not equipped with this kind of support.

5. Conclusions

In conclusion, in children it is not easy to evaluate the effect of a nonpharmacological treatment on metabolic variables that may represent a cardiovascular risk factor. In fact, children are growing organisms in which these parameters change physiologically. Moreover, body weight, a factor strongly associated with metabolic parameters, undergoes important modifications at this age. By using models that correct these confounding factors, our study shows that a change in lifestyle and dietary habits is able to induce an improvement in weight, lipid profile, glycemic and uric acid values. For all metabolic parameters considered, baseline values are strongly associated with those found at follow-up. This means that the more a child presents a metabolic impairment, the more difficult it will be, with the same weight reduction, to bring back its parameters in a normal range. These considerations confirm and emphasize that an early intervention aimed at normalizing the lipid, glycemic and uric acid values is essential for the realization of a real prevention of cardiovascular disease in a world where this is the first cause of death.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu14051034/s1, Figure S1: Flow chart of children excluded from the study and reason for exclusion.

Author Contributions: S.G. and M.G. conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. A.O., G.L. and I.P. collected data. A.O. monitored the nutritional aspects of the intervention. E.T. and L.A. performed data analysis. G.P. reviewed and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Effect of Vitamin D and Docosahexaenoic Acid Co-Supplementation on Vitamin D Status, Body Composition, and Metabolic Markers in Obese Children: A Randomized, Double Blind, Controlled Study

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Abstract: Obese children are at high risk of developing vitamin D deficiency. Omega-3 polyunsaturated fatty acids and their derivatives might have a beneficial effect on vitamin D status of obese children, due to their anti-inflammatory action, and increasing its absorption. This multicenter, randomized, double-blind controlled study aims to investigate the effect of vitamin D and docosahexaenoic acid (DHA) co-supplementation for six months on vitamin D status, body composition, and metabolic markers of obese children with vitamin D deficiency. A total of 108 children were enrolled and 73 children completed the study: 33 were supplemented with an oral dose of 500 mg of DHA and 1200 IU/day of vitamin D3 and 41 were supplemented with 1200 IU/day of vitamin D3 + wheat germ oil. At the end of the study, more than 50% of the subjects improved their vitamin D status. However, co-supplementation was not more effective than vitamin D plus wheat germ oil. Fat mass percentage was significantly reduced, and body mass index improved in both groups, even if all the subjects were still obese at the end of the study. Children receiving both vitamin D and DHA presented a higher increase of DHA levels that could be relevant to prevent inflammatory-associated complications of obesity, but they had no effect on vitamin D levels.

Keywords: fatty acids; obesity; vitamin D; vitamin D deficiency; DHA; dietary supplements

1. Introduction

Obese children are at high risk of developing vitamin D deficiency (VDD) [1], which, in turn, impacts on glucose homeostasis, insulin resistance (IR), and inflammation, thus exacerbating the negative effects of fat accumulation on overall health [2]. The relationship

between obesity and low levels of circulating 25-hydroxyvitamin D (25(OH)D) has not been completely elucidated. Adipose tissue represents the major extra skeletal targets of vitamin D. It plays an important role as a vitamin D storage site, due to vitamin D fat-solubility, resulting in a lower bioavailability in the obese subjects [3]. An impaired hydroxylation in adipose tissue and 25(OH)D accumulation in fat may be hypothesized to explain this relationship. Anotherhypothesis considers low serum 25(OH)D, a lipophilic compound, the result of vitamin D dilution in fat mass [4]. Animal and in vitro studies point out that vitamin D modulates adipose tissue biology, by inhibitory or stimulatory effects on adipocyte differentiation depending on cell type and stage of differentiation [5]. Vitamin D exerts its effect also by regulating energy metabolism gene expression, preventing excess body fatness, and limiting the expression of inflammatory molecules [3,6,7].

VDD appears to predispose also to further metabolic disturbances including the metabolic syndrome [3,8,9]. Insulin resistance and cardiovascular diseases, two conditions in which pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), are critically involved, are recognized comorbidities in obese subjects. The raised level of TNF-α induces insulin resistance in adipocytes and peripheral tissues by impairing the insulin signaling. A condition of chronic low-grade inflammation secondary to the abnormal production of proinflammatory cytokines, including TNF α, is considered the main mechanism leading to endothelial dysfunction in obesity [10].

It is also known that inflammation is associated with low levels of vitamin D [11]. On the contrary, some fatty acids (especially omega 3) have an anti-inflammatory action and vitamin D and DHA co-supplementation have a favorable effect on the metabolic status of patients with diabetes [12,13]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) and their derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to exert anti-inflammatory and inflammation-resolving effects, thus they are suggested to be relevant to both the prevention and treatment of human diseases with an inflammatory component [14]. Therefore, we speculated that co-administration of vitamin D and DHA (an omega-3 fatty acid) might improve the vitamin D status more than vitamin D supplementation plus wheat germ oil (a compound rich in omega-6 fatty acids) in obese children.

Hence, the primary aim of this study was to investigate if the co-supplementation of vitamin D and DHA was more effective than vitamin D plus wheat germ oil to improve the vitamin D status of obese children. The secondary aims were to evaluate the effect of vitamin D and DHA co-supplementation on body composition and metabolic markers.

2. Methods

2.1. Study Design

The study was an investigator-initiated multicenter, randomized, double-blind controlled study. It was conducted from March 2015 to March 2020 at the pediatric outpatient clinic of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milan and of the ASST Rhodense POT Bollate, Milan, Italy. Inclusion criteria were: (1) Body Mass Index (BMI) higher than 95° centile for age [15]; (2) 6 to 14 years of age at the time of enrollment; (3) VDD (circulating 25-hydroxy vitamin D levels <20 ng/mL); and (4) written informed consent of legal caregivers. Exclusion criteria were the presence of malabsorption, ongoing treatment with corticosteroids or anticonvulsants, metabolic alterations of the calcium–phosphorus balance, and other endocrinologic disorders such as thyroid dysfunction, growth hormone deficiency, and endogenous hypercortisolism.

The study was approved by the Ethical Review Board of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano Area 2 (reference number 1 July 2014, 1472/2014). All procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the caregiver of all subjects.

2.2. Intervention

The participants were randomly assigned by a computer-generated randomization sequence to the following two arms:

- GROUP I (vitamin D + DHA): oral administration of vitamin D3 (1200 IU daily) + 500 mg of DHA for 6 months.
- GROUP II (vitamin D + wheat germ oil): oral administration of vitamin D3 (1200 IU daily) + wheat germ oil capsules for 6 months.

Children were instructed to assume the capsules containing DHA or wheat germ oil and vitamin D during the same mealtime. Throughout the duration of the study, all investigators, participants, outcome assessors, and data analysts were blinded to the assigned treatment. The D + DHA and vitamin D + wheat germ oil capsules were identical, indistinguishable by appearance, color, or flavor. Additionally, all patients were included in the same lifestyle intervention program consisting of advice for a healthy diet and regular physical exercise. Nutritional habits were evaluated by a 24-h recall by a dedicated nutritionist during routine visit, after 3 months from the beginning, and at the end of the study. To increase the compliance monthly calls to the parents were performed.

2.3. History, Anthropometric, and Laboratory Parameters

Age, sex, and birth weight were recorded at the enrollment. Furthermore, body height (Harpenden stadiometer), body weight, body composition by bioelectric impedance (Tanita BC 420 MA; Sensor Medics, Milano, Italy), arm circumference, waist circumference, and triceps, biceps, subscapular, and sovrailiac skinfold thickness were measured at enrollment, after 3 months, and at the end of the intervention. The values of BMI and fat mass were transformed into a standard deviation score (SDS).

For the laboratory analysis, a 5 mL blood venous sample in EDTA was collected in fasting conditions at each time point of the study. The following parameters were measured, at the enrollment and at the end of the intervention: glucose, insulin, glycohemoglobin (HbA1c), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), apolipoprotein A (ApoA), apolipoprotein B (ApoB), ApoB/ApoA ratio, parathyroid hormone (PTH), aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), calcium (Ca), and phosphorus (P). Homeostasis model assessment (HOMA) index was calculated as glucose values expressed in mg/dL multiplied by the insulin expressed in milliunits/L divided by 405 [16].

FA acids were measured at the enrollment and at the end of the intervention. For the analyses, a few drops of blood were put on Whatman 903 collection cards BHT (Sigma-Aldrich), pre-treated, and stored at temperature of −20 °C. Cards were cut and transferred into vials (one vial for each sample) for methylation as described by Marangoni et al. [17]. Next, 2 mL of KCl solution (Sigma-Aldrich, Steinheim, Germany) and 330 µL hexane (Sigma-Aldrich, Steinheim, Germany) were added. Samples were first vortexed and then centrifuged at 3000 rpm for 10 min. Finally, hexane layer (the upper layer) was collected from each vial and transferred into gas chromatography vial for fatty acids profile evaluation with fast gas-chromatographer Master GC fast (Dani), equipped with a 15-m fused silica capillary column OmegawaxTM 100 (Supelco). The gas chromatography results were analysed using Clarity software (Data Apex). Each fatty acid was evaluated as percentage of the total FA. The following FA were considered for comparisons: palmitic acid (C16:0) and stearic acid (C18:0) of the saturated fatty acids (SFA); 16:1n7; oleic acid (C18:1n9) of the monounsaturated fatty acids (MUFA); alpha-linolenic acid (ALA, C18:3n3), eicosapentaenoic acid (EPA, C20:5n3), and docosahexaenoic acid (DHA, C22:6n3) of the n-3 PUFA; and linoleic (LA, C18:2n6), dihomo-gamma-linolenic acid (DGLA, C20:3n6), and arachidonic acid (AA, C20:4n6) of the n-6 PUFA. The following FA ratios were calculated as proxy of inflammatory state: AA/EPA, AA/DHA, and n6/n3 the PUFA balance marker (EPA+DHA)/total PUFA [18,19]. The related enzymatic activities were estimated by proxy from the following product/substrate ratios: stearoyl-CoA desaturase (SCD), from C18:1n9/C18:0, (16); fatty acid desaturase (FADS2), from DGLA/LA, and FADS1

from AA/DGLA and from EPA/ALA and DHA/EPA. The related pro-inflammatory activities were estimated by proxy from the following product/substrate ratios, ARA/DHA, ARA/LA, e omega3 index, EPA+DHA. For a subsample of patients, tumor necrosis factor alpha (TNF-α), routinely assessed in obese children at our centers, was analyzed at baseline and 6 months from plasma, with the Quantikine HS Human TNF-alpha Immunoassay (R&D Systems, Minneapolis, MO, USA).

Circulating levels of 25(OH)D were measured by an Abbott chemiluminescent microparticle immunoassay. Reliability and accuracy of the assay were assessed both in the Vitamin D External Quality Assessment Scheme and in the Vitamin D Standardization Program [20,21]. All analyses were performed at the central laboratory of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy.

2.4. Statistical Analysis

Assuming an increase of vitamin D levels of 40% and 55% in the group receiving vitamin D plus wheat germ oil and in the group receiving vitamin D plus DHA, respectively, and the possibility of some dropouts (approximately 10%), a sample size of 108 children is necessary to achieve a power of 80% with a 95% significance level. Data distribution was checked for normality by the Kolmogorov–Smirnov test. Continuous variables were expressed as mean ± SD or as median and interquartile range (IQR). Categorical data were expressed as absolute and relative frequency. Data were analyzed using the per-protocol principle and the values recorded at baseline were compared to values recorded at 6 months. Baseline and follow-up characteristics were tested for differences by Student's t-test or Mann–Whitney test, as appropriate. The change of anthropometric and laboratory values between baseline and 6 months was evaluated using the Wilcoxon test for repeated measures. Difference between proportions were tested using the chi-squared test. Delta differences (∂) between the start and the end of treatment were calculated as $\partial = [(T1-T0)/T0] \times 100$. Univariate correlations were investigated with Spearman's rank correlation test. Logistic regression analysis was used to test the independence of associations between vitamin D levels (≥20 ng/mL vs. <20 ng/mL) at the end of the study and the interventional group, DHA levels, and anthropometric variables. Missing data were handled using complete case analysis. Significance was assumed for $p < 0.05$. The data were analyzed using SPSS (Statistical Package for Social Science v.20 Inc., Chicago, IL, USA).

3. Results

3.1. Baseline Characteristics

A total of 250 patients were screened in the two study centers and 108 were finally enrolled, as reported in Figure 1. Of these, 74 (45 males, 29 females), (median age 11.2 (IQR 3.2) years) completed the study. A total of 34 patients were lost at follow-up (a comparison of the main baseline characteristics of subjects who completed the study and dropouts are reported in the supplementary online materials). The dropouts were due to the refusal of parents or their children to continue the supplementation. Among subjects who completed the study, 33 were in the GROUP I and 41 children were in GROUP II. The two groups showed similar baseline characteristics, as shown in Table 1.

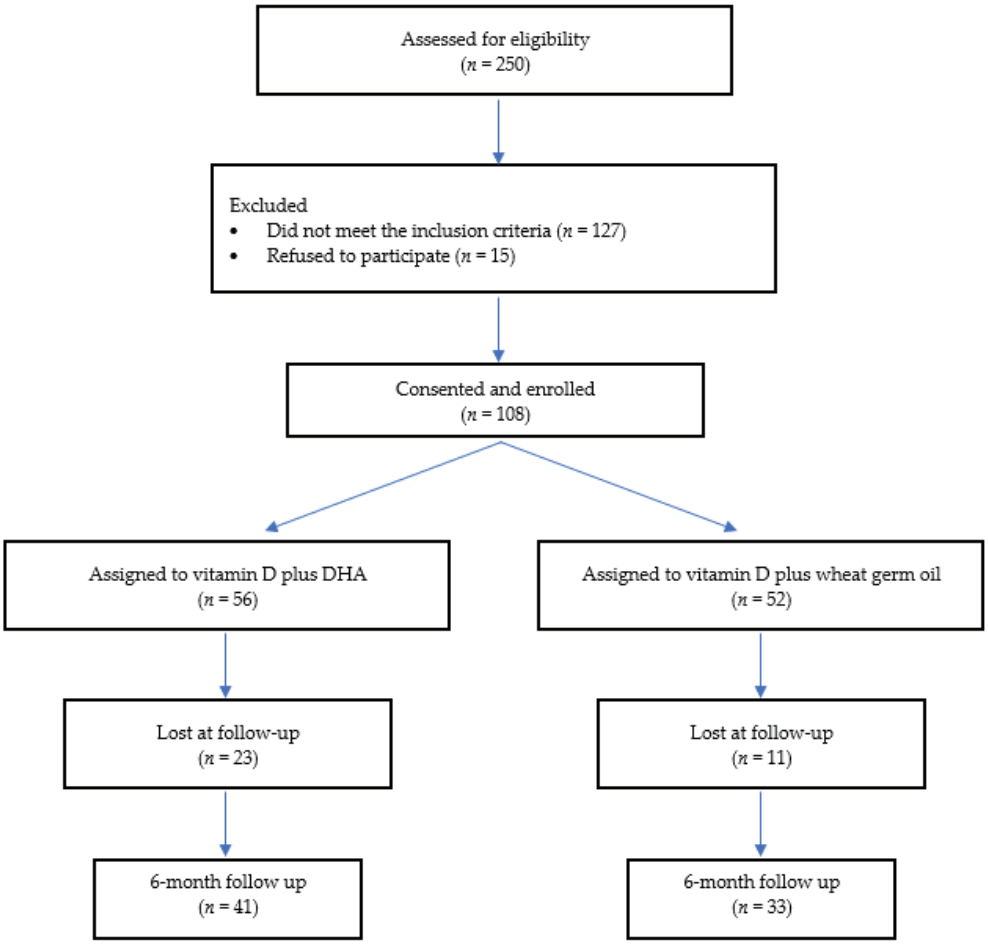

Figure 1. Enrollment flow chart.

At baseline, median values (IQR) of vitamin D were 14.2 (7.8) ng/mL. In both groups, the concentration of vitamin D increased during the study period. At the end of the 6 months, in GROUP I, median vitamin D levels were 21.9. (IQR 11.8) ng/mL and in GROUP II were 23.4 (8.8) ng/mL, thusresulting in a median (IQR) increase by 64.3% and 70% in the two groups, respectively. Only for 4 patients vitamin D concentrations decreased (3 patients in GROUP I, 1 in GROUP II). However, at the end of the study, 28 (38%) patients (16 patients in vitamin GROUP I and 12 in GROUP II) had persistent VDD (median vitamin D 15.7 (IQR 4.1) ng/mL).

Logistic regression analysis showed that vitamin VDD at the end of the study was independently associated with the percentage of fat (OR = 1.10, 95% CI = 1.01–1.21, *p*-value = 0.037) (Table 2). No association was found between VDD and the assigned interventional group.

Table 1. Clinical and anthropometric variables in D + DHA group and vitamin D group after 6 months from the start of the study. Data are expressed as median (IQR).

	Group	Baseline	6 Months
Sex (M/F)	45/29		
Age, years	I	11.9 (3.1)	12.0 (3.1)
	II	11.0 (3.5)	11.6 (3.1)
Gestational Age, weeks	I	37.0 (3.0)	
	II	37.0 (3.0)	
Birth weight, g	I	3250 (767.5)	
	II	3300.0 (730.0)	
Age at obesity diagnosis, years	I	7 (2.0)	
	II	6.5 (3.8)	
Body height, cm	I	150.0 (15.8)	154.0 (15.0) ***
	II	145.0 (15.3)	149.2 (15.0) ***
Body weight, kg	I	61.5 (24.0)	64.7 (23.9) *
	II	56.0 (19.4)	58.5 (20.4) **
SDS BMI	I	2.53 (0.64)	2.5 (0.6)
	II	2.63 (0.70)	2.5 (0.7) **
AM, cm	I	28.3 (3.9)	28.5 (5.0)
	II	27.0 (3.8)	28.0 (4.0)
WC, cm	I	84.3 (16.6)	84.0 (17.0)
	II	81.5 (12.4)	83.0 (10.0)
Biceps skinfold, mm	I	19.8 (8.0)	19.0 (5.3)
	II	19.5 (6.8)	20.0 (6.6)
Triceps skinfold, mm	I	26.0 (7.1)	26.0 (4.6)
	II	24.3 (7.8)	24.0 (4.5)
Sovrailiac skinfold, mm	I	26.0 (7.0)	25.0 (7.5)
	II	24.2 (7.6)	25.0 (8.0)
Subscapular skinfold, mm	I	23.6 (10.7)	21.0 (12.0)
	II	23.3 (6.4)	23.0 (9.0)
Fat Mass, %	I	35.0 (8.3)	35.4 (8.4)
	II	35.3 (5.5)	33.4 (7.5)
Fat Mass SDS, %	I	5.87 (2.45)	1.42 (0.85) ***
	II	6.38 (3.05)	1.36 (0.58) ***
FFM, kg	I	37.7 (11.6)	41.4 (12.8) ***
	II	35.1 (13.2)	39.1 (12.2) ***

Baseline vs. 6 months (Wilcoxon test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. SDS = Standard Deviation; AM = Arm Circumference; FFM = Fat Free Mass; WC =Waist Circumference.

Table 2. Logistic regression showing the association between VDD (dependent variable) and interventional arm, percentage of fat, BMI status and DHA levels at the end of the study.

	OR	95% CI		*p*-Value
Follow-Up				
22:6n3	1.65	0.85	3.21	0.138
Fat %	1.11	1.01	1.21	0.037
BMI SDS	0.78	0.22	2.70	0.694
Group II	1.42	0.46	4.40	0.548

22:6n3 = Docosahexaenoic Acid (DHA); BMI = Body Mass Index; SDS = Standard Deviation.

3.2. Anthropometric, Clinical, and Laboratory Parameters

Table 3 shows the anthropometric and laboratory characteristics for each group at baseline and at the end of intervention The two groups showed a reduction in SDS BMI % (−2.9 in GROUP I, −7.1 in GROUP II). There was a major reduction of fat mass in GROUP

II (−3.5%) than GROUP I (0.62%). GROUP II showed a reduction in median (IQR) levels of ApoA (from 138.5 (25) mg/dL at baseline to 132(29) mg/dL at 6-months $p = 0.018$; ∂ −6.2%). The ApoB/ApoA ratio was significantly increased in both groups (∂: +8.4% in GROUP I and +3.5% in GROUP II, $p = 0.030$). No other significant differences for any anthropometric and laboratory parameters were found at 6 months.

Table 3. Laboratory/biochemical variables in D + DHA group and vitamin D group at baseline and after 6 months. Data are expressed as median (IQR).

	Group	Baseline	6 Months
Glucose, mg/dL	I	85.0 (12.0)	87.0 (10.0)
	II	86.0 (5.0)	85.0 (8.8)
Insulin, mg/dL	I	15.5 (17.5)	17.8 (15.6)
	II	15.7 (9.7)	15.4 (12.0)
HOMA	I	3.0 (4.0)	3.5 (3.3)
	II	3.1 (2.4)	3.2 (2.5)
HbA1c (%)	I	5.3 (0.4)	5.2 (0.4)
	II	5.3 (0.3)	5.3 (0.3)
TC (mg/dL)	I	153.5 (40.8)	154.0 (32.0)
	II	158.0 (31.0)	158.0 (36.5)
HDL-c (mg/dL)	I	51.0 (17.3)	49.0 (17.0)
	II	50.0 (16.0)	49.0 (17.8)
TG (mg/dL)	I	78.5 (60.8)	66.0 (33.0)
	II	69.0 (59.0)	73.5 (57.8)
LDL-c (mg/dL)	I	84.0 (28.0)	83.0 (33.0)
	II	88.0 (35.1)	89.0 (36.7)
ALT (UI/L)	I	21.5 (8.5)	23.5 (10.0)
	II	19.0 (15.5)	26.0 (17.0)
AST (UI/L)	I	23.0 (7.5)	26.0 (6.8)
	II	25.0 (7.5)	28.0 (10.0)
GGT (UI/L)	I	13.0 (8.0)	15.5 (10.8)
	II	13.0 (8.5)	14.5 (7.5)
ApoA (mg/dL)	I	138.5 (25.0)	132.0 (29.0) *
	II	137.0 (25.5)	130.0 (26.3)
ApoB (mg/dL)	I	81.0 (24.5)	79.0 (30.0)
	II	77.0 (26.5)	81.5 (24.5)
B/A	I	0.59 (0.19)	0.58 (0.19) *
	II	0.55 (0.15)	0.57 (0.23) *
PTH (pg/mL)	I	28.1 (27.0)	33.6 (24.8)
	II	36.5 (25.5)	31.8 (24.9)
Calcium (mg/dL)	I	9.76 (0.40)	9.71 (0.37)
	II	9.74 (0.50)	9.73 (0.54)
25OHD (ng/mL)	I	14.0 (7.2)	21.99 (11.8)
	II	15.3 (8.4)	23.4 (8.8) ***

Baseline vs. 6 months (Wilcoxon test): * $p < 0.05$; *** $p < 0.001$. HOMA = Homeostasis Model Assessment; HbA1c = Glycohemoglobin; TC = Total Cholesterol; HDL-c = High-Density Lipoprotein Cholesterol; TG = Triglycerides; LDL-c = Low-Density Lipoprotein Cholesterol; ASL = Aspartate Transaminase; ALT = Alanine Transaminase; GGT = ϒ-glutamyl-transferase; ApoA = Apolipoprotein A; ApoB = Apolipoprotein B; B/A = ApoB/ApoA Ratio; PTH = Parathyroid Hormone; 25OHD = 25-hydroxy Vitamin D.

3.3. Fatty Acids

Table 4 shows the fatty acids concentration at baseline and at the end of the study in the two groups. Median (IQR) DHA % was higher in GROUP I (2.56 (1.15)); thus resulting in an increase of 58% vs. an increase of 35% in GROUP II ($p = 0.010$). Differences were found also for total n3-PUFA, PUFA balance, AA/DHA, n3 derivatives/ALA, DHA/EPA,

DHA/ALA, and EPA+DHA, as shown in Table 4. The concentration of DHA and total n3-PUFA increased in both groups. Consequently, PUFA balance and the following ratios (n3-derivatives/ALA, DHA/EPA, and DHA/ALA) increased, too. In GROUP I, 16:1n7, SCD16, total MUFA, AA/EPA, AA/DHA and n6/n3 decreased. In GROUP II, the concentration of 16:0, AA/DHA, AA/EPA, n6/n3 decreased. No significant correlations of serum 25(OH)D concentrations with 20:4n6; 22:6n3; 20:5 n3 were found either at baseline or at 6 months in both groups (data not shown).

Table 4. Percentage of fatty acids distribution in vitamin D + DHA subjects (Group I) and vitamin D + wheat germ oil subjects (GROUP II) at baseline and after 6 months. Data are expressed as median (IQR).

% of Total Fatty Acids	Baseline		6 Months	
	GROUP I	GROUP II	GROUP I	GROUP II
16:00	23.65 (1.89)	23.61 (1.98)	23.53 (1.75)	23.23 (1.64) *
18:00	11.90 (2.10)	11.74 (1.75)	11.58 (2.30)	11.43 (2.19)
16:1n7	1.42 (0.67)	1.41 (0.69)	1.18 (0.59) *	1.22 (0.81)
18:1n9	18.36 (3.43)	18.60 (3.14)	18.30 (2.56)	17.96 (2.93)
18:1n7	1.30 (0.26)	1.30 (0.27)	1.31 (0.38)	1.31 (0.22)
18:2n6	21.07 (3.24)	21.31 (2.88)	21.34 (3.36)	20.86 (3.80)
20:3n6	1.60 (0.40)	1.59 (0.29)	1.56 (0.31)	1.57 (0.45)
20:4n6	9.41 (3.05)	9.61 (2.24)	9.38 (1.99)	9.89 (1.77)
22:4n6	1.20 (0.56)	1.19 (0.56)	1.02 (0.45)	1.23 (0.49)
18:3n3	0.19 (0.08)	0.19 (0.08)	0.17 (0.08)	0.20 (0.09)
20:5n3	0.30 (0.27)	0.23 (0.19)	0.26 (0.17)	0.29 (0.20)
22:5n3	0.54 (0.24)	0.58 (0.31)	0.47 (0.26)	0.57 (0.20)
22:6n3	1.64 (0.81)	1.65 (0.71)	2.59 (1.15) ***	2.06 (1.35) *** °
Total SFA	39.68 (1.96)	38.56 (3.92)	38.73 (4.53)	38.21 (3.34)
Total MUFA	23.35 (3.20)	23.21 (3.45)	22.97 (2.47) *	22.78 (3.44)
Total PUFA	36.90 (4.16)	37.61 (6.38)	37.49 (6.19)	37.80
Total n6-PUFA	34.14 (3.80)	35.10 (5.69)	34.19 (5.18)	34.81 (5.27)
Total n3-PUFA	2.84 (1.02)	2.67 (0.88)	3.71 (0.99) ***	3.14 (1.68) *** °
LCPUFA	14.97 (4.59)	15.57 (2.40)	15.91 (2.34)*	15.73 (3.19)
PUFA balance	5.50 (2.27)	5.25 (2.00)	7.87 (2.71) ***	5.87 (4.01) *** °
AA/EPA	29.91 (27.15)	35.56 (22.59)	4.16 (25.94) ***	7.78 (23.89) ***
AA/DHA	5.43 (2.73)	5.76 (2.09)	3.40 (1.72) ***	4.75 (4.75) *** °
n6/n3	21.85 (30.05)	21.05 (33.44)	9.35 (3.00) ***	11.00 (4.95) ***
n6-derivates/LA	1.59 (0.22)	1.62 (0.13)	1.60 (0.16)	1.65 (0.15)
n3-derivates/ALA	15.05 (6.49)	14.70 (6.56)	22.37 (12.03) ***	17.51 (9.33) °
DGLA/LA (FADS2)	0.07 (0.03)	0.07 (0.03)	0.07 (0.02)	0.08 (0.03)
ARA/DGLA (FADS1)	5.64 (1.54)	5.89 (1.79)	5.89 (1.42)	6.11 (2.18)
ARA/LA	0.42 (0.16)	0.45 (0.09)	0.44 (0.13)	0.48 (0.13)
EPA/ALA	1.53 (1.38)	1.39 (1.12)	1.65 (1.14) ***	1.44 (1.00)
DHA/EPA	5.55 (4.48)	5.63 (4.50)	10.22 (8.17) ***	7.69 (4.16) *** °
DHA/ALA	9.11 (4.23)	9.33 (4.44)	17.27 (10.69) ***	12.03 (6.68) *** °
SCD(16)	0.06 (0.03)	0.06 (0.03)	0.05 (0.02) *	0.05 (0.03)
SCD(18)	1.55 (0.42)	1.63 (0.39)	1.55 (0.42)	1.55 (0.37)
EPA+DHA	2.01 (0.97)	1.88 (0.83)	2.84 (1.17) ***	2.28 (1.41) *** °

Baseline vs. 6 months (Wilcoxon test): * $p < 0.05$; *** $p < 0.001$. D + DHA group vs. vitamin D at baseline and 6 months (Mann–Whitney test): ° $p < 0.05$. 16:00 = Palmitic Acid; 18:0 Stearic Acid; 16:1n-7 = Palmitoleic Acid; 18:1n7 = Vaccenic Acid; 18:1n-9 = Oleic Acid; 18:2n6 = Linoleic Acid; 20:3n6 = Dihomo-γ-linolenic Acid; 20:4n6 = Arachidonic Acid; 22:4n6 = Adrenic acid; 18:3n3 = Alpha-linoleic Acid; 20:5n3 = Eicosapentaenoic Acid; 22:5n3 = Docosopentaenoic Acid; 22:6n3 = Docosahexaenoic Acid; SFA = Saturated Fatty Acids; MUFA = Monounsaturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids; LCPUFA = Long Chain Polyunsaturated Fatty Acids; AA = Arachidonic Acid; EPA = Eicosapentaenoic Acid; DHA = Docosahexaenoic Acid; DGLA = Dihomo-γ-linolenic Acid; LA = Linoleic Acid; FADS = Fatty Acid Desaturase; ALA = Alpha-linoleic Acid; SCD = Stearoyl-CoA Desaturase.

3.4. TNF-α

In 28 patients (14 per group) TNF-α was measured. The median TNF-α value was 6.6 (IQR 9.6) pg/mL at baseline and 5.2 (7.6) pg/mL at 6 months. In GROUP I, the median TNF-α value was 5.5 (IQR 7.9) pg/mL, and 6.1 (7.9) pg/mL, at baseline and at 6 months,

respectively, and in GROUP II the median TNF-α value was 7.2 (IQR 9.7) pg/mL and 4.6 (7.2) pg/mL at baseline and at 6 months, respectively. There was a significative reduction in TNF-α levels between the two groups ($p = 0.048$).

4. Discussion

This study aimed to investigate the potential benefit of supplementing VDD obese children with vitamin D3 plus DHA as compared to vitamin D supplementation plus wheat germ oil. We hypothesized that the association of DHA with vitamin D supplements could help improve the vitamin D status, body composition, and metabolic markers of this population. Regarding the primary outcome—vitamin D blood levels—more that 50% of the subjects improved their vitamin D status, regardless of the supplementation assigned. Children with persistent VDD at the end of the study were about 38% of the sample. This result could be explained by different reasons. Firstly, in our study vitamin D supplementation was daily based, which may have represented a challenge to some patients in terms of compliance. Secondly, we did not investigate the sunexposure and the season on which the supplementation was provided [22]. Lastly, the dose of vitamin D might be too low to obtain significant results: previous studies reported an increase of serum 25(OH)D after supplementation of 1000–2000 IU/day but not after 600 IU/day in overweight/obese children [23,24]. Castaneda and colleagues [25] found a significantly lower vitamin D increase in obese adolescents compared to non-obese subjects, after supplementation of 2000 IU/day, thus confirming that a higher vitamin D dose in obese subjects is needed to treat VDD.

The secondary aim of the study was to test the effects of vitamin D plus DHA DHA on BMI and body composition. The nutritional outcome was ameliorated, but not significantly changed in terms of BMI among the included subjects, who were still in condition of obesity at the end of the study. This finding is likely due to the length of the study that may not be sufficient to detect significant differences in subjects with obesity [26]. Nevertheless, the nutritional outcome was overall ameliorated in terms of the FM% which was significantly reduced in both groups. This is probably the result of the dietary and lifestyle counseling provided to all subjects at the beginning of study, according to international and national guidelines on nutritional management of obesity in pediatric subjects. As expected, we found that FM% at the end of the study was independently associated with VDD status of the included subjects. Previous studies showed similar results in term of BMI [27], while others suggested a dose-related effect on BMI, waist circumference, and total fat mass [28].

The present study also investigated the fatty acids profile in both groups. To the best of our knowledge, little is known about the effect of PUFA supplementation in obese children. Supplementation with DHA in children with non-alcoholic fatty liver disease showed a decrease of fatty liver [28] and a significant reduction in pericardiac and visceral fat, and also in triglycerides and fasting insulin after 6 months of treatment [29]. As expected, in the group supplemented also with DHA, this n-3 fatty acid was increased after 6 months compared with the subjects supplemented with vitamin D plus wheat germ oil. Specifically, DHA were increased by more than 50%, in the group receiving DHA and only by 35% in the other group. Similar findings were reported by López-Alarcón [30] showing a significantly increased EPA and DHA after 3 months of LC-PUFA supplementation. The increase of DHA also in the group receiving wheat germ oil might be explained by the improvement of dietary habits, such as increased consumption of fish.

In the past decade, evidence suggested that obesity induces low-grade chronic inflammation, affecting the immune and metabolic state [31]. Moreover, markers of oxidative stress and inflammation are shown to be elevated in people with low serum 25(OH)D concentrations; however, results are not always consistent [32–35]. In our study TNF-α levels were found to be slightly, but still significantly, decreased in both groups. A recent study [36] investigated whether dietary fat and/or antioxidant intake influences circulating TNF-α, interleukin-6 (IL-6), CRP, and leptin concentrations. There was a significant increase in CRP, IL-6, and leptin, but not in TNF-α, with increasing adiposity, independent of age.

Another study showed that inflammatory markers are increased in overweight children as young as 6 years old [37]. Moreover, there is growing evidence about the role of total fat intake and specific fatty intake on systemic inflammation: C18:2 might increase IL-6 and other inflammatory cytokines production in the adipose tissue, whereas α-linolenic acid might reduce inflammation [38]. However, we speculate that the decrease of TNF-α in both the studied groups might be associated both to the BMI decrease and the dietary changes.

Previous studies found that n-3 FA treatment was able to attenuate metabolic disorders associated with obesity, by limiting HF-induced glucose intolerance and liver steatosis, and mice fed n-3 FA displayed lower circulating leptin [39]. On the contrary, vitamin D3 supplementation did not enhance the benefits observed with n-3 FA on plasma leptin levels, glucose tolerance, the liver lipid metabolism, or intestinal health. Moreover, the coadministration of n-3 FA and D3 significantly reduced the increase in circulating 25(OH)D following D3 consumption. Some authors hypothesized that n-3 FA–based bile acid micelles have an increased size that would compromise the diffusion of micelles containing D3. They also suggested that n-3 FA supplements may impede the efficacy of D3 supplementation in obesity. Yet, these assumptions warrant further mechanistic investigations and validation studies [39].

A few limitations deserve to be commented. This study compared two active groups without including a placebo group, because our study included children with a deficit of vitamin D who must receive vitamin D according to Italian guidelines [40]. Therefore, it would have been unethical to include a placebo group. Furthermore, the interventional period was limited.

5. Conclusions

In conclusion, this study shows that vitamin D and DHA co-supplementation for 6 months does not lead to a better vitamin D status as compared to vitamin D plus wheat germ oil. We observed a beneficial effect on the DHA levels in children supplemented with this FA. It might be speculated that the anti-inflammatory action of n-3 PUFA may in part compensate for the detrimental outcome of VDD. Understanding the relationship among obesity, vitamin D status, and fatty acids profile is clinically relevant, and the investigation of the underlying pathogenic mechanisms may expand clinical approach to obesity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14071397/s1, Supplementary Table S1: Median (IQR) clinical and anthropometric variables in the total groups of patients who completed the study and in the drop-outs group at baseline; Supplementary Table S2: Median (IQR) clinical and anthropometric variables of patients who completed the study and of the drop-outs patients in vitamin D +DHA group at baseline; Supplementary Table S3: Median (IQR) clinical and anthropometric variables of patients who completed the study and of the drop-outs patients in vitamin D group at baseline.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available upon reasonable request from the corresponding author.

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Review

Nutritional Approach to Prevention and Treatment of Cardiovascular Disease in Childhood

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Abstract: Coronary Heart Disease (CHD) is a major mortality and morbidity cause in adulthood worldwide. The atherosclerotic process starts even before birth, progresses through childhood and, if not stopped, eventually leads to CHD. Therefore, it is important to start prevention from the earliest stages of life. CHD prevention can be performed at different interventional stages: primordial prevention is aimed at preventing risk factors, primary prevention is aimed at early identification and treatment of risk factors, secondary prevention is aimed at reducing the risk of further events in those patients who have already experienced a CHD event. In this context, CHD risk stratification is of utmost importance, in order to tailor the preventive and therapeutic approach. Nutritional intervention is the milestone treatment in pediatric patients at increased CHD risk. According to the Developmental Origin of Health and Disease theory, the origins of lifestyle-related disease is formed in the so called "first thousand days" from conception, when an insult, either positive or negative, can cause life-lasting consequences. Nutrition is a positive epigenetic factor: an adequate nutritional intervention in a developmental critical period can change the outcome from childhood into adulthood.

Keywords: nutrition in childhood; primordial prevention; dietary treatment; hypercholesterolemia; CHD prevention

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1. Introduction

1.1. Coronary Heart Disease and Atherosclerosis

Coronary Heart Disease (CHD) is one of the main causes of death in adulthood in the United States and in Western countries [1,2]. CHD mainly causes morbidity and mortality in adult subjects, but lipid deposition and arterial damage dates back to the early stages of life [3,4]. The atherosclerotic process starts even before birth; post-mortem studies showed that fatty-streaks are already detectable in human fetal aortas, greatly increased by maternal hypercholesterolemia [3,4]. The prolonged and sustained arterial exposure to elevated low-density lipoprotein cholesterol (LDL-C) increases cholesterol deposition and enhances the atherosclerotic process, eventually leading to CHD [5]. The exposure to factors associated with increased cardiovascular risk exacerbates the atherosclerotic cascade [6]. Atherosclerosis is already present in childhood, for this reason prevention of atherosclerosis related diseases should be present and mandatory from the earliest stages of life. Health professionals dealing with children and adolescents are the main actors of this preventive battle, and the knowledge, the detection and the treatment of CHD risk are their cornerstone swords. In this narrative review we focus on nutritional intervention in children and adolescents at high cardiovascular risk, starting from the historical data

of the literature up to the latest indications of the most recent guidelines. In childhood, cardiovascular risk has been often referred to a single disease or condition, whereas in our review we have tried to consider cardiovascular risk and nutritional intervention as the "trait d'union" of a variety of conditions present in childhood and adolescence. We have looked at childhood and adolescence focusing on cardiovascular-disease risk factors, so as to spot and unify all the main conditions at high CHD risk in these age groups. In the near future, further studies will be needed to sharpen even more this approach, thus making nutritional intervention more tailored and effective.

1.2. Paediatric Patients and Cardiovascular Risk

CHD prevention can be performed at different stages of intervention: primordial prevention is aimed at preventing risk factors, primary prevention is aimed at early identification and treatment of risk factors, whereas secondary prevention is aimed at reducing the risk of further events in those patients who have already experienced a CHD event. CHD risk factors can be present since the early stages of life, both on a genetic and on a metabolic basis. The presence of classical severe CHD risk factors and the presence of heart diseases, that make the child more vulnerable to CHD, are both underlying conditions for increased CHD in childhood and/or in the following stages of life. Detection of CHD risk factors and risk stratification is an issue of utmost importance in the management of paediatric patients with cardiovascular risk. In this context, the first step is the knowledge of the conditions that enhance CHD risk in childhood, their detection and their adequate treatment [7]. The extent of the risk for atherosclerotic coronary artery disease, compared to the general population, has been proposed as a model for CHD risk stratification [8]. Children and adolescents with underlying heart diseases are at increased CHD risk as well. In particular, subjects with coronary artery anomalies must be strictly supervised. On a pathogenetic basis, three underlying conditions seem to increase CHD risk: inflammation, insulin resistance and oxidative stress [9]. Inflammation seems to be the main causative condition of CHD risk through elevated triglycerides levels, low high-density lipoprotein (HDL) cholesterol (HDL-C) levels and increase in small LDL-C particles. Inflammation causes oxidative stress and requires an increased glucose intake, thus triggering insulin resistance. Congenital or acquired diseases and conditions can be classified as "high risk", "moderate risk" or "at risk" for CHD, according to the extent of the coronary artery pathology compared to that of healthy population. Conditions that make the subject at high CHD risk include homozygous familial hypercholesterolemia (HoFH), diabetes mellitus type 1 and 2, end stage renal disease, Kawasaki disease with persistent aneurysms, childhood cancer survival. Conditions that cause a moderate CHD risk include heterozygous familial hypercholesterolemia (FH), severe obesity, hypertension, elevated blood level of lipoprotein(a). Diseases that make the affected subject at risk for CHD include obesity, chronic inflammatory diseases, Kawasaki disease with regressed aneurysms and some congenital heart defects. Once the CHD risk stratification has been carried out, it is important to assess the presence of other CHD risk factors and/or comorbidities, such as altered fasting lipid profile, smoking, positive family history for premature CHD in first-degree relatives, elevated blood pressure, overweight or obesity, altered fasting blood glucose, lack of physical activity. The extent and the strictness of intervention, both on a lifestyle and on a pharmacological basis, are determined by the risk stratification and the presence or absence of comorbidities [7,8].

1.3. Classical CHD Risk Factors

Among the classical CHD risk factors, FH is one of the most important and most frequent conditions. FH is a primitive dyslipidemia and is present in 1 per 200–250 subjects in the heterozygous form in the general population. Patients with FH are at increased risk of CHD disease because they have genetic conditions (mutations on LDL-Receptor gene, Apolipoprotein B gene, PCSK9 gene) that result in high LDL-C levels and can cause premature atherosclerosis; they frequently have elevated non-HDL-C levels (expression of

highly atherogenic VLDL particles) and apoB/apoA ratio >1, recently recognized as a new risk factor for atherosclerosis [10]. If undetected and untreated, subjects with FH have increased mortality and morbidity. Early detection and treatment of FH helps "gaining decades of life", as stated in the recent European Atherosclerosis Society Consensus [11]. Phenotypic expression of FH typically occurs in adulthood, so the detection of affected children and adolescents must be based on screening strategies. Unfortunately, knowledge of the "cholesterol problem" is still really poor in the general population, especially among young adults [12]. Lipoprotein(a) is a strong, genetically defined CHD risk factor. Lipoprotein(a) levels are stable from early childhood and high Lipoprotein(a) levels can help to identify FH patients at higher CHD risk [13,14]. Moreover, elevated Lipoprotein(a) levels have been linked to thromboembolic events in children and adolescents [15]. Childhood obesity is defined as body mass index (BMI) higher than 95th centile for age and sex. Obesity is a metabolic and public health emergency in the Western World starting from childhood and involving, in some countries, up to one third of 6–9 years old children [16]. Numerous studies [17–19] have correlated obesity with fatty streaks, atherosclerotic lesions and CHD morbidity and mortality. The American Academy of Pediatrics guidelines indicate children with obesity as children with "independent CHD risk factors" [20]. Severe obesity is defined as BMI higher than 35 kg/m^2 and/or higher than 120% of the 95th centile for sex and age. Children and adolescents with severe obesity are a cluster at extremely elevated CHD risk, as they have subclinical atherosclerosis and endothelial activation [21,22]. Diabetes mellitus, both type 1 and type 2, is related to increased CHD risk. Subclinical atherosclerosis, shown as an increase in carotid intima media thickness, and microvascular damage have been demonstrated in children with diabetes mellitus [23]. Hyperglycemia is the primary mediator of atherosclerosis in subjects with diabetes mellitus, therefore the achievement of optimal glucose control is the first step in CHD risk reduction [24]. However, the maintenance of a balanced diet is of utmost importance for these subjects, as they often tend to assume an excessive intake of lipids and proteins. Hypertension is another risk factor for CHD [25], both isolated or associated to other diseases. Elevated blood pressure has been linked to higher level of carotid intima media thickness and to arterial damage [26].

1.4. High Risk Medical Conditions and Heart Disease

Chronic kidney disease is a vasculopathic state characterized by early abnormalities of vascular and cardiac function [27]. Numerous chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus, are characterized by an increased CHD risk in adulthood. Childhood cancer survivors represent another CHD high risk category. Several studies [28] have shown that childhood cancer survivors have greater insulin resistance and increased arterial stiffness compared with their healthy siblings, and this difference is maintained throughout adulthood. The pathogenesis of this increased cardiovascular risk is related to the vulnerability of these patients and it has a multifactorial basis. Moreover, anthracycline treatment has been linked to dilated cardiomyopathy [29]. Children with congenital heart disease are considered more vulnerable. The prevalence of congenital heart disease is approximately 9 per 1000 live births. The risk of CHD in subjects with congenital heart disease is related to the presence of obstructive lesions of the left ventricle and aorta, cyanotic congenital heart defects and coronary artery abnormalities [30]. An important cause of secondary heart disease is Kawasaki disease, a multisystemic vasculitis that can cause coronary artery aneurysms [31].

2. Nutritional Treatment

Nutritional treatment is the milestone intervention in pediatric patients at increased CHD risk. According to the Developmental Origin of Health and Disease theory, also known as "Barker hypothesis", the origin of lifestyle-related disease begins with conception and proceeds through embryonic, fetal and neonatal stages by the relation between genes

and the environment. In the so called "first thousand days" from conception, an insult, either positive or negative, can cause life-lasting consequences [32]. Barker discovered that ischemic heart disease incidence in adulthood is linked to low birth weight and malnutrition in the earliest stages of life [33]. According to Barker's hypothesis, an individual is programmed towards nutritional thrift during gestation and early postnatal life so as he can survive environmental insults caused by poor nutrition [34]. More recent studies have shown that maternal malnutrition, either qualitative or quantitative, is responsible for shaping future offspring health; in particular, this refers to a maternal low protein and high fat diet [35,36]. According to the developmental programming by early life exposures, children born extremely preterm have higher systolic and diastolic blood pressures already at 2–3 and 6.5 years of age and preterm birth has been linked with higher blood pressure later in life [37,38]; finally, as recently reported, they have an increased risk for ongoing residual kidney injury and chronic kidney disease [39]. In this context, nutrition is a positive epigenetic factor: an adequate nutritional intervention in a developmental critical period can change the outcome in adulthood. This concept has a revolutionary power and supports the idea that nutritional treatment should be considered comparable to drug therapy.

2.1. Scientific Support for Dietary Recommendations

Nutritional intake of saturated fatty acids, trans fatty acids and cholesterol are widely known as main determinants of the increase of cholesterol blood levels and, subsequently, of the development of cardiovascular disease. Other cardiovascular risk factors are implied in this event as well. In the occurrence of many risk factors in one individual, the evidence of atherosclerotic lesions in the aorta and coronary arteries starting from in early childhood becomes more likely [4,17]. Longitudinal studies confirm that there is a tracking of overweight, hypercholesterolemia and hypertension from the earliest years of life into adulthood and that lifestyle and habits as unhealthy diet, excessive caloric intake, lack of physical activity and cigarette smoking play an important role in influencing the above-mentioned risk factors [26]. Intervention studies, aimed at measuring the efficacy and safety of diets poor in total and saturated fat and cholesterol, added further evidence. The meta-analysis of studies in adults suggested that introduction of low saturated-fat and low cholesterol diet lowers blood total and LDL-C levels. Pediatric studies confirmed the safety and efficacy of a low-cholesterol and low-saturated fat diet in children and adolescents. Among these, the Dietary Intervention Study in Children (DISC) was a randomized trial consisting in administration of a low-saturated fat and cholesterol diet for a 3 years period in American children aged 8 to 11 years; the intervention group received a diet with 28% of calories from total fat, 10% of calories from saturated fat and 95 mg per day of cholesterol, while the control group consumed 33 to 34% of calories as total fat, 12.7% of calories as saturated fat and 112 mg per day of cholesterol. No differences in anthropometric parameters nor in serum ferritin levels were found in the two groups; the intervention group showed lower levels of LDL-C and maintained psychological well-being [40]. Likewise, in the Special Turku Coronary Risk Factor Intervention Project for Babies (STRIP) randomized and prospective study, a low-saturated fat and low-cholesterol diet was introduced at a very young age (during weaning, at 7 months of life), with dietary education continued until the age of 20 years [41,42]. As for the intervention group, both studies used diets tailored according to current recommendations for therapeutic lifestyle changes to lower cholesterol levels, with total fat <30% of total calories and cholesterol intake <200 mg per day. Saturated fat intake, although not <7% of total calories, was significantly less than in children assigned to usual care. Non-adverse effects of the recommended intervention diets were observed in growth, neurological development, metabolic function and nutrient adequacy [43–45]. In both studies LDL-C levels were significantly lower among children in the intervention group compared with controls, and children who received nutritional intervention had higher chances of choosing healthy food [46].

2.2. Low Fat Diet in Children

According to Barker et al. [34], granting an adequate growth from birth and throughout childhood may be a fundamental milestone in the prevention of atherosclerosis, as the incidence of CHD is higher especially in males with low birth weight or low weight at 1 year of life. We have also learned that even a little reduction of the mean total and LDL-C values in childhood and adolescence could decrease substantially the incidence of CHD, when prolonged into adult life. It is a general thought that relative fat intake is high (35–55% of energy) during the first year of life, then decreasing toward adult values (30–35% of energy) in the following years. Fat is one of the main sources of energy, mainly in childhood, when growth is remarkably fast. In the first period of life, a great amount of daily energy is used for growth, therefore a high fat intake (40–55% of energy) is probably essential. In the subsequent months and years, the amount of energy required for growth decreases and in older child other components of energy expenditure, such as basal metabolic rate, thermoregulation and, above all, physical activity, become more important. The growth data of the STRIP trial support the safety of a low-saturated fat, low-cholesterol diet administered to infants aged > 7 months and continued from the first year of life throughout childhood, but the precise percentage of fat dietary intake supporting normal growth and development and maximally reducing atherosclerosis risk, is still unknown [43]. Therefore, the appropriate fat intake should be better recommended not a daily, but a several day-range basis.

2.3. Age-Modulated Nutrient Recommendations

The 'programming' hypothesis suggests that the future cardiovascular responses are established either in prenatal age or in response to early feeding exposures in life [33]. Human milk is universally recognized as the "gold standard" for infant nutrition so as every other nutritional strategy must be compared to it [47]. Breast milk contains an elevated amount of saturated fat and cholesterol but low amount of sodium. Infants who are breastfed have higher blood cholesterol levels at one year of age if compared to formula-fed infants, but when they become adults, these data are often inverted and adults who have been breastfed end to have lower blood cholesterol values. [48]. Furthermore, breast-feeding is associated with at least 2 behavioral benefits on cardiovascular risk: a better self-regulation on intake amounts, and a taste evolution that may improve acceptance and preference of healthy foods after weaning and later in life [49]. Evidence suggests that infants who were breastfed tend to have lower blood pressure values i later in childhood [50]. Breastfeeding seems to have a protective effect towards the development of future obesity, as reported in other systematic reviews [51]. The period from complementary feeding to the achievement of a mature diet, from 4–6 months to 2–3 years of age, represents a radical shift in pattern of food consumption. The introduction of solid food as a complement of breastmilk or formula milk should start at about 6 months of age to grant a sufficient amount of micronutrients, but the optimal methods to fulfill this goal are not clearly defined. [52]. Current feeding practices are influenced by small scale studies of infant feeding behavior [53], parental behavior, popular opinions and folk customs. New healthy foods may need to be introduced repeatedly to establish taste preferences [54]. Moreover, it is recognized that children ranging from 2 to 5 years of age are selective in their food choices. After 2 years of age, the amount of calories derived from fat should be gradually reduced to less than 30% of total daily calories. Calories no more introduced as fat should be replaced with grain products, fruits, vegetables, low fat milk products, legumes, lean meat, fish or other protein rich foods. In the age from 2 to 6 years, challenges are related to providing quality nutrient intake and avoiding excessive caloric intake. A significant amount of saturated fat and cholesterol derives from milk and dairy products in this age group, therefore a transition to low-fat milk and other dairy products is important. In the clinical practice, some parents and their children may over-interpret the need to restrict their fat intakes and for this reason it is important to give a higher accepted limit of fat intake; we must also emphasize that these recommendations are for average intake

over several days, so that if fat-rich food are consumed, a compensation can be obtained eating healthier food in the other weekly meals. Since no single food item provides all the essential nutrients in the amounts needed, food variety seems to be a key stone in building an adequate diet. As children grow up, sources of food and influences on eating behavior increase; many meals and snacks are usually consumed away from home and often without supervision. Significant adverse changes occur in older children's food consumption: they usually reduce or skip breakfast and increase intake of snacks, fried and nutrient-poor foods, sweetened beverages, as well as portion size at each meal. In addition, at this age dairy product consumption decreases and there is a shift away from fruit and vegetable consumption. This shift in dietary patterns in adolescence results in calcium, potassium, iron, zinc and vitamins intake below recommended levels, whereas sodium intake is far above recommended intake [55]. Adolescence is a nutritionally vulnerable developmental stage, as growth rate accelerates; therefore, counselling in late childhood and adolescence should be individualized for better dealing with contemporary lifestyles.

2.4. Dietary Recommendations for Children over the Past Twenty Years

In 1998 the American Academy of Pediatrics recommended on whether to promote dietary changes in all healthy children (population approach) or to identify and treat only children who are at the highest risk for the development of accelerated atherosclerosis in early adult life (individualized approach) [56]. In 2000 the Italian Society of Pediatric Nutrition (SINUPE) endorsed a similar document to identify and treat hypercholesterolemia in children in Italy, as shown in Table 1 [57].

Table 1. Dietary recommendations for children with hypercholesterolemia, modified from Giovannini M. et al. [57].

DIETARY RECOMMENDATIONS FOR CHILDREN
GENERAL (population approach)
• No restriction of fat or cholesterol intake for infants of age < 2 years (rapid growth) • Caloric intake should be adequate to support growth and achieve desirable body weight • Nutritional adequacy should be achieved by eating a wide variety of foods
LOW-FAT DIET (for hypercholesterolemia)
STEP ONE DIET
• Total fat $\leq$ 30% (no less than 20%) of total daily calories • Saturated fats < 10% of total daily calories • Polyunsaturated fats $\leq$ 10% of total daily calories • Cholesterol $\leq$ 300 mg/day
STEP TWO DIET
• Total fat $\leq$ 30% (no less of 20%) of total daily calories • Saturated fats $\leq$ 7% of total daily calories • Polyunsaturated fats $\leq$ 10% of total daily calories • Cholesterol $\leq$ 200 mg/day

Specific nutrient recommendations are as follows: no restriction of fat or cholesterol for infants <2 years when rapid growth and development require high energy intakes; nutrition adequacy should be obtained by eating a wide variety of foods; caloric intake should be adequate to support growth and development and to reach and maintain desirable body weight. In the population approach to lower cholesterol levels the Step-One diet is recommended: $\leq$30% and no less than 20% of calories from total fat; <10% of total calories from saturated fat; $\leq$10% of calories from polyunsaturated fat; cholesterol no more than 300 mg per day. This therapeutic diet should be prescribed in a medical setting with monitoring and follow-up provided by a health professional; if children do not reach the desirable cholesterol levels after 3–6 months of such diet, they require counselling to adopt the Step-Two Diet.

This regimen should start with accurate assessment of current eating patterns and instruction by a physician. It includes: no more than 30% and no less than 20% of calories from total fat; ≤7% of total calories from saturated fat; ≤10% of calories from polyunsaturated fat; no more than 200 mg/day of cholesterol. This dietary pattern requires careful planning to ensure adequacy of nutrients, vitamins and minerals and follow up by a qualified nutrition professional [56].

In 2005 the American Heart Association presented the Dietary Recommendations for Children and Adolescents endorsed by the American Academy of Pediatrics with new focuses on both caloric intake and eating behaviors [58]. In the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents the first step proposed for management of children with identified lipid abnormalities was a focused intervention on diet and physical activity, with dietary accommodations that still follow the Step-One and the Step-Two Diet. The use of dietary adjuncts such as plant sterols or stanols was also proposed for children with primary hypercholesterolemia who do not achieve LDL-C goals with dietary treatment alone to avoid the necessity of drug treatment [6].

In the European Atherosclerosis Society (EAS) document for detection and treatment of FH in children and adolescents the Panel recommends a heart-healthy, fat modified diet (<30% calories from total fat, <7% of calories from saturated fat and <200 mg/day of cholesterol), including nutrient dense-foods with appropriate energy to maintain optimal body weight. Intake of fruit and vegetables, whole grains, low fat dairy products, legumes, fish and lean meats should be encouraged; the Mediterranean diet is referred to as an ideal model of heart-healthy diet (Figure 1).

Figure 1. The Nutritional Diet Pyramid for children, modified from Giovannini et al. [57].

The possibility of using functional foods is included; in this regard, foods containing added plant sterols/stanols, psyllium-enriched cereals, garlic extract, omega-3 fatty acids and soy proteins, have been evaluated in small studies in children with hyperc-

holesterolemia. Anyway, no strong recommendations on functional foods utilization in childhood and adolescence are yet available. [11]. In the 2016 European Society of Cardiology (ESC) and EAS guidelines for the management of dyslipidemias, plant sterols/stanols are indicated for adults and children (>6 years) with FH [59]. In Italy, a heart-healthy diet should be in accordance with the Italian Society of Human Nutrition reference values (LARN) [55]. A daily recommended distributions of meals is shown in Figure 2. Recommended macronutrient intake for school-age children are shown in Table 2.

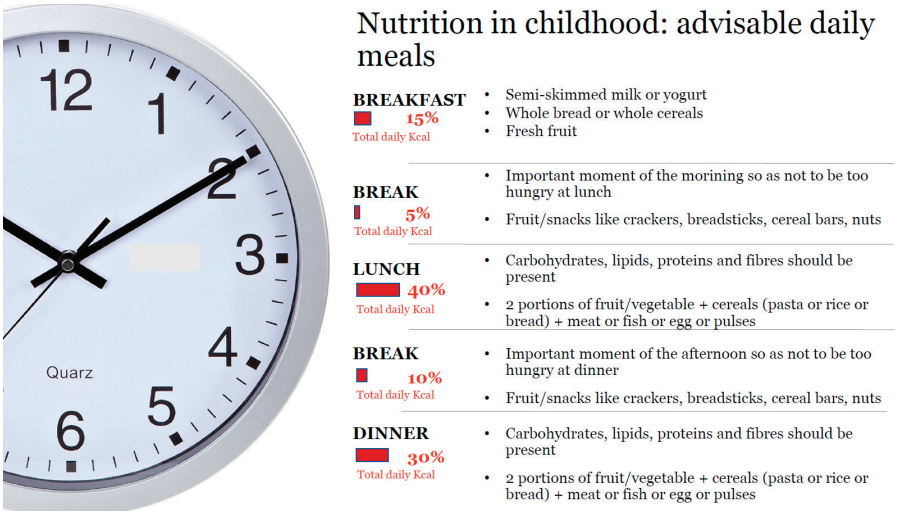

Figure 2. Advisable daily meal distributions in childhood, modified from Giovannini et al. [57].

Table 2. Recommended macronutrients intake for school-age children, modified from SINU [55].

• Adequate energy intake depending on age and sex (1372–2499 Kcal/day)
• Proteins 12–14% of total daily energy (0.94–0.97 g/kg/day)
• Carbohydrates: 55–60% of total daily energy (sugar <15%)
• Fats: 20–35% of total daily energy (saturated-fat <10%, polyunsaturated fats 5–10% of total energy)

Daily energy intake must always be age related; protein intake 0.94–0.97 g/kg per day; carbohydrate and fat intake: 45–60% (sugar < 15%) and 20–35% (saturated fatty acids < 10%, polyunsaturated fatty acids 5–10%) of daily energy intake, respectively; fiber 8.4 g/1000 Kcal. General nutritional recommendations include increasing frequency intake of fruit, vegetables, legumes and fish, meantime decreasing meat consumption and introducing grain food, also according to the principles of the Mediterranean diet [60]. In the last years, the Nordic diet has emerged as a healthy eating option in Nordic countries (Denmark, Sweden, Finland) [61]; it is characterized by the intake of apples, pears and berries, root and cruciferous vegetables, cabbages, whole grain and rye bread as cereals, high intake of fish, low fat dairy products, potatoes and vegetable fats (margarine, vegetable oil). To our knowledge, the long-term effects of this diet on major chronic diseases have only been investigated in adults within Nordic counties [62,63]. Characteristics of Mediterranean Diet can be found in Table 3, comparison of the main feature of Mediterranean and Nordic diet can be found in Table 4.

Table 3. Main characteristics of Mediterranean Diet, derived from Widmer Rj et al. [60].

• Ideal model of diet even in "non-Mediterranean" countries
• High intake of fresh fruits and vegetables
• Daily intake of cereals (such as pasta, bread and others)
• High intake of blue fish
• Low intake of meat, preferring sheep, goat, chicken or turkey
• Daily use of olive oil, rich in unsaturated fatty acids
• High fibre intake
• Moderate amounts of food for meal
• Fresh and seasonal food
• Importance of conviviality

Table 4. Comparison of some aspects of Mediterranean and Nordic diet, derived from Galbete C. [63].

HEALTHY DIET OPTIONS IN EUROPE: NORDIC DIET AND MEDITERRANEAN DIET	
NORDIC DIET	MEDITERRANEAN DIET
• Whole grain/rye bread	• Cereals
• Berries	• Fruits/Nuts
• Apple and pear	• Vegetables
• Fish	• Fish
• Dairy products	• Dairy products
• Root vegetables	• Meat
• Cabbage/cruciferous vegetables	• Legumes and pulses
• Vegetable fats	• Olive oil
• Potatoes	• Alcohol

3. Specific Dietary Interventions

3.1. Dietary Treatment for FH in Children

Early treatment of FH can reduce the negative impact of high LDL-C levels, improving endothelial function, reducing atherosclerosis progression and the risk of cardiovascular disease [11]. First-line treatment of FH is represented by diet and promotion of a healthy habits [6]. Patients and their families should undergo education targeting lifestyle management and should be informed about food choices. A certified pediatrician expert in nutrition should involve the whole family in laying the foundations of a healthy diet. A complete record of dietary habits must be obtained, and recommendations for a diet should be personalized and interpreted for each child to address individual diet patterns. The goal is to early establish correct habits that are most likely to be maintained over time, until adulthood [64,65]. The lipid intake, in particular cholesterol and saturated fats, is a major determinant of blood cholesterol levels. The 2015 EAS guidelines recommended a restriction of saturated fats intake <7% of total calories and daily cholesterol intake <200 mg. In children below 2 years of age, dietary restriction of fat and cholesterol is not recommended to prevent the risk of poor growth and developmental delay, due to their crucial role in brain and cognitive development. In school-age children challenges are related to achieving quality of nutrient intake, meantime avoiding excessive caloric intake. In children below two years of age, saturated fats and cholesterol mainly derive from dairy products, therefore it is important to promote consumption of low-fat milk and other dairy products [58]. The ideal diet should present the following characteristics: protein intake of 12–14% of total daily calories with an animal/vegetal protein ratio of 1:1; carbohydrates (mainly complex type) intake of 55–60% of total daily calories, lipid intake below 30% but not below 25% of total daily calories (saturated fats < 7%, monounsaturated 10–15% and polyunsaturated 5–10%). The goal should be a moderate fat intake, with primary sources of added fats coming from vegetable oils. The dietary scheme should consist of 5 daily meals: breakfast, morning break, lunch, afternoon break and dinner. Daily energy intake should be correctly provided as follows: 20% from breakfast and morning break, 40% from lunch, 10% from afternoon break and 30% at dinner. Weekly food frequency

should be: meat 3 times/week (preferring lean meat), fish 4 times/week rich in DHA (blue fish, cod, salmon, tuna avoiding shellfish and clams), legumes 3–4 times/week, low-fat cheese 1–2 times/week, cold cuts 1–2 times/week and egg once/week [57]. The Mediterranean diet is the ideal model recommended, consisting of fruit and vegetables, grain products (especially whole grains), legumes, poultry and lean meats, fish and olive oil as the principal source of fats and low intake of salt. It encourages steam and oven cooking, limiting the use of fried foods [60,66]. A recent review has assessed the impact of diet on plasma lipids in patients with FH. A total of 19 RCTs (5 of which with pediatric population) encompassing 837 individuals with FH were included. In 10 out of 19 studies, a significant reduction in LDL-C was reported, including 8 dietary supplement interventions, 1 food-base intervention and 1 dietary counselling intervention. This systematic review first highlighted that the lack of effectiveness of diet in modulating LDL-C levels is likely due to biases in study designs rather than a true lack of effects. In fact, most RCTs with a low risk of bias were more likely to report significant reduction in LDL-C [67]. Diet and healthy lifestyle efficacy in children with FH is variable and it is modulated by genetic factors, such as Apolipoprotein E genotype (ApoE genotype E3/E4 is related to higher lipid profile but with a better response to diet intervention) [59]. Diet alone is often not able to achieve target levels for LDL-C and drug treatment is also required [58]. Physical activity should be promoted and sedentary lifestyle should be limited. It is strongly recommended the reduction of associated risk factors: cigarette smoke (also passive), obesity, diabetes and hypertension (11). Smoking habit should be discouraged firstly among parents and other family members, so as to reduce the risk for their children to adopt this habit [68].

3.2. Diet and Nutritional Intervention in Obesity

Obesity is currently one of the most serious global public health problems and robust evidence demonstrates that physical, metabolic, cardiovascular and psychosocial complications are already present in obese children and worsen in adulthood. The WHO European Childhood Obesity Surveillance Program (COSI) revealed that overweight and obesity rates among primary-schoolchildren range from 15–52% in boys and from 13–43% in girls, with higher prevalence in Southern European Countries (2012) [16]. Healthy lifestyle is another milestone for cardiovascular health, as shown in Figure 3.

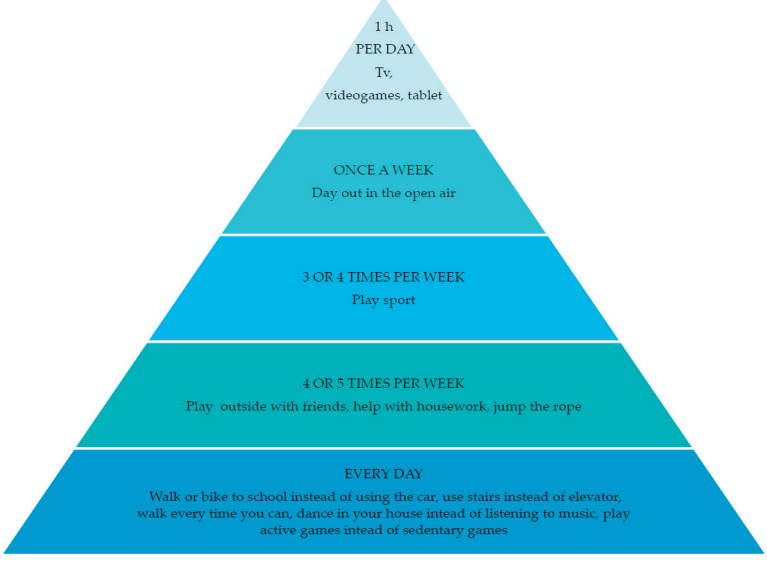

Figure 3. The physical activity pyramid for children, modified from Giovannini et al. [57].

According to the Italian Government Surveillance Project 'Okkio alla Salute' between 2008 and 2019, in Italy the prevalence of obesity decreased from 12% to 9.4%, while overweight prevalence decreased from 23.2% to 20.4% in primary-school children [69]. This is the consequence of numerous interventions promoting healthy lifestyles, such as increasing physical activity and improving eating habits in the schools, implemented at regional and local levels. Obesity is frequently associated with multiple metabolic abnormalities that increase patient's cardiovascular risk: dyslipidemia, insulin resistance and hypertension are the basic components of the metabolic syndrome. According to the International Diabetes Federation, the pediatric metabolic syndrome should be diagnosed in school-aged children (10 years old or more) when abdominal obesity is associated with two or more other clinical features as high blood pressure, elevated triglycerides levels, low HDL-C levels and increased glycemic levels [70]. Genetic, socio-economic and environmental factors play a role as metabolic syndrome drivers with unhealthy eating habits at the first place. A diet characterized by high protein content, saturated fats, refined grains, sugar and salty foods and a low consumption of fruits and vegetables, is recognized to worsen metabolic patterns associated with obesity and metabolic syndrome. Dyslipidemia, defined by one or more serum lipids out of range, represents one of the main comorbidities in pediatric obesity and its prevalence among overweight and obese children is about 46–50.4% [71,72]. In particular, in a study that included 139 children, ranging from 8 to 14 years of age, who were overweight or obese according to BMI z-score, detected dyslipidemia patterns were as follows: hypertriglyceridemia (31.9%), low HDL-C (29.7%), high non-HDL-C (15.8%), hypercholesterolemia (11.9%), high LDL-C (10.7%) [71]. BMI z-score showed a positive correlation with triglycerides (TG) and negative with HDL-C levels. The simultaneous presence of obesity and dyslipidemia (especially high TG levels) is associated with the risk of developing cardiovascular events in adulthood [73]. Specifically, TG/HDL-C ratio plays a decisive role and it can be used as a marker for identifying cardio-metabolic risk factors or signs of organ damage when >2.2 [74]. For this reason, it is recommended to screen overweight or obese children for dyslipidemia every three years since they reach six years of age; if they show a rapid increase in weight or develop other comorbidities, blood testing can be anticipated [6]. The main objective of the treatment of obese children and adolescents is a permanent change in their eating habits and lifestyle. A gradual reduction of BMI should be achieved through changes in diet and lifestyle, so as to obtain a negative caloric balance. As indicated by many expert panels, the whole family involvement and the setting of achievable goals are mandatory. Different authors reported that the prescription of a low caloric diet is not effective in the medium- and long-term management of obese children, being associated to relapses and failures, increased risk of dropout and progression into more complicated forms. The winning strategy starts from the analysis of the eating habits of the child and his family, for instance using a valid tool such as a food diary [75]. The main dietary advices summarized by the 2018 Consensus Position on Pediatric Obesity, issued by the Italian Society for Pediatric Endocrinology and Diabetology, are the following [76]: have an adequate breakfast, avoid eating between meals, have three meals and no more than two snacks per day, limit portions, avoid high-energy and low nutrient density foods (e.g., fruit juices or fast food), increase intake of fruit, vegetables and fiber-rich cereals. Nutritional recommendations for children with obesity can be found in Table 5.

Table 5. Nutritional recommendations for obese children, derived from Valerio G et al. [76].

Main Dietary Advices for Obese Children
• Consume adequate breakfast
• Avoid nibbling/avoid eating between meals
• Have three main meals and no more than two snacks per day
• Avoid high-energy food
• Limit portions
• Increase intake of fresh fruit and vegetables
• Increase intake of fiber-rich cereals

If a low caloric diet should be prescribed, LARN reference values for Italian population have to be respected for different age groups. In selected patients with severe obesity a very low caloric diet may be prescribed for some months (no longer than 10 weeks) under close medical surveillance with the aim to induce rapid weight loss, followed by a less restrictive dietary regimen, balanced in macronutrients. The protein sparing modified fast is an example of very low caloric diet (600–800 Kcal per day, protein 1.5–2 g/kg of ideal weight, carbohydrates 20–25 g/day; multivitamins, minerals and water > 2000 mL per day). Reduced caloric intake (1000–1500 Kcal/day) is achieved through categories of food grouped by nutrient density, it is well accepted and produces a significant improvement of BMI in 8–12 years old children even in a long-term period. Replaced meals are not recommended, since efficacy and safety have not been tested in children and adolescents; hypocaloric diets with low glycemic load, although having an effect on satiety, have not superior effect compared with other dietary approaches in the medium term [77]. The diet effect on these children is evaluated using the BMI-SDS (Standard Deviation Score); a reduction >0.5 in a growing child correlates with better body composition and decreased CHD risk [78]; waist circumference and skinfold thicknesses can be also used to measure fat percentage, but they do not offer other benefits with regard to BMI [79]. Several studies have shown that the discontinuation of weight management programs can discourage families, hinder the action of clinicians and result in inefficient use of clinical resources. Attendance rates and patients' enrollment present similar challenges, which must be addressed in order to optimize the strategy of care. Programs not meeting families' needs and logistical barriers are reported as the most commonly causes of attrition and failure in the management of pediatric obesity [80–82]. Several systematic reviews and meta-analyses on prevention and treatment of overweight and obese children and adolescents indicate that weight control may be obtained by multicomponent intervention focused on life-long changes of dietary habits and lifestyle, involving the whole family, the school and the communities. The effectiveness of these treatment programs on weight reduction on the long term from childhood to adulthood is still unknown and it needs further long-term studies.

3.3. Dietary Complements

In recent years, there is an increasing interest on functional foods and nutraceuticals. These products may act as a support therapy for lowering plasma cholesterol, LDL-C and triglycerides, especially in primary prevention of CHD in hypercholesterolemic subjects, whose blood cholesterol level is not within normal range but not high enough to require pharmacological treatment [83]. In 2011, the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents included for the first time the use of dietary adjuncts such plant sterols or stanols for children with primary hypercholesterolemia who do not achieve LDL-C goals with dietary treatment alone to avoid the necessity of drug treatment [6]. According to current evidence, nutraceuticals could exert significant lipid-lowering activity and their introduction in the dietary intervention has many advantages. Multiple mechanisms, such as inhibition of the absorption, synthesis and regulation of cholesterol metabolism are involved [84]. Nutraceuticals can act simultaneously on multiple stages of lipid-induced vascular damage; therefore, they can improve the lipid-lowering effects when used in combination with diet, pharmacological

treatment or other nutraceuticals. In addition, more, they can have a variety of positive pleiotropic effects, including improvement of arterial stiffness and endothelial dysfunction, as well as anti-inflammatory and anti-oxidative properties. Most of them originate from vegetable products. These compounds have a moderate lipid-lowering action and their use has proved to be safe and frequently well tolerated. Nutraceuticals do not represent an alternative, but a complement to the dietary-nutritional intervention that is the first line approach [85]. The experience of using nutraceuticals in pediatric age is still limited; most of the studies analyze the integration with alimentary fibers or with phytosterols/stanols [86]. Lipid-lowering effect of the fibers is associated to inhibition of the cholesterol absorption. Its intake should mainly derive from a correct intake of fruit and vegetables. In case of persistence of elevated levels of total cholesterol and LDL-C, supplementation with soluble fiber can improve the lipid profile, without significant adverse health effects [87]. Plant sterols are naturally occurring compounds found in plant cell membranes that are structurally similar to cholesterol. They compete with it and inhibit the absorption of cholesterol in the small intestine, resulting in lower plasma LDL-C levels. Sterols can be used in children with mild hypercholesterolemia, but there are no long-term safety data [88]. N-3 polyunsaturated fatty acids (PUFAs) with a double bond in position 3 at the end of the carbon chain, are found in animal (e.g., fish, egg, squid) and plant (e.g., algae, walnut, seed) sources. In recent years, the European Food Safety Authority (EFSA) and the American Heart Association (AHA) have recognized n-3 PUFAs as preventive nutraceuticals for CHD. EFSA established a claim in 2010 indicating that the intake of at least 2 g/day of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has the ability to maintain normal blood TG levels. AHA indicated doses from 2 to 4 g/day of EPA/DHA to reduce TG levels by 25–30%. All these guidelines agree about the high safety of PUFAs [89]. According with data available in the literature and the European Guidelines (EAS 2016) we can suggest the use of functional foods based on fiber and plant sterols in the treatment of children with genetic-familial dyslipidemia from 6 years of age upwards.

4. Conclusions

It is worldwide recognized that cardiovascular diseases are related to dyslipidemia, hypertension, obesity, diabetes mellitus, tobacco use and physical inactivity and the principal causes of these risk factors are adverse behaviors and lifestyles. In the last 20 years, an increasing attention has been given to the importance of nutrition in the first years of life, also including fetal period, as primordial prevention. A prudent diet is central to prevent the development of dangerous serum lipid levels, excessive adiposity and elevated blood pressure. Prevention of the CHD through intervention in childhood is supported by the fact that dietary habits and food preferences are formed early in life and that lifestyle and eating habits set in the family environment since childhood tend to be maintained over time throughout the life span. Nowadays, promoting lifestyle modifications and the pursuit of psychological well-being are universally recognized as major factors that may provide more enduring effects, as well as the reduction of the so-called allostatic load in pediatric patients and their families, thus improving the approach to the treatment of children and adolescents at high cardiovascular risk [90].

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Abbreviations

AHA	American Heart Association
BMI	Body Mass Index
CHD	Coronary Heart Disease
COSI	European Childhood Obesity Surveillance Program
DHA	Docosaexahenoic Acid
DISC	Dietary Intervention Study in Children
EAS	European Atherosclerosis Society
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FH	Familial Hypercholesterolemia
LARN	Italian Society of Human Nutrition reference values
LDL-C	Low Density Lipoprotein Cholesterol
HDL-C	High Density Lipoprotein Cholesterol
HoFH	Homozygous Familial Hypercholesterolemia
PUFAs	N-3 polyunsaturated fatty acids
SINUPE	Italian Society of Pediatric Nutrition
STRIP	Special Turku Coronary Risk Factor Intervention Project for Babies
TG	Triglycerides

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Review

Nutraceuticals in Paediatric Patients with Dyslipidaemia

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Abstract: Coronary heart disease (CHD) is the main cause of death and morbidity in the world. Childhood is a critical period during which atherosclerosis may begin to develop; in the presence of familial hypercholesterolaemia (FH), the lifelong elevation of LDL cholesterol levels greatly accelerates atherosclerosis. Lowering LDL-C levels is associated with a well-documented reduction in cardiovascular disease risk. Current guidelines support the dietary and lifestyle approach as the primary strategy of intervention in children and adolescents with FH. Nutraceuticals (functional foods or dietary supplements of plant or microbial origin) are included in the EU guidelines as lifestyle interventions and may provide an additional contribution in reducing LDL levels when pharmacological therapy is not yet indicated. Meta-analyses of randomised clinical trials have demonstrated that the same nutraceuticals improve lipid profile, including lowering LDL-C, total cholesterol and triglyceride levels. In this narrative review, starting from current scientific evidence, we analyse the benefits and limitations of the nutraceuticals in children and adolescents with dyslipidaemia, and we try to evaluate their use and safety in clinical practice.

Keywords: nutraceuticals; paediatric; diet; familial hypercholesterolaemia; dyslipidaemia

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1. Introduction

Coronary heart disease (CHD) is the first cause of mortality and morbidity worldwide, especially in industrialised countries: in Europe, cardiovascular events account for 45% of mortality in adults, with a prevalence of 49% in females and 40% in males [1].

Dyslipidaemia is an important cardiovascular risk factor: high total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels, low high-density lipoprotein cholesterol (HDL-C) and elevated triglycerides (TG) levels support the atherosclerotic pathways from the first decades of life. An altered lipid profile is a common finding both in the adult and paediatric population, with an estimated prevalence of 10–25% in children and adolescents. Dyslipidaemia is constantly increasing in children and adolescents, together with the epidemic increase of overweight and obesity [2].

It is recognised worldwide that the atherosclerotic process begins early in life and progresses through childhood into adulthood. The cumulative risk of exposure to elevated LDL-C levels accelerates the progression of atherosclerosis: the longer the exposure to high LDL-C, the higher is the CHD risk. In patients with homozygous familial hypercholesterolaemia (HoFH), LDL-C values are extremely elevated starting from birth: in these patients, myocardial stroke and angina pectoris have been described even before the age of ten years. Early detection and treatment of children and adolescents at high CHD risk is a fundamental milestone in CHD prevention: early intervention on dietary habits, lifestyle and, if necessary, pharmacological therapy started in paediatric age can modify the natural history of the disease, thus "gaining decades of life" with a drastic reduction in CHD events in adulthood [3,4].

According to the main Guidelines and Consensus Statements (American Academy of Pediatrics, AAP and European Atherosclerosis Society, EAS), the first step in the treatment of hypercholesterolaemia in children and adolescents are nutritional and dietary approach and the improvement of the lifestyle, as shown in Table 1.

Table 1. Nutritional and lifestyle intervention in paediatric patients with hypercholesterolaemia; adapted from Giovannini et al. [5] and Catapano et al. [6].

- Nutritional treatment is the milestone intervention in paediatric patients at increased CVD risk.
- Dietary-nutritional intervention in children with dyslipidaemia has the main objective to establish correct eating habits that are most likely to be maintained over time until adulthood.
- It is recommended to limit the consumption of foods with high content of saturated fats, as they are the main responsible for the increase in cholesterolaemia.
- A prudent low-fat diet is recommended, encouraging the intake of fruits, vegetables, unrefined grains, pulses, fish and meats.
- The traditional Mediterranean diet represents the ideal model because it proposes a diet rich in these foods, reduced consumption of salt and condiments in the preparation of foods, a preference for extra virgin olive oil and steamed, baked and stewed foods.
- Physical activity should be promoted, and conditions related to the CVD risk should be limited, such as sedentary life, cigarette smoke (also passive), obesity, hypertension and diabetes.

If dietary and lifestyle interventions are not effective on LDL-C reduction and/or in the presence of severe hypercholesterolaemia, pharmacological treatment should be considered [5,7].

The so-called functional foods have recently been introduced in the treatment of dyslipidaemia in adult patients. Dietary supplements or fortified foods have been widely used as a complement of the diet in adult patients with dyslipidaemia, and in the latest EAS guidelines [6], they have also been indicated for paediatric patients with familial hypercholesterolaemia (FH).

The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice and can be defined as "a food (or part of the food) that provides medical or health benefits, including the prevention and/or treatment of a disease".

We have schematically represented the possible use of nutraceuticals in the treatment of dyslipidemia in pediatric age in Figure 1.

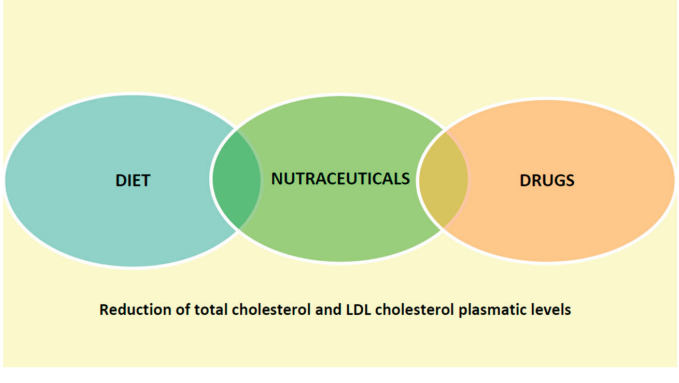

Figure 1. Use of nutraceuticals in dyslipidaemia in childhood.

Based on the current scientific evidence, this narrative review aims to analyse the benefits and limitations of nutraceuticals in children and adolescents with dyslipidaemia and tries to evaluate their use and safety in clinical practice.

We decided to consider the most widely used nutraceuticals in dyslipidaemia treatment. We started from each nutraceutical mechanism of action, and we considered the most important studies conducted on adult subjects. Moreover, we analysed the existing evidence in childhood and reported the main indications for paediatric patients in the present consensus documents.

2. Nutraceuticals and Dyslipidaemias

Nutraceuticals are included in the recent guidelines as lifestyle interventions for adult patients with dyslipidaemia in order to reduce total-cholesterol, LDL-cholesterol and triglycerides plasma levels, especially in those patients with moderately altered lipid profile, as primary prevention, when pharmacological intervention is not yet indicated [6]. Nutraceuticals showed a moderate lipid-lowering action, and their use in adult patients is safe and often well tolerated. They exert their lipid-lowering effect through different mechanisms: inhibition of cholesterol absorption, synthesis and metabolism, thus obtaining a multiple-line intervention, which can be combined with dietetic and lifestyle treatment, other nutraceuticals and pharmacological therapy [8]. Nutraceuticals have multiple pleiotropic effects: they enhance endothelial function, modulate arterial wall stiffness and have anti-inflammatory and anti-oxidative properties [9]. They are often well tolerated also by those patients that are intolerant to statin therapy [10]. Old patients (aged > 75 years) and patients that, despite therapy with statin or ezetimibe, cannot reach LDL-cholesterol target levels could benefit from the association of pharmacological therapy with nutraceuticals [11,12]. According to this evidence, in EAS guidelines [6], nutraceuticals are indicated as lipid-lowering agents for specific categories of both adult and paediatric dyslipidaemic patients above six years of age.

There are few randomised controlled studies, often based on small cohorts, analysing the effect of nutraceuticals in paediatric patients with dyslipidaemia. Fibres and phytosterols/stanols are the most widely studied nutraceuticals in childhood, whereas clinical trials concerning red yeast, soy proteins, probiotics, omega-3 fatty acids and nuts are sporadic and isolated [13].

Nutraceuticals with lipid-lowering effect can be divided into three main categories, according to their action on cholesterol metabolism:

- Inhibitors of intestinal cholesterol absorption;
- Inhibitors of liver cholesterol synthesis;
- Inducers of cholesterol excretion.

However, many nutraceuticals can act through multiple pathways and often with unclear mechanisms of action, with a final positive effect on lipid metabolism and atherosclerosis reduction, as shown in Table 2 [14].

Table 2. Nutraceuticals effect on lipid profile.

	Reduce Total Cholesterol	Reduce LDL Cholesterol	Reduce Triglycerides	Increase HDL Cholesterol
Fibres	+	+		
Phytosterols and stanols	+	+		
Probiotics	+	+		
Red yeast rice	+	+		
Soy and lupins	+	+	+	+
Omega-3 fatty acids			+	

+ means positive effect.

3. Nutraceuticals Inhibitors of Intestinal Cholesterol Absorption

3.1. Fibres

Alimentary fibre is a component of plant foods constituted by carbohydrates resistant to the digestive process in the gastrointestinal tract. Alimentary fibre includes non-amylaceous polysaccharides (cellulose, hemicellulose, gum, pectin), oligosaccharides (inulin, fructo-oligosaccharides) and lignin.

From a functional point of view, alimentary fibre can be divided into four classes [15]:

(1) Insoluble fibre (bran): fibre not soluble in water and poorly fermented in the gut, with a possible mechanical laxative effect.
(2) Soluble fibre (inulin, dextrin, oligosaccharides), non-viscous, rapidly fermented; it does not cause any increase in viscosity and is completely fermented by gut microbiota; it can exert a prebiotic effect, without any laxative effect.
(3) Viscous soluble fibre, rapidly fermented (β-glucan, guar gum, pectin); it creates a viscous gel in water, thus increasing chime viscosity and consequently slowing nutrients absorption. It is quickly fermented in the gut, losing its laxative effect.
(4) Soluble viscous non-fermentable fibre (psyllium, multicellulose): it reduces nutrients absorption thanks to its viscosity and can exert a laxative effect.

The main characteristics of inhibitors of intestinal cholesterol absorption are summarised in Figure 2.

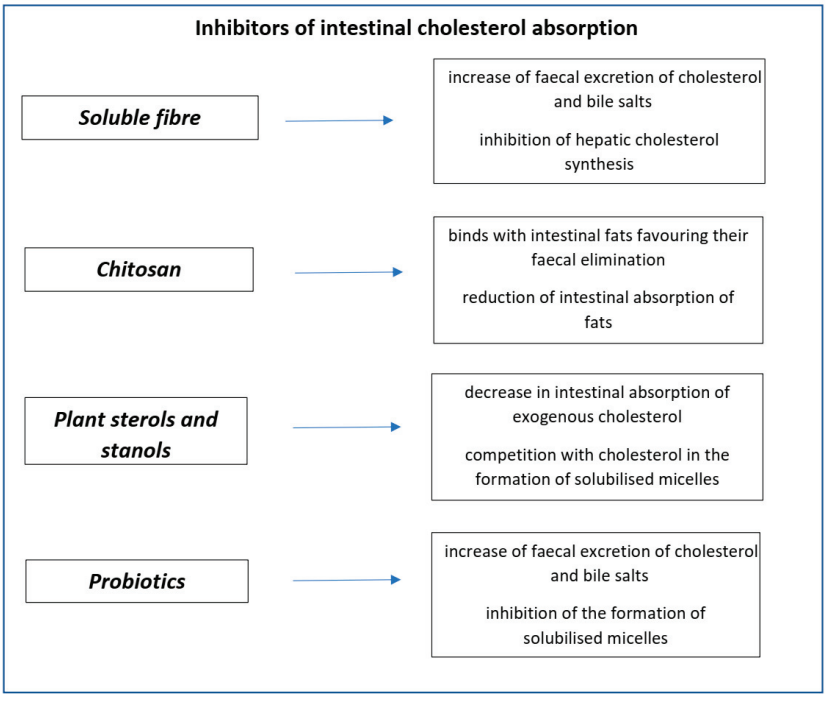

Figure 2. Nutraceuticals' inhibitors of intestinal absorption.

The cholesterol-lowering effect of fibre is mainly due to its viscosity: viscous fibres soluble in water form a gel that binds to bile salts in the gut and increases their excretion with stools. Cholesterol is one of the main components of bile; therefore, an increase in bile salts excretion causes an increased cholesterol use for hepatic bile synthesis. The higher the fibre viscosity, the greater its cholesterol-lowering effect [16]. Moreover, short-chain fatty acids (SCFA), which are derived from gut fermentation of fibre, can have positive effects on lipid profile [17].

The effect of fibre on lipid metabolism has been demonstrated in terms of total and LDL-cholesterol plasma levels reduction [18], and therefore, it has been recognised by the European Food Safety Authority (EFSA) [19]. Observational studies have shown that the habitual assumption of fibre is associated with a reduction in cardiovascular risk [20]. In particular, every 10 g increase in fibre daily assumption (especially if derived from fruits and whole cereals) is related to a reduction of 14% of acute coronary events and a reduction of 27% of death derived for coronary events [21].

Numerous studies have evaluated the effect of fibre assumption on lipid levels [22,23]. Dietary supplementation with oat β-glucan [24,25], psyllium [26,27], pectin [28], guar gum, glucomannan [29] and hydroxypropyl methylcellulose [30] significantly reduces LDL-cholesterol levels.

Psyllium is derived from the peel of a seed called *Plantago Ovata*; if added to cereals, it does not alter their taste or consistency, thus granting good compliance [31].

Oat is a soluble and viscous alimentary fibre derived from *Avena Sativa*, rich in β-glucan [32].

Glucomannan is derived from a tuber called *Amorphophallus Konjac*; it is the fibre with the highest molecular weight and the greatest viscosity, able to reduce LDL-cholesterol levels more effectively than psyllium, bran [16], oat and barley [33].

Fibre assumption in childhood is a debated topic. Food and Drug Administration (FDA) advise an adequate fibre daily intake in relation to the total daily caloric intake (12 g/1000 Kcal), whereas the AAP relates it to age and weight [34]. In clinical practice, for children above three years of age, the recommended daily fibre intake (in grams) is equal to the sum of age in years plus 5 [35–37]. A large general paediatric study on a cohort of 5873 Japanese school children has evaluated that daily fibre intake is inversely related to total cholesterol plasma levels and to the development of overweight and obesity, confirming data already reported in adult patients [38,39].

Psyllium effect on lipid profile has been demonstrated in various studies [36,38,39]. In a 12-week randomised controlled trial in a cohort of 50 children with mild hypercholesterolaemia in CHILD I dietary treatment (lipid daily intake <30% total daily calories, saturated fatty acids daily intake $\leq$10% of total daily calories, cholesterol daily intake <300 mg) or in CHILD II dietary treatment (fatty acids daily intake 7% of total daily calories, cholesterol daily intake <200 mg), treatment with psyllium 3.2 g/day resulted in an 8.9% reduction of LDL cholesterol if compared to the control group receiving only dietary treatment [38]. In another study, in a cohort of 36 children with familial combined hypercholesterolaemia (FCH) on CHILD I dietary treatment, a daily intake of psyllium (2.5–10/day, depending on age) was associated with an 18% and 23% reduction of total cholesterol and LDL-cholesterol levels, respectively [37].

Glucomannan has been successfully tested in a 24-week trial in a cohort of 36 children (aged 6–15 years) with FH. All children were advised to follow CHILD I dietary treatment for one month, and then, they were given glucomannan (1–1.5 g/day, according to the patient's weight). After one month of treatment, their lipid profile was improved, with a reduction in the levels of total cholesterol (5.1%), LDL cholesterol (7.3%) and non-HDL cholesterol (7.2%), compared to pre-treatment values [40]. The same results were found in another study: 40 children treated with glucomannan (2–3 g/day) had a reduction in LDL cholesterol of 30% in females and 9% in males [41]. The efficacy of glucomannan on lipid profile was also analysed in two meta-analyses. In the first one, in a cohort of 531 obese children, the authors did not report any reduction in LDL-cholesterol levels but

a statistically significant reduction in triglycerides levels [29]. In the second study, LDL cholesterol was significantly reduced both in children and in adults [42].

Oat bran effect on lipid profile has been poorly investigated up to now, and there are no recommendations on its assumption for paediatric patients [43]. A cross-over randomised clinical trial analysed the effect of carob seed flour on lipid profile: in a 16-week period, 11 children with FCH, 10 controls and 17 adults were given 8–30 g/day of carob seeds flour, with a consequent 11–19% reduction of LDL-cholesterol levels [44].

Most of the studies carried out on the use of the fibre in paediatric patients highlighted good compliance to the proposed therapy, thanks to the good palatability of the nutraceutical; side effects as diarrhoea and abdominal pain were only occasionally reported [39,40]. However, safety data on long term fibre treatment in paediatric patients are still limited.

In conclusion, fibre intake should mostly derive from an adequate fruits and vegetable intake. In case of persistently elevated total and LDL-cholesterol levels despite good compliance to dietary treatment, supplementation with soluble fibre should be considered in order to improve the lipid profile, with no relevant adverse effects.

3.2. Phytosterols and Stanols

Phytosterols and stanols are plant-derived bioactive components structurally similar to cholesterol. Phytosterols are steroidal alkaloids that differ from cholesterol for their lateral chain, whereas stanols are 5α-saturated derived from phytosterols. Both these compounds are not synthesised by humans, so they have to be introduced with foods, such as fresh fruits, nuts, vegetables, seeds, cereals, pulses and vegetable oils [45].

The use of foods enriched with phytosterols was included in 2011 National Cholesterol Education Program (NCEP) guidelines for LDL-cholesterol reduction. The cholesterol-lowering effect of phytosterols is based on the reduction of the intestinal absorption of exogenous cholesterol: due to their structural homology to cholesterol, phytosterols compete with cholesterol in the formation of solubilised micelles and in the binding with Niemann-Pick C1 Like Protein (NPC1L1) in the enterocytes; this condition is favoured by the highly hydrophobic properties of phytosterols compared to cholesterol. Phytosterols present in the enterocytes are quickly excreted in the lumen by transporters ABCG5 and ABCG8, so plasma phytosterols concentrations are always very low, and they are promptly available in the gut to compete with cholesterol. Moreover, the presence of phytosterols in the intestinal lumen inactivates acyl-CoA-cholesterol-acyltransferase, limiting cholesterol entry into the lymphatic vessels and its transport to the liver. The reduction of intestinal cholesterol entrance and of chylomicrons transport to the liver reduces plasma LDL-cholesterol levels [46,47].

Transversal studies have demonstrated an inverse correlation between natural phytosterols intake and LDL-cholesterol plasma levels [48,49]. Randomised controlled intervention studies have validated the cholesterol-lowering effect of phytosterols: the intake of functional foods containing phytosterols significantly reduces total and LDL-cholesterol plasma levels with an average reduction of 8–10% both in hypercholesterolaemic and in healthy subjects [46]. The effect of phytosterols is dose-dependent for doses <3 g/day, whereas for doses higher than 3 g/day, there is a plateau effect without any further beneficial effect on lipid metabolism [14].

There is little evidence of the effect of phytosterols in paediatric patients; however, the available literature demonstrates that phytosterol intake is associated with a reduction in total cholesterol levels in children with mild hypercholesterolaemia [50] and in children with FH [51,52]. The supplementation with 1.2–2 g/day phytosterols in children with FH in CHILD I or CHILD II dietary treatment has determined a further 10% reduction of LDL-cholesterol levels. An increased dose of phytosterols (2.3 g/day) was associated with further LDL-cholesterol reduction [51,52]. Treatment with phytosterols and stanols is usually well tolerated, and no major adverse effects have been reported so far, even if long term follow-up data are not yet available [14].

3.3. Chitosan

Chitosan is derived from chitin deacetylation. Chitin is a polymer that protects insects and shellfish, granting hardness and resistance to their shells. Chitosan can bind to lipids, thus reducing their intestinal absorption and promoting their elimination with the stools [53].

A meta-analysis of six randomised controlled trials on 416 adult patients with hypercholesterolaemia concluded that chitosan has a significant effect on total cholesterol reduction (-11.6 mg/dL, $p = 0.002$), but no effect on the other lipid parameters [54]. On the contrary, other studies highlighted the effect of chitosan on LDL cholesterol, HDL cholesterol and triglycerides [55]. These contrasting data suggest the need for further studies to better understand the lipid-lowering effect of chitosan [14]. Chitosan is usually well tolerated; transient minor adverse effects, such as abdominal pain, vomiting or diarrhoea, have been seldom reported [56]. Based on the mechanism of action, chitosan use seems to be promising, but data regarding its effects in the paediatric age are still lacking.

3.4. Probiotics

Probiotics are vital micro-organisms that, when taken in adequate amounts, confer a health benefit to the host. In the last years, some trials have supported the clinical use of probiotics as lipid-lowering agents. However, available studies are heterogeneous in terms of length, strains of probiotics, dose, clinical characteristics of study participants and type of carriers [14]. The mechanisms of action of probiotics on lipid metabolism are still unclear and not yet fully defined. Probiotics seem to interfere with gut cholesterol, binding to it or incorporating it in their cell membrane [57]. Lactobacillus acidophilus and Lactobacillus bulgaricus contain some enzymes able to catalyse cholesterol transformation, promoting cholesterol excretion with stools [58]. Other probiotics reduce the entero-hepatic circulation of bile salts through the activation of bile salt hydrolase. Some strains of Lactobacilli and Bifidobacteria can enzymatically de-conjugate bile acids, increasing their excretion and promoting cholesterol systemic hepatic mobilisation for bile salts de novo synthesis [59]. Other probiotics can influence intestinal pH, micelle formation, cholesterol transport and the transport of lipoproteins and of cholesterol esters [60]. These mechanisms are only hypothetical, so additional data are necessary to confirm which probiotic has a better lipid-lowering effect.

In adult patients with hypercholesterolaemia, the administration of different probiotic strains caused a reduction in total and LDL-cholesterol levels [61].

In a randomised, double-blind, placebo-controlled, cross-over trial in a cohort of paediatric patients with hypercholesterolaemia (total cholesterol $\geq 90^{\circ}$ centile for age and sex), the oral intake of a three-strain Bifidobacteria probiotic blend resulted in an improvement in the lipid profile with a reduction of 3.4% of total and of 3.8% of LDL cholesterol [62]. The use of probiotics is considered safe and with no adverse effects [14].

4. Nutraceuticals Inhibitors of Liver Cholesterol Synthesis

Nutraceuticals that mainly act through the inhibition of hepatic cholesterol synthesis are red yeast rice, policosanols, bergamot and garlic. Their characteristics are summarised in Figure 3.

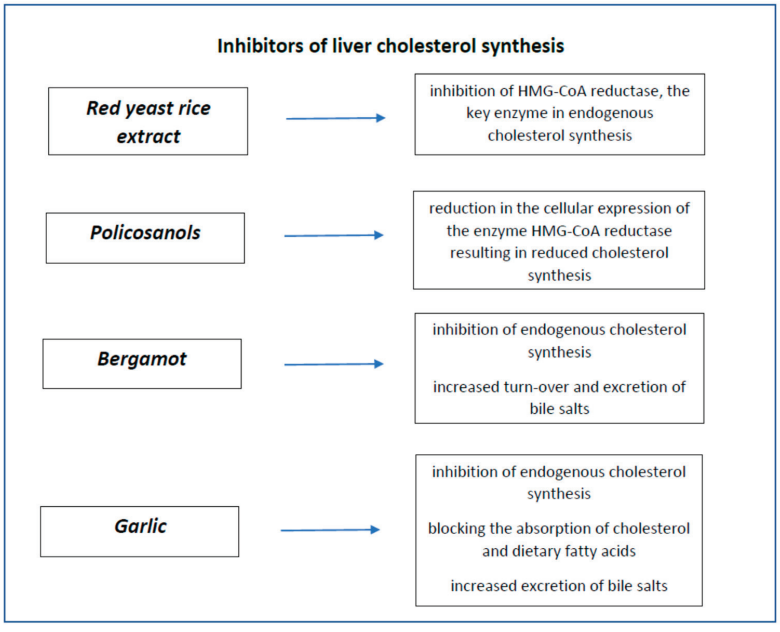

Figure 3. Nutraceuticals' inhibitors of liver cholesterol synthesis.

4.1. Red Yeast Rice

Red yeast rice (RYR) is obtained by the fermentation of a specific yeast (*Monascus purpureus*, *M. pilosus*, *M. floridanus*, *M. ruber*) in rice (*Oryza sativa*); it has been widely used in China in the past centuries to flavour foods [63].

Monascus purpureus ferments red rice and produces a substance called Monacoline K, which can inhibit the hepatic activity of HMGCoA reductase and, therefore, endogenous cholesterol synthesis. Monacolin K is structurally and functionally similar to lovastatin [64]. Cholesterol-lowering effect of RYR is only partially due to Monacolin K, as RYR contains at least other ten different kinds of monacolin, phytosterols able to reduce cholesterol gut absorption, fibres and niacin, that can have a lipid-lowering effect as well [63,64].

RYR has been proven to be effective and safe in patients with mild or moderate hypercholesterolaemia. The first prospective, double-blind, placebo-controlled study on the effect of RYR on lipid profile was conducted in the USA in 1999. Patients with hypercholesterolaemia not on pharmacological treatment were randomised to receive RYR 2.4 g/day or placebo for 12 weeks; at the end of the study, LDL-cholesterol levels were reduced by 22% compared to a reduction of 5% in the placebo group, with no significant side effects [65]. The efficacy and safety of RYR have been recently confirmed in a meta-analysis involving 20 studies: LDL-cholesterol reduction in a 2–24 months period of treatment was comparable to that obtained with a moderate-intensity statin (pravastatin 40 mg, simvastatin 10 mg or lovastatin 20 mg), and the incidence of hepatic, muscular or kidney adverse effects was similar to those reported in the placebo group [66].

RYR is one of the few nutraceuticals also studied to determine its efficacy in the secondary prevention of cardiovascular events: patients treated with RYR had a 20% reduction in LDL-cholesterol plasma levels and a 45% reduction of coronary events compared with the placebo group [67].

Commercial products containing RYR are still under analysis for their safety profile, as there is a large variety in the content of monacolin K, and the presence of a mycotoxin called citrinin has been reported [68,69].

According to EFSA claim in 2011, RYR with content of monacolin K ≤10 mg/die can be used in adult patients at mild to moderate cardiovascular risk, with LDL cholesterol higher no more than 25% of the therapeutic goals, despite dietary and lifestyle interventions [6,70].

There are few studies on the use of the RYR in paediatric patients. In a clinical trial conducted by Guardamagna et al., a paediatric population aged 8–16 years with mild to moderate dyslipidaemia treated for 8 weeks with RYR containing 3 mg/day of monacolin K showed an 18.5% and 25.1% reduction of the total and LDL cholesterol, respectively; no particular side effects were reported. However, monacolin is similar to lovastatin; therefore, a careful clinical and biochemical follow-up is mandatory when it is used in paediatric patients. The purity of the product and the absence of citrinin must be granted as well [69].

In conclusion, RYR could be a therapeutic alternative for paediatric patients with hypercholesterolaemia at high risk, but it must be accompanied by close clinical and biochemical monitoring under medical supervision [70,71].

4.2. Policosanols

Policosanols (PCS) are a mixture of long-chain alcohols extracted from plant waxes, sugar cane, rice bran and potatoes [72]. PCS have been widely used in Cuba in the last decades as lipid-lowering agents. Several studies demonstrated that PCS derived from sugar cane have better lipid-lowering activity than phytosterols, similar to statins, but with a better effect on HDL-cholesterol level and few side effects [73]. The mechanism of action of PCS is yet partially unknown, and it is probably due to a reduction in cellular expression of HMGCoA reductase, with a consequent reduction in cholesterol synthesis. The cholesterol-lowering effect seems to be dose-dependent in a dose range from 2 to 40 mg/day [74]. Recently, the lipid-lowering effect of PCS has been questioned in studies conducted in Europe and in the USA [75]. In 2011, EFSA rejected a claim in favour of PCS for lack of evidence [76].

There are few data on the use of PCS in paediatric patients with dyslipidaemia. In a study conducted by Guardamagna et al., the association of PCS and RYR showed a reduction of the total- and LDL-cholesterol levels, with good compliance and few adverse effects [71]. In conclusion, there is no evidence on efficacy and safety of PCS in paediatric patients, and PCS are not currently recommended in children [76].

4.3. Bergamot

Bergamot is the common name of Citrus bergamia Risso, a citrus rich in flavonoids, some of which have a statin-like lipid-lowering activity as they inhibit HMGCoA reductase in the liver [77]. Bergamot also has an anti-oxidative effect, and it can promote cholesterol stool excretion by reducing intestinal cholesterol absorption and increasing bile acid turnover [78]. There are few studies on the lipid-lowering effect of bergamot. Gliozzi et al. reported a reduction in LDL-cholesterol plasma levels (comparable to that obtained with rosuvastatin 10 mg/day) in patients with dyslipidaemia treated with 1000 mg/day bergamot for a four-week period [79]. In another study, a dose-dependent lipid-lowering effect was reported in patients treated with bergamot, both in patients with mild hypercholesterolaemia (LDL cholesterol: 130 mg/dL) and in those with combined dyslipidaemia (hypercholesterolaemia and hypertriglyceridaemia), with no adverse effects [80].

Up to now, studies with bergamot supplementation with doses ranging from 500 mg to 1500 mg/day have a good safety profile and no side effects reported [78]; this makes it interesting for possible use in children, but there is a lack of specific data in the literature.

4.4. Garlic

Garlic (*Allium sativum*) is a functional food with multiple health benefits. Garlic's main active compound is called allicin. When fresh garlic is chopped or crushed, the enzyme alliinase converts alliin into allicin, which is responsible for the aroma of fresh garlic; the allicin generated is unstable and quickly changes into a series of other sulfur-containing

compounds such as diallyl disulfide. Allicin can inhibit HMGCoA reductase, reducing endogenous cholesterol synthesis [81].

A meta-analysis of 39 RCT has reported garlic intake in a two-month period in patients with mild to moderate hypercholesterolaemia and showed a 9 mg/dL reduction of LDL-cholesterol levels [82]. Another study highlighted a good antihypertensive effect of garlic [83], whereas Jung et al. reported a significant association between garlic intake and improvement in LDL/apoB ratio with the reduction in apoB [84]. Garlic also has anti-platelets properties [85].

In conclusion, a 6 g garlic daily intake can be useful in the treatment of moderate hypercholesterolaemia, probably mainly due to garlic anti-platelets and anti-hypertensive properties rather than a direct lipid-lowering effect. Garlic is usually well tolerated, and adverse effects are mild, mainly gastrointestinal. However, the high dosage required and peculiar garlic aroma often interfere with patients' compliance [86]. There is no available evidence in paediatric patients.

5. Nutraceuticals Inducer of LDL Cholesterol Excretion

Some nutraceuticals exert their lipid-lowering action through an increase in LDL-cholesterol excretion, promoting an increase in LDL-receptor expression and its half-life on the hepatocyte surface.

Soy, lupins and berberine are the most studied among this class of functional foods. Green tea extracts are in this category as well.

Their main characteristics are summarised in Figure 4.

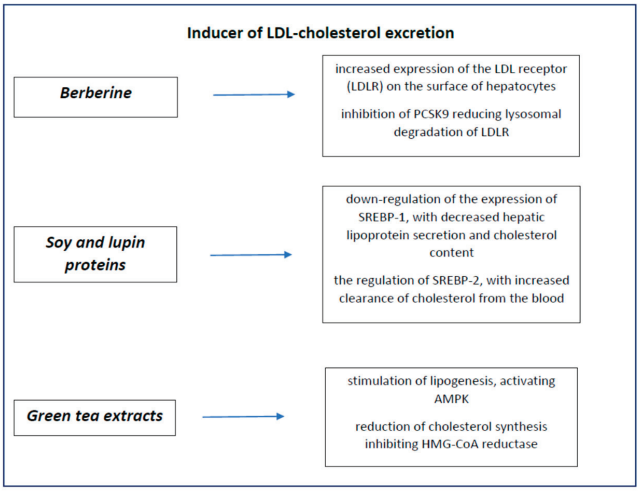

Figure 4. Nutraceuticals' inducer of LDL-cholesterol excretion.

5.1. Berberine

Berberine is a quaternary ammonium salt from the protoberberine group of benzylisoquinoline alkaloids found in such plants as Berberis, such as *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric), *Mahonia aquifolium* (Oregon grape), *Hydrastis canadensis* (goldenseal), and *Coptis chinensis* (Chinese goldthread) [87]. Berberine is usually found in the roots, rhizomes, stems and bark [87].

Berberine cholesterol-lowering effect is determined by several mechanisms. Berberine promotes an increase in the expression and in the half-life of LDL receptor (LDL-R) on hepatocyte surface [88]; it also enhances the increase of LDL-R promoter transcriptional activity and the stabilisation of its mRNA [89]. Moreover, in vitro studies show that

berberine inhibits proprotein convertase subtilisin/kexin type 9 (PCSK-9) activity, reducing lysosomal LDL-R degradation and increasing its availably in the liver [90].

In the first study on the effect of berberine intake on lipid profile in a cohort of patients with hypercholesterolaemia, berberine intake was related to a reduction both in LDL cholesterol and in triglycerides [89]. Berberine's lipid-lowering effect has been analysed in three meta-analyses [91–93] that showed similar results: berberine allowed a 25 mg/dL reduction in LDL cholesterol, a significant reduction in triglycerides and a slight increase in HDL-cholesterol levels. Berberine main side effects, which occur at high dosages, are gastrointestinal, such as constipation, diarrhoea, abdominal pain and sour taste [93]; these characteristics make it unattractive in children.

5.2. Soy and Lupins

Soy is a bean (*Glycine max*) derived from an Asian plant, grown for its multiple nutritional properties; it is very rich in protein (36–46%), essential amino acids, lipids (18%), soluble carbohydrates (15%) and fibres (15%). Soy contains a lot of micronutrients, such as soy lecithin (0.5%), sterols (0.5%) and tocopherols (0.02%). Soy nutritional values and positive health effects have been studied for years, based on epidemiologic data suggesting an inverse relationship between soy intake and cardiovascular disease [94].

Soy lipid-lowering effect is mainly due to isoflavones, which increase LDL-R expression in the liver. Isoflavones can also bind to estrogen receptors, promoting an estrogen-like activity, thus affecting lipid metabolism directly through the modulation of the lipogenesis or indirectly influencing appetite and the energy balance [95]. Multiple mechanisms are involved in the lipid-lowering effect of the soy [96]: lecithin and sterols reduce the intestinal cholesterol absorption, β-glucan increases the bile salts excretion [96,97] with a consequent reduction in lipoprotein hepatic secretion, reduction in cholesterol synthesis, and increase in biliary acids stool excretion [98–100]. Bioactive peptides, such as gamma conglutinine (Cy), present both in soy and in lupins, have a lipid-lowering effect through LDL-R activation in the liver [101].

Descovich et al. described a reduction in total cholesterol levels in a cohort of 127 patients with hypercholesterolaemia treated with soy proteins [96]. Various meta-analyses have confirmed the soy lipid-lowering effect. In 2015, in a study involving 35 clinical trials with a total of 2670 patients, soy intake was associated with a reduction of 2% in total cholesterol, 3% in LDL cholesterol and 4% in triglycerides and an increase of 3% in HDL-cholesterol levels [102].

Lupins are beans with low salt content, low glycaemic index and no phytoestrogens. Lupins are composed of proteins (30–35%), fibres (30%), carbohydrates (3–10%) and lipids (6%), 81% of which are polyunsaturated fatty acids.

Lupins' lipid-lowering effect has been described in several studies. Baehr et al. reported a positive effect on lipid profile (increase in HDL cholesterol and reduction in LDL cholesterol) in 33 subjects with hypercholesterolaemia treated with lupins (25 g/day) for a two-month period, followed by one month of wash-out [103].

There are few studies on soy and lupin effect on the lipid profile in paediatric patients. In a group of 16 children with FH on the CHILD I diet, the intake of soy milk (0.25–0.5 g/kg/day) was associated with a reduction in total cholesterol, LDL cholesterol and Apolipoprotein B (7.7%, 6.4% and 12.6%, respectively) [104].

In a recent study, the effect of soy intake on lipid profile was analysed in a cohort of paediatric patients with FH treated for a 13-week period. In the intervention group, LDL-cholesterol levels were reduced by 10% compared to pre-treatment values [105].

Chronic use of high quantity of soy, rich in isoflavones, could interfere with thyroid function and with fertility. Moreover, soy and its derivates are rich in phytic acid, which can reduce calcium, magnesium, copper, iron and zinc absorption.

Lupins have a good safety profile, and minor adverse effects have been reported. However, lupins' lipid-lowering action is dose dependent, and this could interfere with long-term compliance [14].

5.3. Green Tea Extracts

Some trials on green tea suggest that it can have a protective effect on cardiovascular disease [106]. Green tea is rich in antioxidants, such as polyphenols, which are cardioprotective compounds. Furthermore, green tea can promote lipogenesis through AMP-activated protein kinase (AMPK) activation, and it can reduce cholesterol synthesis through HMGCoA reductase inhibition. Green tea may also interfere with micelle formation, thus reducing intestinal cholesterol absorption [14]. Green tea catechins have an inhibitory effect on apical biliary salt transporter in the ileum, so they can reduce bile salts re-uptake and increase liver expression of LDL receptors [107].

Some studies have reported the efficacy of green tea extracts on LDL cholesterol and on systemic blood pressure reduction [108]. Green tea is usually well tolerated, but if it is used for a long time and at a high dose, it can interfere with the intestinal absorption of iron and folate; for this reason, its use in children is unsafe.

6. Nutraceuticals with Mixed Action

In this category, we have nutraceuticals that exert their lipid-lowering and anti-atherogenic action with multiple mechanisms, often not completely known. Polyunsaturated long-chain fatty acids and curcumin are two of the most studied ones. Their characteristics are shown in Figure 5.

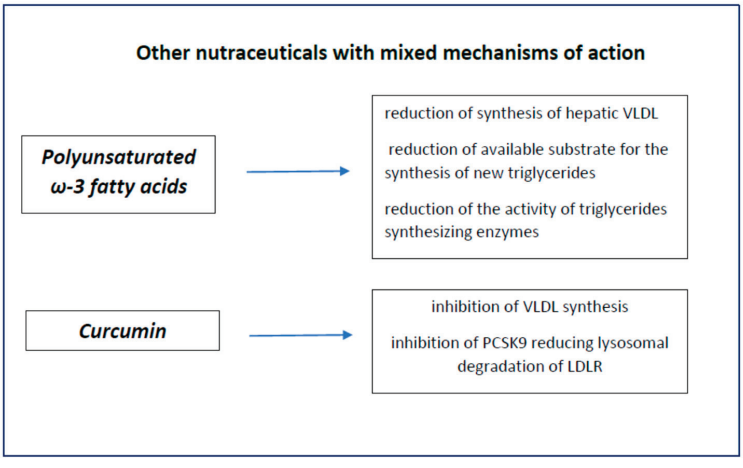

Figure 5. Nutraceuticals with mixed action.

6.1. Omega-3 Polyunsaturated Long Chain Fatty Acids

Polyunsaturated fatty acids (PUFA) are fatty acids that have more than one double C=C bond in their molecule. With regard to cardiovascular prevention, omega-3 and omega-6 PUFA are the most studied, as they can modulate triglycerides, LDL cholesterol and inflammatory markers implied in the atherosclerotic process [109–111].

Omega-3 fatty acids are present in nature both in animals, such as fish, krill, eggs and squid, and in vegetables, such as seaweeds, nuts, flax seeds and sage. Omega-3 PUFA have positive effects on cardiovascular health, as suggested by epidemiological and intervention studies. In the last few years, EFSA [112], AHA (American Heart Association) [113] and FSANZ (Food Standard of Australia and New Zealand) [114] have recognised omega-3 PUFA as functional foods for cardiovascular disease. EFSA suggests that a 2 g/day supplementation with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can maintain triglycerides plasmatic levels within normal range, whereas AHA suggests a 2–4 g/day supplementation with DHA and EPA to reduce triglycerides by 25–30% [113].

Omega-3 PUFA play their triglycerides lowering effect through different mechanisms: they reduce hepatic VLDL synthesis, act as false substrate in triglycerides synthesis, reduce the activity of enzymes involved in triglycerides synthesis, enhance fatty acids beta-oxidation and reduce fatty acids endogenous synthesis [115].

The positive effects of omega-3 fatty acids on cardiovascular risk factors have been confirmed by many authors. Eslick et al. published a very comprehensive meta-analysis involving 47 RCTs with approximately 16,500 patients with hypercholesterolaemia. The authors evaluated the effect on cardiovascular risk of a 3.5 g/day supplementation with EPA/DHA over 24 weeks; they found significantly reduced (14% compared to pre-treatment values) triglycerides, slightly reduced (2.5 mg/dL) LDL-cholesterol and unchanged HDL-cholesterol levels [116]. Later on, Leslie et al. confirmed these results in subjects with a normal lipid profile or with moderate dyslipidaemia, reporting a 9–26% reduction of triglycerides levels with a 4 g/day omega-3 PUFA intake and a 4–51% reduction with a 1–5 g daily intake [117].

The DART study, published in 1989, involved 2033 male patients with recent myocardial stroke randomised to different diets. After a two-year follow-up, the group treated with omega-3 showed a 29% reduction in mortality rate compared to the control group, and this reduction was mainly attributed to a decrease in cardiovascular events [118]. Approximately ten years later, in the GISSI study, 11,324 patients with recent myocardial stroke were enrolled. They were randomised to omega-3 PUFA and/or vitamin E for 3.5 years: after six months, no modification in the lipid profile was detected, while after twelve months, patients treated with omega-3 PUFA showed a 15% reduction both in global mortality and cardiovascular mortality (ictus cerebri and myocardial stroke); in the intervention group, death related to acute cardiovascular events was reduced by 45% [119]. In the JELIS study, the effect of EPA in addition to statin therapy was evaluated in 18,645 patients with hypercholesterolaemia: after 4.6 years follow up, a 19% reduction in major cardiovascular events was reported in the group that received EPA, but no modification on LDL or HDL cholesterol was detected [120]. These are the main trials analysing the hypothesis that supplementation with PUFA, alone or together with pharmacological therapy, can promote a reduction in individual cardiovascular risk and in major cardiovascular events.

However, the cardioprotective effect of PUFA has been questioned in a few meta-analyses [121,122]: different dietary habits of the studied populations have been highlighted (the intake of omega-3 PUFA is 15 times less in Europe compared to Eastern Countries, such as Japan), as well as different doses and follow up periods, and these biases might have determined mixed results [123].

In the past few years, two intervention studies have analysed the effect of omega-3 PUFA. In the REDU-CE-IT study, involving approximately 8000 patients, the authors evaluated the supplementation with eicosapent ethile, a high resistance purified formulation of EPA, on triglycerides reduction and on cardiovascular events in association with statin therapy. Study participants were patients with cardiovascular disease, diabetes or other cardiovascular risk factors, with triglycerides levels ranging from 150 to 500 mg/dL and LDL cholesterol ranging from 40 to 100 mg/dL. The intervention group received 4 g EPA every day and, after a 4.8 year follow up, showed a 25% reduction in the primary endpoints, that is to say, a compound of cardiovascular death, non-fatal myocardial stroke or cerebral stroke, coronary revascularisation or unstable angina [124]. The STRENGHT Study (Cardiovascular Outcomes with Omega-3 Carboxylic Acids (Epanova) in Patients with High Vascular Risk and Atherogenic Dyslipidaemia) was designed to evaluate the effect of DHA plus EPA in patients at high cardiovascular risk, in association with statin therapy, but it was prematurely discontinued for the lack of end points achievement [125].

There are few data on PUFA effect on lipid profile in the paediatric population. ESPGHAN has recently evaluated the positive effect of PUFA on global health in children [126], whereas PUFA effect on lipoproteins in children with dyslipidaemia were described by Engler et al. in the EARLY study [127]. Dangardt et al. reported an improvement in vascular function and a reduction in inflammation in obese adolescents treated

with omega-3 PUFA [128], whereas Nobili et al. documented a reduction in hepatic steatosis in children with non-alcoholic fatty liver disease [129].

Gidding S et al. reported a reduction in triglycerides levels in children and adolescents (42 subjects, mean age 14 years) treated with fish oil containing 4 g/day DHA and EPA, with no significant LDL-cholesterol modification but with a significant anti-thrombotic effect (reduction in plasmatic fibrinogen and in plasminogen activator) [130].

Del Bò et al. recently documented an increase in omega-3 and omega-6 PUFA in erythrocyte cell membranes in paediatric patients with dyslipidaemia on dietary treatment with good compliance; patients were given hemp (3 g/day 1.4 g linoleic acid, 0.7 g di alpha linolenic acid) for eight weeks, with a 14% reduction in LDL-cholesterol levels compared to the control group [131]. Further studies are needed to consolidate this evidence.

PUFA have few adverse effects and a good safety profile, but they are derived from fish, and their taste is not always well accepted. Seaweed derived omega-3 PUFA should be considered in order to obtain better compliance.

6.2. Curcumin

Curcumin is the main phenolic compound of the aromatic rhizome of an Asian plant, *Curcuma domestica* (or *C. longa*), belonging to the ginger family. Curcumin has multiple properties, including cholesterol-lowering activity, anti-oxidative and anti-inflammatory effects [132]. The lipid-lowering mechanism of curcumin are unclear: curcumin seems to inhibit NPC1L1 transporter expression through transcription factor SREBP2 [133] and to enhance cholesterol efflux through ABCA1 activation [134]. Curcumin can also increase the number of LDL receptors through inhibition of PCSK9 activity [135] and modulate microRNA [136]. The effects of curcumin on lipid profile are not always univocal: in a meta-analysis conducted by Sahebkar et al., curcumin was not associated with significant modifications of LDL cholesterol, HDL cholesterol or triglycerides [137], whereas in another study involving patients with metabolic syndrome who were given curcumin 1 g/day, LDL cholesterol, triglycerides and Lp(a) were all reduced, and HDL cholesterol increased [138]. These results have been confirmed in other trials that have also highlighted an anti-diabetic effect of turmeric [139].

Curcumin has a good safety profile, but it has low bioavailability, as it is slightly water-soluble; thus, new formulations are nowadays under evaluation to facilitate its use [140].

No data are yet available in paediatric patients.

7. Nutraceuticals in Combined Therapy

Scientifical evidence that supports the cholesterol-lowering effect of some nutraceuticals has led to the development of multi-activity products (foods or supplements) containing different bioactive compounds so as to obtain an additive lipid-lowering action. The use of nutraceuticals with multiple metabolic actions on cholesterol metabolism brings about an improvement in cardiovascular risk profile, in particular in primary prevention for those patients with low-to-moderate hypercholesterolaemia not on target, as well as in patients with statin-associated side effects, who cannot be treated with high statin doses [141].

The association of natural products with different mechanisms of action can potentiate their effect, acting simultaneously on different biochemical pathways: they can reduce intestinal cholesterol absorption and/or increase cholesterol excretion (soluble fibres, glucomannan, phytosterols, probiotic), or they can increase cholesterol hepatic re-uptake (soy, berberine) or they can inhibit HMGCoA reductase activity, thus limiting endogenous cholesterol synthesis (monacolins, polycosanols, soy and bergamot) [71].

There are many associations of nutraceuticals: red yeast rice and polycosanols, red yeast rice, polycosanols and berberine, red yeast rice and phytosterols, and so on. These associations have been tested with good results in adult patients, but there are few data for paediatric patients. The available evidence in paediatric patients seems promising, but further and more robust studies are needed on this topic.

8. Nutraceuticals and Pharmacologic Therapy

Many studies have highlighted an additive and complementary effect of nutraceuticals both on lifestyle and pharmacological therapy. Their association with the lipid-lowering drugs could help achieve the target lipid plasma levels with lower dosages and fewer adverse effects [142]. Nutraceuticals can be used in combined therapy, especially in those patients who do not tolerate a high dose of statins, with poor adherence to the therapy [143].

The main associations proposed are statin and PUFA; statin and fibres; ezetimibe, red yeast rice, polycosanols and berberine; statin and silymarin; ezetimibe and silymarin; statin/ezetimibe and phytosterols. However, it is important to acknowledge that these associations have been tested in the short-term and limited cohort studies, and their long-term efficacy is not yet fully convincing. Moreover, further studies are needed before adopting these associations in paediatric patients.

9. Final Considerations

The use of nutraceuticals with cholesterol-lowering effect, both as functional foods and as supplements, is an interesting strategy for paediatric patients, but it may have some risks. As a matter of fact, trials on nutraceuticals have been frequently carried out on a limited study population, so further multicentric studies on larger cohorts are needed (Table 3).

Table 3. Characteristics of studies about the effect of nutraceuticals in paediatric subjects with dyslipidaemia.

Nutraceuticals	Type of Study	Aim	Casuistry	Dose and Duration of the Intervention	Effects	Reference
Psyllium	DB-CO-RCT	Lipid-lowering effect of cereals added to psyllium	32 children Age: 6–18 years Inclusion criteria: LDL-C $\geq$ 90th percentile	8-week diet: 58 g of cereals added to psyllium (6.4 g) or to placebo	$\downarrow$ TC: -5% $\downarrow$ LDL-C: -6.8%	Davidson MH et al., Am J Clin Nutr 1996 [36]
	RCT	Reduction of CT and LDL-C after integration with psyllium	36 children Age: 3–17 years Inclusion criteria: FH	Age $\leq$ 7 years: 5 g/die Age $\geq$ 7 years: 10 g/die Duration: 8.0 $\pm$ 1.1 months	$\downarrow$ TC: -18% $\downarrow$ LDL-C: -23%	Glassman M et al., AJDC 1990 [37]
	SB-RCT	Effectiveness of psyllium in CT and LDL-C reduction	50 children Age: 2–11 years Inclusion criteria: LDL-C $\geq$ 110 mg/dL	CHILD I: all groups. Intervention: cereals containing 3.2 g of psyllium. Duration: 12 weeks	$\downarrow$ TC: -9.6% $\downarrow$ LDL-C: -15.7% $\uparrow$ HDL-C: $+9.96\%$	Williams CL et al., J Am Coll Nutr 1995 [38]
	DB-RCT	Effectiveness of psyllium on LDL-C in Brazilians children and teenagers with dyslipidaemia	51 children Age: 6–19 years Inclusion criteria: TC $\geq$ 175 mg/dL	CHILD II: 6 weeks. Intervention group: 7 g/die of psyllium Control group: 7.0 g/die of cellulose	$\downarrow$ TC: -7.7% $\downarrow$ LDL-C: -10.7%	Ribas SA et al., Br J Nutr 2015 [39]

Table 3. *Cont.*

Nutraceuticals	Type of Study	Aim	Casuistry	Dose and Duration of the Intervention	Effects	Reference
Glucomannan	DB-CO-RCT	Efficacy and tolerability of supplementation with glucomannan	36 FH children Age: 6–15 years Inclusion criteria: baseline values of CT > 90°percentile by gender and age	CHILD I diet Duration: 4 weeks 2 cps/day of Glucomannan or placebo Duration: 8 weeks Wash-out: 4 weeks	↓ TC: 5.1% ↓ LDL-C: 7.3%	Guardamagna O et al., Nutrition 2013 [40]
	DB-RCT	Lipid-lowering effects of glucomannan in combination with CP or PC	132 children Age: 3–16 years Inclusion criteria: TC ≥ 170 mg/dL, 1 parent with CT 240 mg/dL, or familiarity for CVD	Randomised assignment to 5 neutraceuticals and 1 placebo (only resistant starch) 8-week treatment groups Duration: 8 weeks	GM + CP: ↓ LDL-C: −16% GM + PC: ↓ LDL-C: −10%	Martino F et al., Atherosclerosis 2013 [41]
Phytosterols and stanols	DB-CO-RCT	Lipid-lowering effects of sterols	30 children Age: 6–9 years Inclusion criteria: TC ≥170 mg/dL, LDL-C ≥110 mg/dL	Intervention group: milk +1.2 g/day of sterols vegetable. Control group: skim milk Duration: 8 weeks	Intervention group: ↓ TC: −4.5% ↓ LDL-C: −11.1% Control group: TC: +1.4 ↓ LDL-C: −0.9%	Ribas SA et al., NMCD 2017 [50]
	DB-CO-RCT	Lipid-lowering effects of stanols	38 children Age: 7–12 years Inclusion criteria: "Definite" diagnosis or "possible" diagnosis of FH	CHILD I + 1.6 g of stanols or placebo Duration: 8 weeks	↓ LDL-C 10.2% ↓ TC and ApoB 7.4%	Amundsen AL et al., Am J Clin Nutr 2002 [51]
	CT	Effects of sterols on LDL-C levels	64 children 25 LDL-C ≥ 130 mg/dL 34 LDL-C ≤ 130 mg/dL Age: 4.5–15.9 years	CHILD II Intervention group: yoghurt (2 g/die sterols) Duration: 6–12 months	Intervention group: ↓ LDL-C: −13%	Garoufi A et al., IJP 2014 [52]
Red yeast rice	DB-CO-RCT	Efficacy and safety of a combination of red yeast rice extract and policosanols	80 children Age: 8–16 years	CHILD I red yeast rice 200 mg/die + policosanols 10 mg/placebo Duration: 8 weeks Wash-out: 4 weeks	↓ TC: 18.5% ↓ LDL-C: 25.1% ↓ ApoB: 25.3%	Guardamagna O et al., NMCD 2011 [71]

Table 3. *Cont.*

Nutraceuticals	Type of Study	Aim	Casuistry	Dose and Duration of the Intervention	Effects	Reference
Soy	RCT	The effect of integration with protein of soy on lipoproteins	23 children Age: 4–18 years Inclusion criteria: FH	Step 1: diet Duration: 3 months Step 2: diet + soya protein 0.25 g/kg in replacement animal protein. Duration: 3 months	Step 1: ↓ TC: −12.3% ↓ LDL-C: −11.8% ↓ ApoB: −10.6% Step 2: ↓ TC: −7.7% ↓ LDL-C: −6.4% ↓ ApoB: −12.6%	Weghuber D et al., Br J Nutr 2008 [104]
	RCT	The effect of soy on LDL-C levels	17 children Age: 5–13 years Inclusion criteria: FH	Soy group: soy-enriched fat modified diet Control group: fat modified diet Duration: 13 weeks	LDL-C decrease: statistically significantly greater in the soy group	Helk O et al., Clin Nutr 2020 [105]
Omega-3 polyunsaturated long-chain fatty acids	DB-CO-RCT	The efficacy of fish oil in lowering TG and impacting lipoprotein particles	42 children Age: 10–17 years Inclusion criteria: TG ≥ 150 mg/dL and < 750 mg/dL, LDL-C < 160 mg/dL	Intervention group: 4 g daily of fish oil Control group: placebo Duration: 8 weeks	TG decrease: greater in the intervention group	Gidding SS et al., J Pediatr 2014 [130]
	RCT	The effectiveness of hempseed oil in the modulation of hyperlipidaemia and evaluation of fatty acid composition of red blood cells	36 children Age: 6–16 years Inclusion criteria: hyperlipidaemia primitive and compliance to the alimentary indications	Control group: CHILD I Intervention group: hempseed oil 3 g/die Duration: 8 weeks	Intervention group: ↓ RC SFA: −5.02% ↓ RC MUFA: −2.12% ↑ PUFA $n-3$: +1.57% ↑ PUFAs $n-6$: +5.39% ↑ Omega 3 Index: 1.18% ↓ LDL-C: 14.2%, Control group: ↓ LDL-C: −4.94%	del Bo' C et al., Food Res Int. 2019 [131]

DB—double blind; SB—single blind; CO—cross-over; RCT—randomised controlled trial; CT—controlled trial; ↓—decrease; ↑—increase; FH—familial hypercholesterolaemia; TC—total cholesterol; LDL-C—low-density lipoprotein cholesterol; HDL-C—high-density lipoprotein cholesterol; TG—triglycerides; GM—glucomannan; CP—chromium-polynicotinate; RC—blood red cells; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids.

In clinical practice, the availability of nutraceuticals as supplements without medical prescription could result in uncontrolled use such as auto-prescription, therapy discontinuation and/or excessive dose, with a consequent reduced therapeutic effect and/or increased adverse events. However, we must not forget that the effects on lipid profile are closely related to their continuous intake within a defined therapeutic programme [144].

Furthermore, functional foods are expensive compared to traditional foods and drugs, and this could be a major limitation for their long-term use [145].

In conclusion, paediatric lipidologists should consolidate a good alliance with their patients so as to avoid improper and uncontrolled use of nutraceuticals.

Despite the fact that randomised controlled trials on nutraceuticals use in paediatric patients are few and conducted on a limited number of patients, a beneficial short- and medium-term effect on lipid profile has been observed, especially with regard to total- and LDL-cholesterol plasma level reduction. Soluble fibres and phytosterols are the most studied nutraceuticals in children, as they seem to be well tolerated with no relevant adverse effects.

According to the available scientific evidence and to EAS guidelines, we can suggest the use of functional foods containing fibres and phytosterols for children with genetic dyslipidaemia, starting from six years of age [6].

Nutraceuticals containing fibres and phytosterols should always be considered as a complement to the dietary [146] and lifestyle intervention, which remains the milestone approach in the treatment of dyslipidaemia in paediatric patients [147]. Nutraceuticals should be used for a short period and in those children who do not tolerate pharmacological therapy or who cannot yet receive it due to age limitations.

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Abbreviations

AAP	American Academy of Pediatrics
AHA	American Heart Association
CHD	Coronary heart disease
DHA	Docosahexaenoic acid
EAS	European Atherosclerosis Society
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EU	European Union
FCHL	Familial combined hypercholesterolaemia
FH	Familial hypercholesterolaemia
HDL	High-density lipoprotein
HMGCoA	HydroxyMethylGlutaryl CoA
LDL	Low-density lipoprotein
NCEP	National Cholesterol Education Programme
NPC1L1	Niemann–Pick C1-like protein
PUFA	Polyunsaturated fatty acids
RYR	Red yeast rice
SCFA	Short-chain fatty acids
TG	Triglycerides

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Obesity-Related Hypertension in Pediatrics, the Impact of American Academy of Pediatrics Guidelines

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Abstract: The prevalence of primary hypertension in pediatric patients is increasing, especially as a result of the increased prevalence of obesity in children. New diagnostic guidelines for blood pressure were published by the American Academy of Pediatrics (AAP) in 2017 to better define classes of hypertension in children. The aim of our study is to evaluate the impact of new guidelines on diagnosis of hypertension in pediatrics and their capacity to identify the presence of cardiovascular and metabolic risk. **Methods:** Retrospective clinical and laboratory data from 489 overweight and obese children and adolescents were reviewed. Children were classified according to the 2004 and 2017 AAP guidelines for systolic and diastolic blood pressure. Lipid profile and glucose metabolism data were recorded; triglyceride/HDL ratio (TG/HDL) was calculated as an index of endothelial dysfunction. Hepatic steatosis was detected using the ultrasonographic steatosis score. **Results:** Children with elevated blood pressure increased from 12.5% with the 2004 AAP to 23.1% with the 2017 AAP criteria ($p < 0.001$). There was a statistically significant increase in children with high blood pressure in all age groups according to the new cut-off values. Notably, the diagnosis of hypertension according to 2017 AAP criteria had a greater positive association with Hepatic Steatosis (rho 0.2, $p < 0.001$) and TG/HDL ratio (rho 0.125, $p = 0.025$). **Conclusions:** The 2017 AAP tables offer the opportunity to better identify overweight and obese children at risk for organ damage, allowing an earlier and more impactful prevention strategy to be designed.

Keywords: obesity; hypertension; children; cardiovascular risk

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1. Introduction

Over the last years, there has been increasing interest in childhood hypertension and greater recognition that many adult cardiovascular diseases have their origin in childhood. The childhood obesity epidemic had led to an increase in the prevalence of hypertension and its consequences in the young [1]. A recent examination of blood pressure (BP) and lipid levels in United States children clearly showed that the prevalence of elevated BP was grater in overweight and obese children that in the population as a whole [2]. Thus, it is evident that obesity represents one of the most important risk factor for development of primary hypertension even in pediatric population.

Pediatric hypertensive patients are usually asymptomatic, making this condition frequently misdiagnosed, but may present early manifestations of organ damage: 40% of patients among hypertensive children suffer from left ventricular hypertrophy and increased carotid intima-media thickness, an early marker of atherosclerosis [3]. Children with primary hypertension often remain hypertensive even in adulthood with possible irreversible consequences on health status: it has been reported that pediatric patients with high blood pressure have a risk of hypertension in adulthood increased by about 2.4 times [3]. In a large Italian cohort of 415 children and adolescents referred for obesity, 23.6% showed elevated blood pressure, according to criteria belonging to fourth report, the reference at the time of the study [4].

At the time, given the lack of outcome data, the current definition of hypertension in children and adolescents is based on the normative distribution of BP in healthy children. The lack of reliable data on the long-term consequences represents the major limitation of all attempts to define arterial hypertension in the pediatric age. Although it may seem intuitive that a non-physiological situation can lead to long-term damage and the more and more evidence is supported by the literature, it is important to design specific studies in pediatric populations to answer the question. The American Academy of Pediatrics (AAP) revised and published in 2017 new guidelines for the diagnosis of hypertension in children and adolescents, replacing the previous reference values published in 2004 [5,6]. New cut-offs have been proposed for the diagnosis in children <13 years while fixed thresholds independent of age, sex and height have been proposed for subjects ≥13 years of age. In particular, the revised guidelines have identified the diagnostic cut-off considering only the population of normal-weight children.

The aim of our study is to evaluate the impact of new guidelines on diagnosis of hypertension in pediatrics and their capacity to identify the presence of cardiovascular and metabolic risk.

2. Methods

2.1. Study Population

We retrospectively analyzed the data of 489 children and adolescents, all Caucasian, admitted to Pediatric Outpatient Clinic of University of Foggia (Italy) for overweight including obesity, in the period between February 2008 and December 2013. Overweight was defined as body mass index (BMI) ≥ 85° and <95° percentile and obesity as a BMI ≥ 95° percentile for age and sex, according to the growth curves of the Center for Disease Control [7].

The presence of secondary forms of excess weight or other chronic diseases has been considered a criterion for exclusion. Anamnestic information, auxological data (weight, height, waist circumference and pubertal stage) were collected for all subjects according to standard procedures [8].

Since BMI is gender- and age-dependent, the z-score BMI was calculated for all analyzed subjects, which is a measure completely independent from these variables. According to CDC 2000 standards, the LMS method was used (statistical method to normalize data distribution, where L = Box–Cox power, M = generalized mean of data and S = standardized variation coefficient) [9]. The waist circumference/height ratio (WtHr, waist to height ratio) was evaluated for each patient, being an index of visceral adiposity [4,10].

Systolic and diastolic blood pressure was obtained for each child. The blood pressure measurement was performed in a seated patient, after at least five minutes of rest, with differential cuffed aneroid sphygmomanometer (auscultatory method) according to the age and constitution of the child. Three blood pressure measurements were taken for each patient and the average value recorded for the analysis [6,11].

In a first time, absolute values were converted to age-, gender-, and height-specific percentiles, using tables provided by the 2004 National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents (2004 AAP)

and based on these data, we calculated the blood pressure z-score [5]. The same data were then analyzed in accordance with the new 2017 AAP criteria [6].

For children with abnormal values of blood pressure, second-level clinical-laboratory and instrumental investigations were carried out to exclude secondary hypertension. Only data of children without evidence of secondary hypertension were included into the study. The study was approved by the Ethics Committee of the University Hospital of Foggia.

2.2. Laboratory Analysis

Venous blood samples from each subject were taken in the morning, after at least 10 h of fasting. Blood glucose was determined using the gluco-oxidase method.

Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were determined using automated enzymatic methods (Unicel DxC800 SynchronTM System, Beckman Coolter, Fullerton, CA, USA). Insulin was measured by the ELISA method (Elecsys, Roches diagnostics, Mannheim, Germany). The HOMA index (Homeostasis Model Assessment) was calculated as an insulin resistance index [12].

The triglycerides/HDL ratio was also calculated as a marker of endothelial dysfunction [13,14].

2.3. Hepatic Ultrasound

All subjects underwent liver ultrasound examination after a minimum of 10 h of fasting, using a high-resolution ultrasound system (LOGIQ® 7, GE Medical Systems, Milwaukee, WI, USA). The level of liver echogenicity was graded according to the ultrasonographic steatosis score [14]. Subjects were classified as "with and without hepatic steatosis" according to ultrasonographic findings [15,16].

2.4. Statistical Analysis

Results are expressed as mean ± standard deviation (SD) with 95% confidence interval for continuous variables, as percentages for categorical and discrete variables. For the non-Gaussian distribution parameters, instead, the median and the interquartile range were used. The Kolmogorov–Smirnov test was applied to test the hypothesis of normality of the data. The data were analyzed by t-Student test, Mann–Whitney U test, χ^2 test. The calculation of Spearman's rho coefficient was used to evaluate the degree of association between variables. Values of $p < 0.05$ were considered statistically significant.

3. Results

The study population consisted of 489 children, mean age 9.4 ± 2.5 years (range 3–15.8 years): 270 boys (mean age 9.4 ± 2.5 years, range 3–15.8 years) and 219 girls (mean age 9.3 ± 2.5 years, $p = 0.668$). Their mean BMI z-score was 2.3 ± 0.49 (range 1.64–5.62), in particular 2.4 ± 0.6 for boys and 2.2 ± 0.3 for girls ($p = 0.001$).

The median value of systolic pressure z-score in the total population was 0.33 (IQR −0.19–0.89): in male subjects 0.38 (IQR −1.45–1), in female subjects 0.35 (IQR −0.23–0.88, $p = 0.257$). The diastolic pressure z-score was 0.4 ± 0.79 in the total population: 0.4 ± 0.8 in males and 0.38 ± 0.78 in females ($p = 0.531$).

BP Values Classification According to 2004 and 2017 AAP Criteria

According to the 2004 AAP criteria, 84.7% of values showed normal systolic blood pressure, 5.9% were in pre-hypertensive state and 9.4% were classified as systolic hypertensive state. Regarding the diastolic pressure: 87.7% of values were in normal range, 5.7% were in pre-hypertension status, 6.5% were in the range of diastolic hypertension. From the overall statistical analysis conducted: 87.5% of values were in the normal range, 6.1% suggested systolic hypertension, 3.3% diastolic hypertension, 3.1% systodiastolic hypertension according to 2004 AAP cut-offs.

According to the latest guidelines (2017 AAP): 71.2% values showed normal systolic blood pressure, 13.5% were in the range of higher systolic blood pressure, 12.5% of hy-

pertensive Stage 1, 2.9% of hypertensive Stage 2. Regarding diastolic pressure: normal diastolic values were found in 78.9% 9.2% were in high diastolic range, 10.6% in Stage 1, and 1.2% in Stage 2. In particular: 76.9% systodiastolic values were in normal range 11% were in hypertensive range only for systolic, 7% only for diastolic and 5.1% for both.

Then there was a shift from 12.5% to 23.1% of total hypertensive values from the old to the new classification ($p < 0.001$), in particular from 6.1% to 11% for systolic blood pressure only, from 3.3 to 7% for diastolic blood pressure only, from 3.1 to 5.1% for systodiastolic hypertension range.

At the valuation with the new cut off: only 87.9% of the values classified as not hypertensive according to 2004 AAP remained so, while 52 values reclassified as hypertensive (equal to 12.1%, average age 10.1 $\pm$ 2.6 aa, found in 30 males and in 22 females): 28 values reclassified as systolic arterial hypertension, 20 values as diastolic hypertension and 4 as systodiastolic hypertensive state.

In particular, by analyzing the population according to quartiles by age, listed in Table 1, we found in the 2004 AAP classification the following subjects harboring values in hypertensive range: 16.5% in I, 11.4% in II, 10.6% in III, 11.5% in IV ($p = 0.489$). With the 2017 AAP classification, instead, 22.3% in I, 20.3% in II, 22.8% in III, 27% in IV ($p = 0.647$).

Table 1. Percentile according to age in our population.

Percentile	Age (yrs)
25	7.7
50	9.4
75	11

The subjects recognized has having high blood pressure according to AAP 2017 but classified has normotensive in AAP 2004 were 7/121 in the I quartile (5.8%), 11/123 in II (8.9%), 15/123 in III (12.2%), 19/122 in IV (15.6%). The increase in the percentage of subjects carrying abnormal values according to the quartile by age, was statistically significant at the trend test ($p = 0.009$). Of the 52 with abnormal values with new classification, 17.3% suffered from hepatic steatosis. Children with ultrasonographic signs of hepatic steatosis are listed in Table 2.

Table 2. Children with ultrasonographic signs of hepatic steatosis according to sex.

Sex	No Hepatic Steatosis	Hepatic Steatosis	Total
Males n (%)	247 (91.5)	23 (8.5)	270
Females n (%)	206 (94.1)	13 (5.9)	219
Total n (%)	453 (92.6)	36 (7.4)	489

Values falling in the hypertensive range according to 2004 AAP showed positive and statistically significant correlation with WtHR (rho 0.147, $p = 0.001$), with liver steatosis (rho 0.131, $p = 0.004$), but not with HOMA index ($p = 0.181$) nor with TG/HDL ratio ($p = 0.153$).

Values falling in the hypertensive range according to 2017 AAP had positive and statistically significant correlation with WtHR (rho 0.122, $p = 0.004$), with HOMA index (rho 0.103, $p = 0.022$), with liver steatosis (rho 0.2, $p < 0.001$) and with TG/HDL ratio (rho 0.125, $p = 0.025$). Data on clinical and metabolic parameters of children divided according to AAP 2004 and AAP 2017 blood pressure values are expressed in Table 3.

Table 3. Median value (inter quartile range) and subjects with liver steatosis in children classified according to AAP 2004 and AAP 2017 blood pressure values.

Classification	AAP 2004 Hypertension		AAP 2017 Hypertension	
	No	Yes	No	Yes
WHtR	0.56 (0.53–0.6)	0.59 (0.55–0.65)	0.56 (0.53–0.6)	0.57 (0.54–0.64)
TG/HDL	1.6 (1.1–2.5)	1.8 (1.2–2.5)	1.6 (1.1–2.4)	1.8 (1.2–2.7)
HOMA	2.7 (1.9–3.8)	3 (1.9–4.3)	2.7 (1.9–3.7)	3.2 (1.9–4.3)
Liver Steatosis %	6.1	16.4	4.5	16.8

4. Discussion

Hypertension is one of the main causes of mortality worldwide [17]. In the past it was considered a concern for adulthood only, but the current evidence has instead allowed us to understand that essential hypertension can also affect the pediatric age [18]. The atherosclerotic process, as now widely validated in the literature, can start early in children and high blood pressure levels are part of the etio-pathogenetic mechanism of vascular alterations which, as clearly known, are then responsible for the main causes of mortality and cardio-vascular morbidity in adulthood [19,20].

Paying attention to blood pressure in children and adolescents allows, in fact, to operate large-scale prevention of cardiovascular disease [21,22]. There are also several combinations of factors that play a pathogenetic role in the onset of essential hypertension and these risk factors are divided into "non-modifiable", such as age, gender and familiarity, and "modifiable" factors, first of all obesity [23]. For this reason, AAP has decided to re-evaluate the diagnostic cut-offs for hypertensive status in pediatrics, considering only normal-weight patients for the construction of new nomograms [6]. Thus, the limits of the diagnosis of hypertension in pediatric age have been significantly reduced, with substantial and important changes.

Within the same population, as we demonstrated in our results, the percentage of values falling in range of hypertension increased significantly from 12.5% of the 2004 AAP criteria to 23.1% for the 2017 AAP criteria. The 2017 AAP tables, therefore, allowed us to classify more values as hypertensive than the previous assessment. In particular, the highest increase involved older children within our sample. This type of intervention improves, therefore, the recognition of children requiring closer clinical follow-up in order to make the necessary lifestyle changes and improve their health outcomes [24].

In fact, in our analysis the finding of hypertensive values according to the 2017 AAP criteria was more significantly associated with cardio-metabolic risk parameters such as liver steatosis (rho: 0.2 vs. 0.131) and, in particular, had a statistically significant association with HOMA index, an insulin resistance parameter, and with the TG/HDL ratio, an indirect endothelial dysfunction index within the same population of overweight or obese children [13,14]. This association was not detected using the 2004 classification.

Our data show that even in younger children, those of I quartile with age <7.75 years, we found a percentage of more than 20% of subjects harboring abnormal values, while the percentage was even close to 27% in IV quartile subjects. Since the most frequent form of hypertension in pediatrics is the secondary one, especially in younger children, it is suggestive to imagine that the increase of hypertensive values found in in our population is not only related to the increased sensitivity of the new diagnostic guidelines, but also to the spread of the epidemic of obesity with a consequent change in the epidemiology of hypertension in pediatric age [25]. The use of the new guidelines seems to offer the possibility to better screen the pediatric population, recognizing the subjects at higher cardiovascular risk and with signs of endothelial dysfunction [26]. A rather high percentage of overweight children had hypertensive status according to both 2004 AAP and 2017 AAP guidelines. A prevalence of hypertensive arterial hypertension in overweight children

is reported from 4 to 23% (4–14% in overweight and 11–23% in obese subjects), mainly confirmed by our results.

The strengths of the study were to investigate the correlation of BP values with objective markers of metabolic or vascular dysfunction (i.e., liver steatosis, HOMA index, TG/HDL ratio), not limiting the study to a retrospective observation according to new criteria. Furthermore, the population was ethnical homogenous, allowing to avoid any confusion factor belonging to different prevalence in non-European populations.

The major limit is the single evaluation of BP. In effect, a correct diagnosis of BP state (i.e., normal, pre-hypertensive and hypertensive) need at least three consecutive evaluations to classify the subject [6]. The retrospective design does not allow such analysis. The reasoning therefore revolves around the concept of abnormal value of blood pressure and not of hypertensive subject, for methodological correctness. In fact, this specificity that can at first be interpreted as a limit in the study (the fact of not being able to classify subjects as normal and hypertensive in terms of clinical diagnosis) can also be understood as a force of the study. In fact, the correlation with the markers of metabolic and endothelial risk is already evident in subjects with only one pathological measurement (and that therefore will not necessarily be confirmed as hypertensive to a subsequent deepening). It is, therefore, legitimate to think that the correlation between pathological blood pressure values and organ damage can be even stronger by restricting the analysis to patients with consistently high values. This is the purpose of future research. Moreover, our analysis was set on obese and overweight children so we cannot say that the results can be extrapolated to the general pediatric population, including normal weight subjects. In conclusion, the 2017 AAP tables offer the possibility to identify earlier children at risk for organ damage, allowing the structuration of more incisive prevention strategy.

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Lower Intake of Saturated Fatty Acids Is Associated with Improved Lipid Profile in a 6-Year-Old Nationally Representative Population

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Abstract: To strengthen the organization of new national dietary surveys and interventions in childhood, our aim was to study macronutrient intake and blood lipid profile at 6 years of age by comparing results from two earlier population-based cohorts. Subjects were $n = 131$ and $n = 162$ in the years 2001–2002 and 2011–2012, respectively. Three-day weighed food records were used to estimate diet and calculate nutrient intake. Total cholesterol, HDL-cholesterol and triacylglycerol were measured in serum and LDL-cholesterol was calculated. The average intake of saturated fatty acids (SFA) and trans FA was lower in 2011–2012 than 2001–2002 (13.3E% vs. 14.7E%, $p < 0.001$, and 0.8E% vs. 1.4E%, $p < 0.001$, respectively), replaced by a higher intake of unsaturated fatty acids. Total cholesterol and LDL-cholesterol were significantly lower in 2011–2012 than 2001–2002 (4.6 vs. 4.4 mmol/L, $p = 0.003$ and 2.8 vs. 2.5 mmol/L, $p < 0.001$, respectively). In a multiple linear regression model, one E% increase in SFA intake was related to a 0.03 mmol/L increase in LDL cholesterol ($p = 0.04$). A lower intake of saturated and trans fatty acids, replaced by unsaturated fatty acids, may have contributed to an improved lipid profile in a healthy 6-year-old population. Biological data for analysis of blood lipids are important in national dietary surveys in healthy children to monitor important health outcomes of interventions.

Keywords: blood lipids; childhood; diet quality; dietary surveys; fatty acids; nutrition

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1. Introduction

A recent global review reported that the saturated fatty acid (SFA) intake of 2–7-year-old children was generally above the recommended maximum values, especially among European children [1]. While the European Food Safety Authority recommends that SFA intake should be "as low as possible", the upper level of recommended SFA intake for children ranges from 8% of energy (E%) by FAO/WHO to 10E% by the Scientific Advisory Committee on Nutrition in the UK and the Nordic Nutrition Recommendations [2–5]. Recent literature is, however, scarce on the assessment of SFA intake in childhood and the main health outcome associated with SFA, i.e., an unfavourable blood lipid profile.

Monitoring dietary intake is of great importance for developing strategies to improve dietary habits and health. According to the American Heart Association, unhealthy diets are a major challenge for cardiovascular health promotion in children and solid fats are among the most overconsumed nutritional factors in childhood [6]. A systematic review and meta-analysis on the health effects of fatty acid intake in children concluded that reducing SFA intake between 2 and 19 years of age significantly decreased total cholesterol

(TC) and low-density lipoprotein (LDL) cholesterol, with no evidence of adverse effects [7]. Total and LDL-cholesterol are established markers of the most common cause of death in Europe, i.e., cardiovascular disease (CVD) in adults [8,9] and the association between SFA and CVD is widely known [10]. However, the studies in the systematic review and meta-analysis included participants both with a wide age range and with hyperlipidaemia, so translation of the findings to healthy children should be done with a degree of caution.

National dietary surveys among children rarely include blood sample collection, limiting the ability to study how dietary intake affects blood lipids on a population level. While all age groups in childhood are important, starting school is a turning point in a child's life and may be an important time for public health information and strategy [11].

The aim of this analysis was to compare macronutrient intake and blood lipid profile in two earlier population-based and nationally representative cohorts of healthy children, studied cross-sectionally at 6 years of age, and study possible associations between fatty acid intake and blood lipids. This research question is important for the preparation of new national dietary surveys and interventions in childhood.

2. Materials and Methods

2.1. Subjects

This study includes data from two national dietary surveys on healthy Icelandic 6-year-old children, including measurements of blood lipids. Participants in the studies were randomly selected in infancy and recruited into population-based longitudinal cohort studies on diet, growth, and health outcomes. The age of 6 years was chosen as it is the year of starting school in the country. Births were distributed over a whole-year period, residency was in all parts of the country and the participants (who represented 4–6% of live born infants in the country for the respective years) were statistically representative of the infant population of the whole country according to Statistics Iceland. The inclusion criteria in both studies were singleton birth, gestational length of 37–41 weeks, birth weight within the 10th and 90th percentiles, no birth defects or congenital long-term diseases, Icelandic parents, and mother's participation in regular antenatal care. The methods in the two studies have previously been published in detail [12,13]. The families who had been in the cohorts in infancy until 12 months of age were invited to participate in follow-up studies when the children were 6 years old, n = 180 in 2001–2002 (cohort I) and n = 219 in 2011–2012 (cohort II) [14,15]. There was no difference between the children invited to the follow-up and the original infant cohorts when it came to the children's mean weight and length at birth and 12 months, infant dietary intake and parents' age, education, and body mass index (BMI). Informed written consent from the parents was obtained in infancy and at follow-up, and all individual information was processed with strict confidentiality. The studies were approved by the Icelandic Bioethics Committee, the Icelandic Data Protection Authority, and the Local Ethical Committee at Landspitali University Hospital.

2.2. Dietary Assessment

To assess diet and nutrient intake, all foods and fluids consumed were weighed for three consecutive days (72 h) on accurate electronic scales (PHILIPS HR 2385, Koninklijke Philips Electronics N.V, Wien, Austria). The parents or other caregivers were advised to record each food item separately and give precise information about the type of food, cooking procedure and time of serving, and to weigh and register all leftovers. All data were entered into ICEFOOD, an Icelandic calculating program designed for national dietary surveys among adults and children. Nutrient losses due to food preparation were included in the calculations. The consumption of foods and food categories were estimated in grams per day and nutrients were estimated from information about chemical content from food codes and recipes.

2.3. Serum Lipids

Fasting blood samples were taken from the children's antecubital fossa. All blood samples were analysed for serum TC, high-density lipoprotein (HDL) cholesterol and triacylglycerol (TAG). TC and TAG were analysed using an enzymatic colorimetric test (Cholesterol CHOD-PAP, Roche Diagnostics, Mannheim, Germany). HDL cholesterol was measured using the same method after precipitation and centrifugation. LDL cholesterol was calculated from the serum TC, TAG and HDL concentrations expressed in mmol/L using the Friedewald formula [16], which is considered valid if TAG concentrations do not exceed 4.52 mmol/L [17]. Lipid levels were classified according to guidelines for cardiovascular health and risk reduction in children and adolescents by the US National Heart, Lung, and Blood Institute [18]. Cut-off points for acceptable and high levels, respectively, were <4.40 mmol/L and ≥5.18 mmol/L for total cholesterol, <2.85 mmol/L and ≥3.37 mmol/L for LDL cholesterol, and <0.85 mmol/L and ≥1.13 mmol/L for TAG [18].

2.4. Anthropometrics and Covariates

Height and weight were measured at the Children's Hospital at Landspitali University Hospital. Subjects wore lightweight clothing and no shoes. Height was measured to the nearest 0.1 cm, using an Ulmer stadiometer, Busse design (Nersinger Straβe 18, Elchingen, Germany). Weight was measured to the nearest 0.05 kg using a Taniter BWB-620 electronic scale (2625 South Clearbrook Drive, Arlington Height, IL, USA) in cohort I and Marel Model M1100-C2 Weighing Instrument (Marel hf, Austurhraun 9 210 Gardabaer, Iceland) in cohort II. Using calculated BMI, children were classified as being normal weight or overweight/obese according to the International Obesity Task Force (IOTF) cut-off points defined to pass through a BMI of 25 at the age of 18 [19]. Cut-off points of 17.55 and 17.34 for overweight/obesity were applied for 6-year-old boys and girls, respectively.

Information on parent's age, BMI and education were obtained from questionnaires.

2.5. Statistical Analysis

Descriptive analyses (mean and standard deviation, or ratios and percentages for binominal variables) were used for describing the characteristics of study participants. Independent sample t-test or the X^2 test were used for testing for differences between continuous and dichotomous variables, respectively, in cohorts I and II. In cohorts I and II, mean differences (Δ) in fatty acid intake and 95% confidence interval (95% CI) were reported. The relationship between macronutrient intake and blood lipids was examined with multivariate linear regression. Since the directionality of the associations was the same in both cohorts, the cohorts were combined for more statistical power. In these analyses, we included as covariates energy intake (continuous), study (binary), sex (binary) and BMI (continuous) at 6 years. The macronutrients examined were total fat, SFA, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), protein, carbohydrates, added sugar and dietary fibre. For examining the univariate association between total dietary fat intake and LDL-cholesterol in more detail and to relax the condition of linearity we used a restricted cubic-spline with two knots [20]. As these are secondary analyses, the sample size depends on the available data. A p value of <0.05 was considered statistically significant. The statistical analyses were performed using SAS version 9.2 and SAS Enterprise Guide (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Characteristics of Subjects

Complete three-day food records were returned by 131 subjects (72%) in cohort I and 162 subjects (74%) in cohort II. Blood samples were collected from 137 (76%) and 145 subjects (66%), respectively. The characteristics of the subjects and their parents are presented in Table 1. In cohorts I and II, 18% and 12% of participants, respectively, were classified as overweight/obese.

Table 1. Characteristics of the 6-year-old participants and their parents in two cohorts conducted 10 years apart.

Variables	Cohort I (n = 137) Mean (SD)	Cohort II (n = 145) Mean (SD)	Mean Difference Δ (95% CI)
Child			
Age (months)	72.3 (1.6)	73.4 (3.2)	1.1 (0.5, 1.7) *
Weight (kg)	23.0 (3.4)	23.0 (3.7)	−0.1 (−0.9, 0.8)
Height (cm)	119.0 (4.4)	120.0 (4.9)	1.0 (−0.0, 2.1)
BMI (kg/m^2)	16.1 (2.3)	15.9 (1.8)	−0.2 (−0.6, 0.3)
Parents			
Mother's age (years)	35.7 (5.4)	36.3 (4.9)	0.6 (−0.7, 2.0)
Father's age (years)	37.7 (5.9)	38.9 (5.9)	1.2 (−0.4, 2.8)
Mother's BMI (kg/m^2)	25.5 (4.4)	24.8 (4.9)	−0.6 (−2.0, 0.7)
Father's BMI (kg/m^2)	26.5 (3.2)	26.2 (3.2)	−0.3 (−1.4, 0.8)
Mother's education ≥ 12 years, [1] n (%)	70 (74)	121 (81)	0.7 (0.2, 1.3)
Father's education ≥ 12 years, [1] n (%)	76 (80)	111 (76)	1.4 (0.8, 2.5)

* $p < 0.05$. [1] Presented as number and percentages. Δ = mean difference; BMI = body mass index; CI = confidence interval; SD = standard deviation.

3.2. Macronutrient Intake and Blood Lipids

As shown in Table 2, mean intake of SFA and trans fatty acids (TFA) was lower in cohort II than cohort I ($p < 0.001$), replaced by a higher intake of MUFA and PUFA. Additionally, intake of added sugar decreased and intake of fibre increased between the studies, also when taking energy into account (mean difference (95% CI) between cohorts I and II: 0.3 (0.1, 0.4) g fibre per MJ. Total cholesterol and LDL-cholesterol were lower in cohort II than cohort I ($p = 0.003$ and $p < 0.001$, respectively). There was a borderline significant trend ($p = 0.06$) towards a higher HDL-cholesterol concentration in cohort II. Further analysis revealed that the observed differences were driven by normal-weight children. Statistical power to detect significant differences between the two cohorts in the group of overweight/obese children was very low and only significant for trans fatty acids. In cohorts I and II, 17% and 13% of participants, respectively, were classified as having high TC levels, 9% and 6% of participants, respectively, were classified as having high LDL cholesterol levels, and 4% and 3% of participants, respectively, were classified as having high TG levels.

Table 2. Intakes and serum lipid concentrations of the 6-year-old participants in the two cohorts conducted 10 years apart.

Variables	Cohort I Mean (SD)	Cohort II Mean (SD)	Mean Difference Δ (95% CI)
Intake of energy and energy-giving nutrients	n = 131	n = 165	
Energy (kcal)	1494 (308)	1543 (324)	49 (−25, 122)
Total fat (E%)	33.1 (5.4)	32.2 (4.9)	−0.9 (−2.1, 0.3)
SFA (E%)	14.7 (3.1)	13.3 (2.7)	−1.3 (−2.0, −0.7) *
TFA (E%)	1.4 (0.5)	0.8 (0.3)	−0.7 (−0.7, −0.6) *
MUFA (E%)	9.5 (1.7)	10.1 (1.8)	0.6 (0.2, 1.0) *
PUFA (E%)	3.8 (1.2)	4.7 (1.5)	0.9 (0.6, 1.2) *
Omega-3 PUFA (E%)	0.9 (0.4)	1.2 (0.6)	0.3 (0.2, 0.4) *
Omega-6 PUFA (E%)	2.9 (0.9)	3.4 (1.2)	0.5 (0.3, 0.8) *
Omega-6/Omega-3 PUFA ratio (%)	3.6 (1.3)	3.2 (1.2)	−0.4 (−0.6, −0.1) *

Table 2. *Cont.*

Variables	Cohort I Mean (SD)	Cohort II Mean (SD)	Mean Difference Δ (95% CI)
Protein (E%)	15.6 (2.8)	15.4 (2.9)	−0.3 (−0.9, 0.4)
Carbohydrates (E%)	50.8 (5.3)	50.3 (5.5)	−0.5 (−1.8, 0.7)
Added sugar (E%)	12.7 (4.3)	11.2 (4.5)	−1.6 (−2.6, −0.5) *
Fibre (g)	11.1 (3.2)	13.2 (4.0)	2.1 (1.3, 3.0) *
Serum lipid concentration	*n* = 137	*n* = 145	
Total cholesterol (mmol/L)	4.6 (0.6)	4.4 (0.6)	−0.2 (−0.4, −0.1) *
LDL-cholesterol (mmol/L)	2.8 (0.5)	2.5 (0.6)	−0.3 (−0.4, −0.1) *
HDL-cholesterol (mmol/L)	1.5 (0.3)	1.6 (0.3)	0.1 (0.0, 0.2)
TAG (mmol/L)	0.6 (0.2)	0.6 (0.2)	0.02 (0.0, 0.1)

* $p < 0.05$. Δ = mean difference; CI = confidence interval; HDL = high density lipoprotein; LDL = low density lipoprotein; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids, SD = standard deviation; SFA = saturated fatty acids; TAG = triacylglyceride; TFA = trans fatty acids.

3.3. Relationship between Macronutrient Intake and Blood Lipids

As shown in Table 3, a one percent increase in the contribution of saturated fat to total energy intake was associated with a 0.03 mmol/L ($p = 0.04$) higher LDL-cholesterol concentration in the merged dataset of cohorts I and II, adjusted for potential confounding factors. When analysing the association separately in each cohort, the corresponding regression coefficient was 0.02 (−0.01, 0.04) in cohort I and 0.04 (0.01, 0.05) in cohort II. Similarly, the directionality of the associations observed in cohorts I and II were the same, although a slightly stronger association for SFA was observed for cohort I. Multivariate linear regression, adjusted for gender, energy intake, BMI, and study, showed that LDL levels were significantly higher when SFA intake was between 15–17E% compared to less than 11E% (β = 0.3 $p = 0.003$). The exact age of the participants was not associated with blood lipids in these children and further adjustment for age (in months) did not have any impact on the regression coefficient shown in Table 3.

Table 3. The relationship between macronutrient intake and total and LDL-cholesterol assessed by linear regression analysis for both cohorts of 6-year-old children combined.

	Total Cholesterol (mmol/L)				LDL-Cholesterol (mmol/L)			
	Unadjusted		Adjusted [1]		Unadjusted		Adjusted [1]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Total Fat (E%)	0.02	0.006, 0.03 *	0.02	0.004, 0.03 *	0.02	0.004, 0.03 *	0.02	0.003, 0.03 *
SFA (E%)	0.04	0.01, 0.06 *	0.03	0.002, 0,05 *	0.03	0.01, 0.06 *	0.03	0.002, 0.05 *
MUFA (E%)	0.01	−0.03, 0.06	0.02	−0.02, 0.07	0.01	−0.02, 0.05	0.03	−0.01, 0.07
PUFA (E%)	0.02	−0.03, 0.07	0.04	−0.01, 0.09	0.00	−0.05, 0.05	0.03	−0.02, 0.07
Protein (E%)	0.006	−0.02, 0.03	0.01	−0.02, 0.04	0.01	−0.01, 0.03	0.01	−0.01, 0.04
Carbohydrates (E%)	−0.02	−0.03, −0.003 *	−0.02	−0.03, −0.01 *	−0.01	−0.03, −0.002 *	−0.02	−0.03, −0.01 *
Added sugar (E%)	−0.003	−0.02, 0.01	−0.01	−0.03, 0.01	−0.01	−0.02, 0.01	−0.02	−0.03, 0.001
Fibre (g)	−0.01	−0.03, 0.01	−0.01	−0.04, 0.01	−0.01	−0.03, 0.01	−0.01	−0.03, 0.02

* $p < 0.05$. [1] Adjusted for sex, energy intake, BMI, and cohort (I or II). β = beta coefficients; CI = confidence interval; HDL = high density lipoprotein; LDL = low density lipoprotein; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids, SD = standard deviation; SFA = saturated fatty acids; TAG = triacylglyceride; TFA = trans fatty acids.

When examining the association between SFA and LDL in more detail using restricted cubic spline, we observed that the increase in LDL appears to take off at around 13% SFA with the increase in LDL levelling off at around 17% SFA (Figure 1). However, only 39 out of 293 subjects had intake above 17%, so larger samples size would be needed to draw robust conclusions on whether the increase in LDL does level off at this intake.

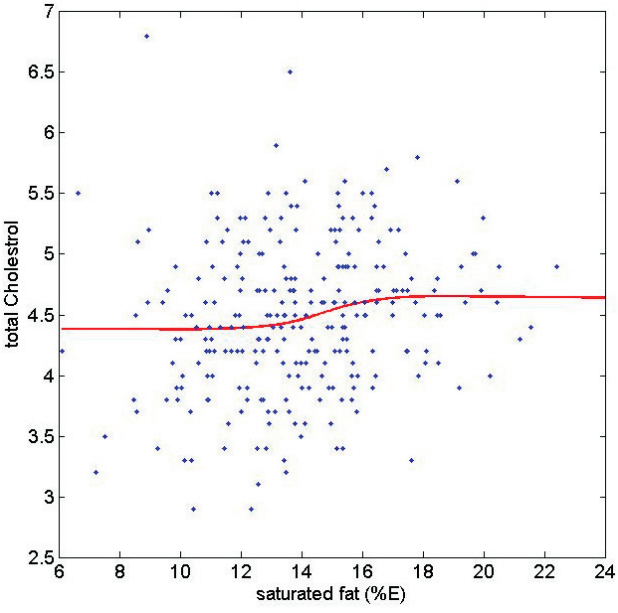

Figure 1. The association between saturated fatty acids and LDL-cholesterol examined by restricted cubic spline for both cohorts of 6-year-old children combined.

Table 4 shows the distribution of children categorised as having acceptable, borderline–high, and high TC and LDL-cholesterol across quartiles of SFA intake. The proportion of children with acceptable/ideal cholesterol is shifted from being 59% and 74% for TC and LDL in the lowest SFA quartile, respectively, to 27% and 46% in the highest SFA quartile, respectively. This shift is primarily driven by an increased proportion of children with borderline–high TC and LDL. After adjustment for covariates, the association was no longer significant for LDL-cholesterol ($p = 0.12$), while the association remained significant for TC ($p = 0.03$).

Table 4. Distribution of children according to classification of lipid levels across quartiles of saturated fatty acid intake for both cohorts of 6-year-old children combined.

	Total Cholesterol (mmol/L)				LDL-Cholesterol (mmol/L)			
SFA (E%)	**Acceptable**	**Borderline**	**High**	***p*-Value**	**Acceptable**	**Borderline**	**High**	***p*-Value**
Q1 (<12E%)	59%	30%	11%		74%	18%	8%	
Q2 (12–14E%)	49%	35%	15%		62%	31%	8%	
Q3 (14–16E%)	51%	34%	15%		60%	41%	9%	
Q4 (>16E%)	27%	55%	18%		46%	48%	6%	
unadjusted [1]				0.02				0.03
adjusted [2]				0.03				0.12

[1] Chi square test. [2] Adjusted for sex, energy intake, BMI, and cohort (I or II) using logistic regression analysis.

4. Discussion

We hypothesise that the observed lower intake of SFA and TFA among the 6-year-olds in cohort II may have contributed to the improved lipid profile compared with cohort I studied ten years earlier. Our findings among healthy, mostly normolipidemic children, are in line with the conclusion from a systematic review and meta-analysis among children with a wide age range [7]. In the study cohorts, intake of SFA and TFA decreased by 9% and 45%, respectively, which is in line with changes in the adult population in Iceland for

the same time, i.e., from 2002 to 2010–2011 [21]. While current food regulations limit the presence of TFA in the food supply, which has proven an effective CVD preventive measure for whole populations, including children [22,23], unpublished data from a new national dietary survey among Icelandic adults in 2019–2021 show a rise in SFA intake. It may be expected that children follow the same patterns. Planning a new Icelandic dietary survey in children including biomarkers of nutritional status is of utmost importance.

In a WHO systematic review and analysis, it was reported that including cis-PUFA (predominantly linoleic acid and alpha-linolenic acid) or cis-MUFA (predominantly oleic acid) in a diet as an exchange for a mixture of SFA had more favourable effects on blood lipid levels than a replacement with a mixture of carbohydrates. In particular, cis-PUFA instead of SFA decreased total and LDL cholesterol and triglycerides [24]. There are, however, different metabolic pathways of the SFAs [24–26], and therefore the effects vary on the blood lipid profile. In the WHO analysis, the ratios of both the total cholesterol to HDL cholesterol and the LDL cholesterol to HDL cholesterol were raised by the saturated fatty acid, lauric acid (C12:0), alone, as compared with carbohydrates [24]. Additionally, longer SFAs such as myristic acid (C14:0) and palmitic acid (C16:0) increase the blood lipids, and have unfavourable effects, as well as lauric acid (C12:0), while stearic acid (C18:0) and short SFAs of 4–10 carbons, do not [24–26]. Dairy is an important part of young children's diet and dairy foods include fatty acids, e.g., myristic acid (C14:0), with unfavourable effects on the lipid profile, but also, albeit in lower concentrations, odd chain fatty acids (OCFA) 15:0 (pentadecanoic acid) and 17:0 (heptadecanoic acid), which may have a different meaning for health [25]. Current evidence is that replacing dairy fat with polyunsaturated fat from plant-based foods has health benefits [25]. The effects of SFAs on the control of blood cholesterol levels have been suggested to be through a biological feedback mechanism. Gu and Yin (2020) describe the mechanism as SFAs diminish cholesterol intracellularly and by that give a signal about lowering cholesterol, which triggers the biosynthesis of cholesterol [26]. Therefore, when cholesterol lowers in cells, the plasma LDL-cholesterol is raised. The step of lowering intracellular cholesterol is by a suppression of LDL endocytosis mediated by LDL receptors and retarding cholesterol transport from plasma membrane to the endoplasmic reticulum. This system effectively regulate the synthesis by sensing cholesterol fluctuations in cells by activating sterol regulatory element-binding protein 2 pathway and degrading 3-hydroxy-3-methyl-glutaryl coenzyme A reductase [26].

It is not certain to what extent a reduction in cholesterol in children will decrease later risk of coronary heart disease, but significant tracking from childhood to adulthood has been reported in many studies [18]. For example, in the Bogalusa Heart Study, children with LDL cholesterol above 3.35 mmol/L (corresponding to our classification as high LDL levels) had significantly higher prevalence of adult dyslipidaemia compared with children with LDL cholesterol below 2.84 mmol/L [27]. In our cohorts, 9% and 6% of children in cohort I and II, respectively, were classified as having high LDL levels. It has been estimated that a reduction of one mmol/L in cholesterol will reduce CHD mortality rates by approximately 50% in middle-aged individuals [8]. According to a study of the Icelandic Heart Association, cholesterol concentration decreased from 6.01 mmol/L to 5.14 mmol/L in the adult population from 1981 to 2006 [28]. At the same time, the contribution of saturated fatty acids plus trans fatty acids to total energy intake of adults reduced from 19.0E% to 15.2E% [21,29]. CHD mortality rates did also decline considerably in Iceland during that time [28], by 80% in men and women. The decline was mostly attributed to risk factor reductions, i.e., cholesterol, smoking and physical inactivity. The 0.3 mmol/L reduction in average LDL cholesterol concentration in 6-year-olds seen in the present study on the population level may be of clinical relevance, as it will have decreased the number of children considered to be at risk of adult dyslipidemia. Even though recent scientific publications and recommendations confirm the former knowledge and advice on lowering SFA and TFA to improve lipid profile in childhood [1,7] and adults [30], there are still doubts about the influences of exchanging SFA for PUFA [31]. Further, there are different ways to present SFA in a diet, and an increase in the percentage intake of SFA may improve

the lipid profile provided the diet is a weight loss diet focusing on lower energy intake with lower intake of refined carbohydrates [32].

Childhood is further a critical period of establishing dietary habits that tend to track until later in life, when they may affect CVD risk [33,34]. Therefore, both continuous monitoring of dietary habits and establishment of healthy dietary habits in infancy and childhood are vital [35,36]. Further, addressing food and nutrition literacy and adapting nutritional advice to local intake patterns rather than specific macronutrient intake may improve children's adherence to dietary guidelines [37,38]. In children, as in adults, blood lipids are considered to be regulated by an interplay of genetic, dietary and lifestyle factors [39]. Weight regulation and increased physical activity may also be beneficial for blood lipids [40]. For the age group studied, i.e., 6-year-olds starting school, we believe it may be a good time for teaching about food and nutrition and health promotion.

There are several limitations associated with the interpretation of the results from two independent cohort studies conducted 10 years apart. However, the present study brings important messages to health authorities implementing different strategies in order to improve health at the population level. The results can therefore be considered of value when developing and supporting concepts in public health nutrition related to improved fat quality. Given the relatively low number of subjects in the present analysis, the confidence intervals in our estimation of the association between one E% increase in the intake of saturated fat and cholesterol intake is broad. However, our results are in line with the results presented by large cohort studies and intervention studies as well as the recent meta-analysis on a wider age range of participants.

In conclusion, this study showed improved dietary fat quality and blood lipid profile among Icelandic 6-year-old children in a nationally representative cohort studied in 2010–2011 (cohort II) as compared to 2001–2002 (cohort I). Lower intake of saturated fat and trans fatty acids, replaced by unsaturated fat, might have contributed to improved lipid profile at the population level. This shows the relevance of including health-related biomarkers in national dietary surveys in childhood for following and better understanding trends in public health and to be better able to give scientifically based advice and prepare interventions in childhood.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Icelandic Bioethics Committee, the Icelandic Data Protection Authority, and the Local Ethical Committee at Landspitali University Hospital.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the nature of the original ethical approvals.

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Review

Effectiveness of Nutritional Strategies on Improving the Quality of Diet of Children from 6 to 12 Years Old: A Systematic Review

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Abstract: Dietary habits, that are formed during childhood and consolidated in adulthood, are known to influence the development of future chronic diseases such as metabolic syndrome or type 2 diabetes. The aim of this review was to evaluate the effectiveness of nutritional interventions carried out in recent years focused on improving the quality of the diet of the child population. A systematic search of the PubMed and Scopus databases was performed from January 2011 until September 2021. A total of 910 articles were identified and screened based on their title, abstract and full text. Finally, 12 articles were included in the current systematic review. Of those, in six studies the intervention was based on the provision of healthy meals and in the other six studies the intervention focused on modifying the school environment. Six of the studies selected included other components in their intervention such as nutritional education sessions, physical activity and/or families. A wide variety of methods were used for diet assessments, from direct method to questionnaires. The results suggest that interventions that modify the school environment or provide different meals or snacks may be effective in improving children's dietary patterns, both in the short and long term. Further research is necessary to evaluate the real effectiveness of strategies with multidisciplinary approach (nutritional sessions, physical activity and family's involvement).

Keywords: diet quality; dietary pattern; children; nutritional strategy

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1. Introduction

Dietary habits are formed during childhood and consolidated in adulthood. They may also have influence in the development of future chronic diseases such as metabolic syndrome, type 2 diabetes, cardiovascular disease and mortality [1–3]. Therefore, different institutions, including the World Health Organization, recommend the establishment of healthy eating habits at an early age as a method to prevent chronic diseases [1–4]. Moreover, poor dietary habits are associated with being overweight and obesity, including comorbidities such as fatty liver disease, dyslipidemia, diabetes, asthma, sleep apnea and cardiovascular disease among others [5]. In addition to health consequences, childhood obesity can affect children's social and emotional well-being and self-esteem [5] as well as educational achievement [6]. Furthermore, it is associated with a lower quality of life [5] and with a higher risk of obesity in adulthood [7].

However, it has been observed that the majority of children of Western populations do not achieve the goals of dietary guidelines, as they consume low amounts of fish, fruit, vegetables and foods high in fiber, and large amounts of foods high in sugar and saturated foods [8].

Diet-related risk factors are a major cause of death and disease worldwide. In the case of children, it has been observed that a high intake of sodium, saturated fat, meat, fast food and soft drinks is negatively associated with cardiovascular health while a high intake of vitamin D, fiber, mono- and poly-unsaturated fatty acids, dairy products, fruits and vegetables is positively associated [9]. Additionally, it has been observed that a high intake of sugar, and particularly the intake of sugar-sweetened beverages and added sugar, is a risk factor for the development of obesity, dyslipidemia, type 2 diabetes mellitus [10] and caries [11], among others.

In addition, there are certain risk eating behaviors associated with different diseases. For example, it has been observed in children and adolescents that skipping breakfast is a risk factor for obesity [12] and metabolic diseases [13]. It is associated with a worse lipid profile, blood pressure levels and insulin resistance too [13].

Changes in lifestyle of young people around the world have been observed in the last decades, leading to changes in their dietary patterns. These changes, in part, appear to be due to the impact of globalization and urbanization on eating patterns. A greater trend has been observed for the consumption of snacks, meals away from home, fast foods and sugar-sweetened beverages. In short, there is a trend towards the consumption of highly processed foods with low nutritional density [14–16]. In addition, it has been observed that adolescents from different regions of the world (North America, Europe and Oceania) do not follow the nutritional recommendations for fruits, vegetables, legumes and sodium, nor do they follow a Mediterranean diet pattern [16]. For this reason and given that eating habits are established in childhood, it is really important that before reaching these stages, adolescence and adulthood, an adequate eating pattern is consolidated.

Dietary patterns have been shown to be strongly influenced by family environment [3] and food availability [3,17]. First, regarding the family environment, it has been observed that the main factors that affect children's eating behaviors are: availability and accessibility of healthy foods, frequency of family meals, parental intake and parenting practices [18,19]. Regarding the availability and accessibility of healthy foods, it has been observed that the intake of fruits and vegetables increases when these two food groups are available at home even when the taste preferences towards them is low [18]. It has also been observed that one of the key elements in increasing the intake of soft drinks is their availability at home together with taste preference [20]. Parental intake and frequency of meals is another essential element. In this context, parents play a fundamental role in the development of children's eating habits because children tend to modulate their eating behavior based on their parent's habits [21]. Besides, having a family dinner is related with higher quality diets, characterized, among others, by a higher consumption of fruits and vegetables and a lower consumption of fried foods, soft drinks and food high in saturated and trans fats [22]. With regards to parenting care practices, it has been observed that children living in authoritative homes eat healthier, are more physically active and have a lower BMI compared to children who were raised in other types of homes such as authoritarian, permissive/indulgent or not involved/neglectful [23]. Moreover, family eating patterns, healthy eating rules at home, and parents' healthy lifestyles have been shown to influence young people's intake of fruits, vegetables, calcium and dairy products and dietary fats [24]. Moreover, it has been stated that those nutritional strategies involving parents in the promotion of healthy eating in children improves the quality of children's diets as well as reducing the prevalence of childhood obesity [21].

On the other hand, regarding the availability of food, it is known that neighborhoods that offer access to a high nutritional quality food improve the diet and weight of the people who live in those neighborhoods. In that sense, it has been shown that access to foods and beverages with a high energy content has negative consequences on the health of people around the world. In addition, the increase in the price of fruits and vegetables in contrast to that of processed products has made the access to these not-so-healthy products very easy. Given this trend in food prices, the population group that has been most affected are those with low incomes [17]. Therefore, to achieve the improvement of the population's

dietary and lifestyle patterns, it is necessary that healthy foods are available, identifiable and affordable for all, regardless of the income level and the area of residence (rural or urban) [18].

In addition, schools are one of the places where children spend most of the day [25,26]. A large part of this population has one of their main meals, lunch, there. Approximately 40% of the total energy intake is consumed in schools [27,28], where there are other sources of food and drinks as well, apart from the cafeteria service, such as vending machines, school stores or food stands [29]. Generally, the type of food coming through these channels is low in nutrients and dense in energy [28]. On the other hand, schools are places where children are exposed to physical exercise, where many of them provide health education and a healthy environment and allows access to a large number of children [26]. In this context, these centers can promote healthy eating by increasing the availability or limiting different foods based on their nutritional quality and teachers can promote healthy eating through nutrition education in the classroom [29]. Therefore, schools are key places for the development of strategies focused on improving eating habits [25,26]. A systematic review suggested that school food and nutrition policies (nutrition guidelines, healthy food price interventions, and fruit and vegetable subscription or distribution plans) might be effective in improving the school feeding environment and the dietary intake of children. However, these strategies were not effective on body weight [30]. Nevertheless, the current scientific evidence on the effectiveness of this type of strategy is still limited [31].

Nowadays, scientific evidence on interventions that improve the nutritional quality of the child population remains limited. No agreement has been reached on the characteristics of the intervention that is responsible for said effectiveness. The reviews on this topic have focused exclusively on the school environment or are not recent. Additionally, these papers are not consistent in their results [32–34]. The present review intends to increase the scientific evidence on this topic and provide updated data, since the problem of low-quality diets in children is a frequent health problem. Therefore, interventions that are truly effective in the proposed population could be developed and implemented.

The aim of the present review was to evaluate the efficacy of different types of interventions carried out in children between 6 and 12 years old focused on the improvement of the quality of the diet in recent years. As secondary objective, to identify and analyze the essential components involved in the change of the nutritional quality of the diet, the magnitude and impact of the results obtained on the diet and the other possible effects that could derive from this improvement on the diet.

2. Materials and Methods

2.1. Search Strategy

A bibliographic search was carried out in two online databases PubMed and Scopus databases from the January 2011 until September 2021. The following groups of keywords were used for the research: (1) "nutrition* intervention" AND "diet* quality" AND "(school meal OR canteen OR food)" and "child"—(2) "lifestyle* intervention" AND "diet* quality" AND "(school meal OR canteen OR food)" AND "child"—(3) "nutrition* intervention" AND "school meal" AND "diet*" AND "improve" AND "child". To appropriately conduct the article search, the following filters were applied in the databases: year (last 10 years), language (English) and type of article/document (Pubmed: clinical trial; Scopus; article).

Articles published in English, carried out in children and that looked for associations between different types of interventions in which one of its components was nutritional and focused on diet/lunch/school meals and the improvement of the quality of the diet or dietary pattern, which in addition were clinical trials or used a similar approach, were selected.

The research was carried out using the PICO strategy (patient, intervention, comparative, results). The question according to the PICO scheme was: "In children, what type of intervention is found to be effective improving the quality of the diet or the dietary pattern compared to control?"

2.2. Inclusion and Exclusion Criteria

All studies carried out in children could be included. Taking into account that many of the characteristics of both the nutritional status and the diet of children change significantly throughout growth, it was decided that a study stage would be established between 6 and 12 years. Those studies whose age of participants was within this range were included. The population that is the object of the current review is the child population. Therefore, all articles studying results on children who met the established age range were selected, regardless of their nutritional status (normal nourished, malnourished or obese). Only children from special education schools were excluded because these children usually present pathologies that require specific nutritional care. Furthermore, all the included studies had to evaluate as one of their results the quality of the diet or dietary pattern (primary outcome of this review), thus being able to determine whether a change occurred after the intervention. Articles involving different intervention approaches were included, both isolated nutritional interventions and those that, in addition to the nutritional component, presented other components such as nutrition education and/or physical activity in their intervention. This second type of intervention was named by the authors as lifestyle interventions. Regarding the studies design only prospective controlled studies were selected.

2.3. Data Extraction

Data were extracted in a standard way. For each included study, the following information was obtained: title, author name, year of publication, study design, subjects' characteristics, sample size, dietary interventions' characteristics, diet assessment methods and main results.

2.4. Search Summary

A total of 910 articles were identified from two electronic databases: PubMed and Scopus. Of all these articles identified, 266 were duplicates, so they were removed, resulting in a total of 644 articles. After a screening based on the title, 97 articles were eligible for abstract screening, of which 30 articles were qualified for a full-text review. After complete revision of the text, 18 articles were eliminated for not meeting the inclusion and exclusion criteria mentioned above, such as the age of the participants, that a nutritional intervention will be carried out on diet, lunch or school meals and that one of the results measured the improvement in the quality of the diet or dietary pattern thus assessing the change after the intervention. In addition, two other articles were identified through articles from the previous search. Finally, 12 articles were selected for the final review. The present review followed the PRISMA guidelines and Figure 1 shows the PRISMA Flowchart of the study [35,36].

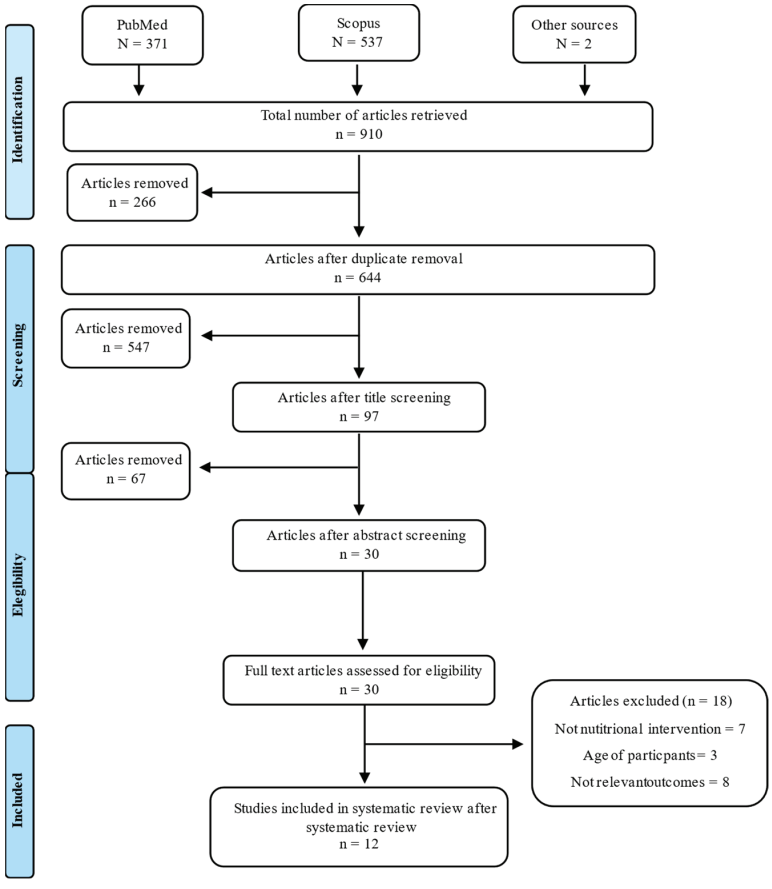

Figure 1. Flow diagram of identification, screening and selection process for included articles.

3. Results

The main characteristics of the included studies are described in Table 1. From a geographical point of view, four studies were conducted in Europe [37–40], six in the United States [41–46], one in China [47] and another in Australia [48]. In relation to the study design, ten were randomized controlled trials [37,39,41–48], one non-randomized trial [40] and one longitudinal quasi-experimental trial [38].

Regarding the sample size of the studies, it ranged from less than 100 to more than 10,000. In this context, one study presented a sample size of <100 participants [41], five studies had a sample size between 100 and 1000 [37,40,42,44,45], another five had a sample size between 1000 and 5000 [38,39,43,46,47] and another one had a sample size greater than >10,000 participants [48].

As established in the inclusion and exclusion criteria, the target population for this review was children aged between 6 and 12 years. In this context, the age of the study participants met this age range, although some studies established 5 years old as the lower eligible age, while the upper range could be up to 15 years old.

Table 1. Characteristics of the studies included in the systematic review.

Reference	Study Design	Sample Size	Age	Intervention Description	Diet Assessment	Results
Murphy et al., 2011 [39]	Cluster, randomized controlled trial 1 year. PSFBI	4472	9–11 years	Free school breakfasts containing milk-based drinks or products, cereal (not sugar coated), fruit and breads compared with their usual breakfasts.	Dietary recall questionnaire.Questionnaire about child's dietary behavior.	Higher consumption of healthy food items in intervention group than control group. Intervention group had more positive attitudes towards breakfast eating than control group.
Williamson et al., 2012 [46]	Cluster, randomized 3-arm controlled trial. 3 academic years. LA Health	2021	7–12 years	(1) Primary Prevention (PP): school environment modification including changes in cafeteria food service and physical education program. (2) Primary + Secondary Prevention (PP + SP): added a classroom/Internet component. (3) Control (C).	Digital photography of food selections and food intake.	No differences between PP + SP and PP and C were found for changes in BMI, body fat, food intake, physical activity or sedentary behavior. Both intervention groups combined (PP + (PP + SP)), were associated with a decreased in body fat and dietary fat intake in comparison to control group.
Brauchla et al., 2013 [41]	Cluster, randomized controlled trial. 2 months.	81	7–11 years	Two high-fiber snacks per day total of 10–12 g of dietary fiber compared with their usual snacks.	24 h dietary recalls via telephone. 8-question Child Regularity Questionnaire. Snack frequency questionnaire.	No differences were observed in terms of energy content, macronutrients, fiber, food groups and punctuation of Regularity Questionnaire between both groups.
Andersen et al., 2014 [37]	Cluster-randomized, controlled, unblinded, cross-over trial. 3 months. OPUS School Meal	834	8–11 years	Free healthy school meals: mid-morning snack, an ad libitum hot lunch meal and afternoon snack compared with their usual packed lunches.	WebDASC: food record tool.	Higher intake of potatoes, fish, cheese, vegetables, eggs and drinks and a lower intake of bread and fats during intervention period compared to control period. Lower energy density of food and beverages during intervention period than in control period. No differences were observed in energy intake during intervention period compared to control period. Higher energy intake from protein (0.9%) and lower energy intake from fats (0.9%) in intervention period. Higher intake of vitamin D (42%) and iodine (11%) in intervention period.

Table 1. *Cont.*

Reference	Study Design	Sample Size	Age	Intervention Description	Diet Assessment	Results
Li et al., 2019 [47]	Parallel, two-arm, cluster-randomized controlled trial. 1 year. CHIRPY DRAGON	1641	6–7 years	In the intervention school the following components were carried out while controls schools continued with usual practice: 1. Education workshops, healthy behavioral challenges and quizzes for families and children. 2. Provision of school lunch 3. Family friendly games at school and home. 4. 1-h physical activity on campus every day.	Short form of SFFQ from University of Leeds. Day in Life Questionnaire.	Higher daily intake of fruit and vegetables and proportion of children consuming at least 5 daily portions of fruit and vegetables in intervention group than in control group. Lower weekly consumption of sugar-sweetened beverages and unhealthy snacks in intervention group compared to control group. Lower proportion of children with screen-based sedentary behavior and higher proportion of children engaging active sport in intervention group compared to control group. More favorable score in questionnaire of quality of life in intervention group than in control group. Decrease in BMI z-score and waist circumference in intervention group compared to control
Cohen et al., 2014 [42]	Randomized, controlled trial. 1 academic year CHANGE	432	6–12 years	Daily access to a food service offering healthier school breakfasts and lunches. Healthy habits acquisition curricula. Different components to promote healthy lifestyle changes for parents and community encouraged during and after the school day.	Block Food Screener	Higher consumption of vegetables and fruits and vegetables combined in intervention schools compared to control schools. No difference was observed in fruit, legume, whole grain or dairy intake. Lower glycemic index of the diet in intervention schools compared to control schools.
Cullen et al., 2015 [43]	Randomized, controlled trial. 6 months.	1876	5–14 years	Intervention and control schools served the same menu, with the difference that intervention group could select one fruit and two vegetables servings per day and control group could only select a total of two servings of fruits and/or vegetables.	Direct observation in the cafeterias during lunch periods.	In elementary intervention schools, increase in the consumption of total vegetables, starchy vegetables and other vegetables and decrease in the consumption of calories juice, whole grains and protein foods compared to control schools. In intermediate intervention schools, increase in the consumption of fruit, total vegetables, starchy vegetables, legumes and decrease in the consumption of calories, total grains and whole grains compared to control schools.
Wolfenden et al., 2017 [48]	Randomized controlled trial. 1 year	57 schools (mean number of students: 256 in intervention group and 253 in control group)	5–12 years	Implementation of a healthy canteen policy that required schools to eliminate unhealthy items from the regular sale and increase those healthy ones.	Direct observations of mean energy, total fat and sodium per student purchase were assessed during one school day.	Intervention schools were more likely to have menus without unhealthy items and to have at least 50% of menu items classified as healthy than control schools. Student purchases from intervention school canteens were lower in total fat but not in energy or sodium than control schools.

Table 1. *Cont.*

Reference	Study Design	Sample Size	Age	Intervention Description	Diet Assessment	Results
Lee et al., 2018 [44]	Group-randomized controlled trial. 1 academic year. OSNAP	400	Mean age: 7–6 years in control group and 7–8 years in interven-tion group.	Implementation of menus that increased the frequency of fruits; reduced the frequency and servings of juice; removed foods with partially hydrogenated oils; and included more whole grain foods. Healthy habits promotion and nutrition sessions for directors, support staff, families and children	Direct observation and digital photography of type, size and brand of all food and beverage items served each day.	Decrease in the consumption of juice, beverage calories, foods with trans fats, total calories and increase in the consumption of whole grain in intervention group than in control group.
Trude et al., 2018 [45]	Group-randomized controlled trial. 8 months. BHCK	509	9–15 years	Environment modification program and increase in the availability of healthy beverages, snack and cooking methods. Nutrition sessions for children. Promotion of healthy eating through social networks.	CIQ: tool for measuring youth purchasing behavior. BKFFQ: to collect youth food and beverage intake.	There was an increase in the purchase of healthier foods and beverages of 1.4 more items per week in intervention group compared to control group. A decrease in the percentage of calories from sandwiches, sweets and desserts was observed among the 13- to 15-year-olds in the intervention group compared to the control group of the same age range. No differences were found in fruit and vegetables intake.
Vik et al., 2020 [40]	Non-randomized trial 1 academic year. School Meal Project	164	10–12 years	Free healthy school meal at lunch in the intervention group compared with normal lunch in the control group.	FFQ: validated questionnaire used in the Fruits and Vegetables Make the Marks-project.	Higher weekly intake of vegetables on sandwiches adjusted for baseline intake in intervention group compared to control group.
Bartelink et al., 2019 [38]	Longitudinal quasi-experimental trial. 2 years HPSF	1676	4–12 years	(1) Full intervention: free healthy school lunch and mid-morning snack each day + structured PA sessions after lunch. (2) Partial intervention: structured PA sessions after lunch. (3) Control	Questionnaires to assess dietary behaviors and intake filled out by parents and children. Child lunch questionnaire.	Higher intake of vegetables and dairy products and lower intake of grains and butter in the full intervention compared to control. In the partial intervention, lower intake of vegetables, dairy products and butter compared to control intervention. Decrease in time spent sedentary and increase in time spent in light PA in the full intervention compared to control intervention. Improve in children's healthy dietary behaviors in full intervention compared to control intervention.

PSFBI: Primary School Free Breakfast Initiative; PP: Primary Prevention; PP + SP: Primary Prevention + Secondary Prevention; C: control; LA Health: Louisiana Health study; OPUS School Meal: Optimal well-being, development and health for Danish children through a healthy New Nordic Diet School Meal Study; WebDASC: Web-based Dietary Assessment Software for Children; CHANGE: Creating Healthy, Active and Nurturing Growing-Up Environments study; USDA: United States Department of Agriculture; OSNAP: Out of School Nutrition and Physical Activity study; BHCK: B'more Healthy Communities for Kids study; CIQ: Child Impact Questionnaire; BKFFQ: Block Kids 2004 Food Frequency Questionnaire; CHIRPY DRAGON: Chinese Primary School Children Physical Activity and Dietary Behavior Changes study; SFFQ: Short food frequency questionnaire; HPSF: Healthy Primary School of the Future study; PA: Physical activity; FFQ: Food frequency questionnaire.

In relation to the intervention type, six studies were nutrition interventions [37,39–41,43,48] and the other six were lifestyle interventions [38,42,44–47]. Regarding the nutritional component of all the interventions, there were two main models: provision of healthy meals during school hours [37–41,47] and modifying the children's food environment [42–46,48]. In interventions in which a healthy meal was provided, there were differences regarding the meal intake; one study focused on breakfast [39], two on lunch [40,47], another two on lunch and mid-morning snack [37,38] and one that did not focus on any particular intake but rather provided two snacks to be consumed throughout the day [41]. In interventions in which children's food environments were modified, in four of them the intervention was focused on modifying the school environment [42,43,48,49], while the two other interventions stressed the change outside school, such as community recreation centers, corner stores and carryout restaurants [45] or after-school sites [44]. In the case of the four strategies based on schools, in two of them the modification of the school environment was based only on the modification of the school food service and in the remaining two, in addition to that change, other actions were carried out aiming at the promotion of health such as: changes in the school physical education program [46], modification of the type of food offered by vending machines [46] and the incorporation into the school curriculum of training activities on healthy eating and physical activity [42]. In the case of the two studies that focused their interventions on modifying the environment outside of school, apart from increasing the availability of healthier food in different retailers, healthy eating habits were also promoted through social networks [45], text messages, posters and handouts promoting healthy food items were placed in all intervention stores [45] and brochures with nutritional messages for families [44].

In addition, six of the studies included other components in their intervention, such as sessions of healthy nutrition and habits or physical activity. In the case of the sessions about healthy nutrition and habits, these were aimed at different groups: children participating in the study [45], children and their families [47] or the school community [42,44]. With regards to physical activity, although in some of the studies physical activity was promoted as part of the healthy habits sessions, in two of the studies physical activity sessions were included as part of the intervention, either daily or a couple of times a week [38,47].

The duration of the intervention period varied depending on the study: the shortest period was two months [41] and the longest was two years [38]. The majority of the studies, eight of the twelve selected, had a duration equal to or greater than one academic year [38–40,42,44–48]. All studies performed final measurements at the end of the intervention, while none of the studies performed longer-term follow-up measurements.

Different diet assessment methods were used to collect food information: in four studies direct observation methods were used to determine the type and quantity of food consumed [43,44,46,48] and in the remaining eight studies different questionnaires were used [37–42,45,47]. Different types of direct observation methods were used: digital photography of the food and drink selected and consumed [46], observation and recording by trained staff of the type and quantity of food and drink selected and consumed [43], direct observation only of the food selected without assessing the real intake made [48] or a combination of both methods, observation and recording by the study staff plus digital photography [44].

Out of the 14 questionnaires used to assess diet among all studies, excluding the two questionnaires that only recorded the consumption of food provided in the study [38,41], eight of them were questionnaires validated in the target population (children) [37,39–42,47], one was a validated questionnaire but not in the target population (validated in adolescents) [45] and three were not validated questionnaires [38,39,41].

In relation to questionnaires' type there were: three 24 h dietary recall [39,41,42], one food record of the last seven days [37], three food frequency questionnaires [40,45,47], one questionnaire that focused only on the consumption of fruits and vegetables [47], another that raised general questions about the intake of different food and beverage groups and

dietary behavior [38], two questionnaires about dietary behavior [39,45] and one about gastrointestinal health [41].

With regards to the main result of the studies, expressed as change in the quality of the diet or the dietary pattern, in ten studies an improvement of diet quality was observed after the intervention [37–40,42–45,47,48], while in two studies no change in the participants' diet quality was observed after the intervention [41,46]. Regarding those studies in which improvements on the dietary pattern were observed, in all of them the improvement was mainly focused on an increased consumption of foods considered as healthy (fruits, vegetables, cereals, dairy products, fish, white meats and water) and a decrease in those considered as unhealthy (pastries, processed meats, commercial juices, sugary drinks and unhealthy snacks such as chips). One of the common improvement factors among the studies was a higher consumption of fruits and vegetables. Six studies reported a higher intake of fruits and vegetables: three studies only observed improvements in the consumption of vegetables [37,38,40] and the remaining three studies in the consumption of both groups, fruits and vegetables [42,43,47]. In another two studies, a higher consumption of foods considered healthy was observed, among those that included fruits and vegetables in that classification; however in these results, it was not differentiated which specific food groups had increased their intake [39,48]. Another factor among the studies is the generalized decrease in the consumption of juices and sugary drinks [37,43,44,48].

In relation to dietary macronutrient distribution, an improvement was observed in four studies [37,43,44,48]: in two of them a decrease in fat intake was observed [37,48] and in the remaining two a decrease in total calorie intake [43,44]. Additionally, in the study carried out by Andersen et al., 2014 [37], these other effects were observed: increase in protein intake, increase in dietary fiber consumption that borderline statistical significance and changes in micronutrient content observing an increase in the intake of vitamin D and iodine. In the remaining studies, no nutritional improvement in the diet was observed.

Changes in physical activity and sedentary attitudes were observed in two of the studies. In the study conducted by Li et al. 2019 [47] a higher proportion of children who carried out some type of activity during the weekend and a lower proportion of children with sedentary behaviors were observed at the end of the intervention. Similar to these results, the study by Bartelink et al., 2019 [38] observed a decrease in the percentage of time that children spent sedentary and an increase in the time they spent in moderate activity.

Furthermore, in some studies other secondary results were observed such as: a decrease in BMI z-score and waist circumference [47], lower glycemic index of the diet [42], better punctuation on the quality of life questionnaire [47] or lower energy density of food and beverages [37]

There was a lack of information on the process of implementation of the study and evaluation of the intervention [37–39,41–46]. Some of the studies also did not specify the study design in detail, such as study enrollment rate [41–43], the baseline dietary characteristics of the participants [43,46], or the attrition rate [38,40,43]. These factors present a possible risk of bias in the studies, but in general most of them reported all the study data, so the evidence from the reviewed studies is strong.

4. Discussion

This review tried to summarize the evidence on the effectiveness of different interventions focused on improving the quality of the children's diets in order to be able to develop future interventions that produce a real and long-term impact on the diet of the child population. Of the evaluated studies, ten out of twelve, showed positive associations between specific interventions and an improvement on diet [37–40,42–45,47,48].

In those studies, in which improvements were observed on the dietary pattern, one of the common improvement factors among the studies was a higher consumption of fruits and vegetables [37,38,40,42,43,47]. The low intake of these two food groups is one of the most worrisome in this target population (children) as it can be particularly difficult to change their consumption. Despite this, Evans et al., 2012 [50] in their review of school in-

terventions focused on increasing the consumption of fruits and vegetables, concluded that these interventions were successful in increasing the consumption of fruits, but not vegetables. Therefore, the results obtained in this review are encouraging and show that there are nutritional strategies capable of increasing the consumption of one of the food groups that are least accepted in childhood, vegetables. Another common improvement factor between studies was a decrease in the consumption of juices and sugary drinks [37,43,44,48]. This was another of the key dietary factors in children associated with multiple diseases and whose intake had increased in recent times [51,52].

Improvements in the macronutrient content of the diet were also reported in four of the reviewed studies [37,43,44,48]. Given that most of the selected studies observed changes in dietary intake tending towards a healthier eating profile, it is foreseeable that in these cases, improvements in caloric and macronutrient content would occur.

In only one of the reviewed studies, changes were observed in the anthropometric variables. The study by Li et al. 2019 [47] reported a significant reduction in the BMI z-score and in waist circumference after one year of intervention. In this context, Alman et al., 2015 [53] observed that the decrease in meals away from home was associated with better diet quality and a reduction in BMI and body fat percentage, and that the relationship between the decrease in the number of meals away from home and the improvement in anthropometric variables was mediated by the improvement in diet quality. This statement is also supported by the results obtained in the study carried out by Williamson et al., 2012 [46] in which no change was observed in the dietary pattern and therefore in the anthropometric variables. Consequently, it is possible that if the rest of the studies anthropometric and body composition measurements had been taken, changes in the dietary pattern would have led to improvements in these variables.

Moreover, two studies found an improvement in physical activity and reduction in sedentary behaviors [38,47]. This emphasizes the importance of carrying out interventions that do not focus only on improving a specific healthy habit, but rather the development of nutritional strategies focused on the acquisition of a healthy lifestyle as a whole, which includes physical activity. In only these two studies, one of the components of the intervention consisted of physical activity sessions [38,47]. Related to this, recent investigation suggests that dealing with two health behaviors grouped together could produce an indirect or synergistic effect, whereby the probability of improving one health behavior increases when an individual has already successfully changed the other health behavior [38]. Therefore, strategies with this multidisciplinary approach (nutrition and physical activity) might be more effective.

However, two of the studies did not show any effect on dietary pattern [41,46]. First, in the study carried out by Brauchla et al., 2019 [41], in which two fiber-rich snacks were provided to the participants in the intervention group, the authors pointed out that the participants in that group did not consume snacks usually, so despite no major dietary changes, the children were probably replacing refined grains with whole grains, which is a positive dietary change. This must be added the small sample size, less than 100 participants. Second, in the study carried out by Williamson et al., 2012 [46], there was a low rate of implementation of these strategies, a modification program of the school environment, so this could explain that no changes would have observed.

An interesting point to highlight is one of the results of the study conducted by Murphy et al. 2011 [39]. In this study, despite having observed that the nutritional strategy impacts on one of the meals of the day, producing a greater consumption of healthy foods at breakfast, no effect was observed on the rest of the meals of the day. These results would have a greater impact and benefit for the participants if they were able to produce improvements in the overall dietary pattern.

With regards to the main characteristics of the studies, most of them had large sample sizes, specifically ten of the selected studies involved more than 400 participants, which allows reliability in the results obtained. However, there were large differences in the sample size of the studies. Perhaps the small sample size of the remaining two studies

is due to the fact that the study was carried out in rural areas where there is generally a smaller population [39,40]. In the case of the studies carried out in schools, it is also important to note that although some studies had a high number of participating schools, in some cases the number of children enrolled in the study was really low, as is the case of the studies carried out by Cohen et al., 2014 [42] and Wolfenden et al., 2017 [48]. Thus, these results should be interpreted with caution.

Another of the main characteristics is the duration of the intervention period. In general, the selected studies presented long intervention periods (in seven of them it was at least one academic year), which suggests that these strategies could be effective in the long term. This would be the case of the studies carried out by Wolfenden et al., 2017 [48] and Bartelink et al., 2019 [38] whose duration is one and two years, respectively. Given that these studies are carried out in the school environment, it is to be assumed that at least during the summer vacation period the intervention was not continued.

In this context, it is necessary to emphasize that the effects obtained in the interventions tend to fade over time [54–56]. It would have been interesting if the selected studies had evaluated these long-term effects after the intervention was completed, for example during the following academic year. In this sense, it could be evaluated if the new healthy habits acquired are maintained over time and especially once the intervention is finished, since this is the ultimate objective of any study. A recent study carried out in an adolescent population that evaluated the long-term effect of a school-based intervention on the nutrition and physical activity on BMI, found that after two years of the intervention it was still being effective, mainly in obese participants [57]. Similar results were observed in Mihas et al., 2010 nutritional intervention study on BMI one year post-intervention [58]. In both studies, no dietary variables were determined, but since improvements in weight were observed, it is more than likely that there were also improvements in diet.

Regarding the type of intervention carried out, most of the included studies focus on schools or on children's environments, but neither of them focused on both areas. This would be another point to be developed in future interventions in children in order to determine if developing an intervention based on both the school and children's environments produces greater effects on diet and on other series of concomitant variables such as anthropometric and body composition. It would be interesting to assess, for example, the effect of interventions that not only modify the school food service but also the rest of the food services in the environment such as recreation centers, restaurants and/or supermarkets, so that access to healthy food is facilitated in all the areas in which the child relates.

Six of the selected studies counted the provision of healthy and free food as one of the main elements of their intervention [37–41,47]. Facilitating access to this type of food can be key to the success of this type of intervention. In this regard, you do not limit yourself to facilitating the availability of this type of food (as in the case of the modification program of the food served in the school cafeteria) but you directly give them access to them. Thus, children can try foods that they normally do not consume and gradually change their food preferences towards a healthier profile. Different authors support this statement that the fact that children have access to free food not only improves the nutritional quality of their diet, especially in low-income families, but also represents a unique opportunity to modify food preferences, by facilitating access to new foods [59,60].

Related with that, school meal programs have proven to be effective in improving the nutritional quality of children's diets [61]. Likewise, recent studies suggest that students who bring lunches from home have poorer nutrition compared to those students who consume the foods provided in school meal programs [62]. This highlights once again the importance of schools and specifically school food services in nutritional interventions carried out in the child population. The data seem to suggest that both the provision of food and the modification of food proportions in school lunch services are an effective measure to improve the quality of the diet.

There were two different types of interventions in the selected studies: nutritional interventions and lifestyle interventions. The data suggest that lifestyle interventions are more effective by encompassing more aspects and not focusing solely on nutrition. As mentioned above, focusing on more than one health habit results in them enhancing their effects on each other. A recent review on the effect of interventions for the prevention of obesity in children and adolescents concluded the same, that interventions that combine diet and exercise are more effective [63].

Only three of the studies took into account families in their intervention, developing different activities or educational sessions for parents with the aim of promoting a healthy diet and lifestyle [42,44,47]. In a recent systematic review on the effectiveness of family-based nutritional interventions in improving the diet of children, it was concluded that these types of strategies have a high potential in improving the quality of the diet, mainly being effective in reducing dietary fat and increasing the consumption of fruits and vegetables [64].

There was no consistency in the method for diet assessment. These ranged from direct methods (observation and photography) [43,44,46,48] to different questionnaires filled out by participants and/or parents [37–42,45,47]. Direct methods have demonstrated to be reliable and accurate in school studies [49,65]. There are validated methods in the child population of all types of questionnaires used. There is no consensus on which method should be used to evaluate children's diets, there are different types and there are validated models of all of them. It is true that the use of questionnaires presents limitations, since the data obtained may be biased among others. However, it has been shown that school-aged children are able to respond to data about themselves such as those related to health, as early as six years old [66]. Besides, in three studies questionnaires to assess eating behavior of children were included, which is useful to assess, among others, the attitude children have towards food [38,39,45]. Perhaps, a good way to evaluate diet globally is to use a combination of both methods. In future studies, the possibility of incorporating diet scores into the evaluation methods should be considered, such as the Kidmed Index, which is a quick and validated method to assess the quality of a child's diet [67].

None of the selected studies considered biochemical variables such as lipid or glycemic profile. In a 2013 systematic review on the effect of nutritional and physical activity interventions on weight and metabolic outcomes in obese children and adolescents, the authors concluded that these strategies were effective on these metabolic variables, especially on HDL cholesterol and fasting insulin levels [68]. Thus, it would have been interesting if some of the selected studies had included biochemical parameters of the lipid and glycemic profiles of the children after improving the nutritional quality of their diets throughout the intervention study. It is possible that improvements had been observed in some of the parameters analyzed, especially in those studies with longer intervention periods.

The main risk of bias in these types of studies is that although these types of studies usually have large sample sizes, the families that participate are often concerned about the health of their children. Thus, in some cases it can be difficult to reach the population that would really benefit from these interventions. For this reason, it is important to evaluate the baseline characteristics of the participants because participating children might generally follow a good quality diet since their parents are very concerned about their diet, so the change produced after the end of the study may not be as big as expected.

At last, there are several limitations of this systematic review. Firstly, not all of the selected studies are randomized controlled trials. Second, most of the result are based on self-reported data. The present systematic review also has strengths. First, it presents a comprehensive and updated evaluation of this topic, analyzing and evaluating each of the components that can make these strategies effective. Second, the large sample size and duration of the intervention of the studies selected shows that the data are reliable.

5. Conclusions

The results of the present review suggest that interventions that modify children's environments or provide different meals or snacks are effective in improving the dietary pattern of the child population in the short and long term. However, it would be interesting to develop interventions that include all the following components in order to produce a greater impact on the diet and health of the child population: (1) strategies that not only focus on improving the diet, but on the acquisition of a healthy lifestyle as a whole, thus incorporating physical activity into the intervention (2) strategies that focused on school and on children's environments which includes, among others, involving parents in the intervention, due to the important role they play in the diet and habits of children (3) strategies that incorporate nutritional education such as healthy eating sessions (4) strategies that evaluate long-lasting effects once the intervention is finished to be able to determine whether the beneficial results obtained are maintained over time. On the other hand, it would also be interesting for these strategies to evaluate other possible effects such as improvements in weight and body composition or in biochemical markers.

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nutrients

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Review

The Effects of Nutrition on Linear Growth

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Abstract: Linear growth is a complex process and is considered one of the best indicators of children's well-being and health. Genetics, epigenetics and environment (mainly stress and availability of nutrients) are the main regulators of growth. Nutrition exerts its effects on growth throughout the course of life with different, not completely understood mechanisms. Cells have a sophisticated sensing system, which allows growth processes to occur in the presence of an adequate nutrient availability. Most of the nutritional influence on growth is mediated by hormonal signals, in turn sensitive to nutritional cues. Both macro- and micro-nutrients are required for normal growth, as demonstrated by the impairment of growth occurring when their intake is insufficient. Clinical conditions characterized by abnormal nutritional status, including obesity and eating disorders, are associated with alterations of growth pattern, confirming the tight link between growth and nutrition. The precise molecular mechanisms connecting nutrition to linear growth are far from being fully understood and further studies are required. A better understanding of the interplay between nutrients and the endocrine system will allow one to develop more appropriate and effective nutritional interventions for optimizing child growth.

Keywords: nutrition; growth; children

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1. Introduction

Linear growth is recognized as a reliable indicator of a child's general health. Growth pattern varies during life, being particularly fast during fetal life and the first two years of life, then slowing during childhood until puberty, when growth spurt occurs [1].

Though growth potential is genetically determined, growth pattern is deeply influenced by endocrine and environmental factors, including psychosocial distress and nutrient availability. These factors act in an extremely sophisticated interplay, differentially intervening during the various phases of growth. Nutrition seems to be particularly relevant during fetal life and the first year of postnatal life, whereas the endocrine control becomes predominant during childhood and puberty.

Nutrition in early life has not only an immediate effect on growth but also affects future health. Undernourished fetuses and infants are more likely to be short adults, to have increased cardiometabolic risk in adulthood, to give birth to smaller infants, to have lower educational achievement and to experience a lower economic status in adulthood [2,3].

Although nutritional control of growth is predominant during the first phases of life, nutrition remains an important regulator of growth even during childhood and adolescence [1]. In low-income countries, approximately 25% of children younger than five years, present linear growth restriction due to malnutrition [4,5]. Mechanisms regulating weight and linear growth appear to be interconnected, as supported by the observation that in

undernourished children linear catch-up growth occurs when 85% of weight for height has been recovered [6].

Though the causal link between nutrition and growth failure has been questioned [7,8], with some studies reporting beneficial effects of nutritional interventions on linear growth, and others showing poor or no effectiveness [9], overall the available data support the concept that nutrition plays a major role in linear growth.

The effects of macronutrients (proteins, fats and carbohydrates), representing the immediate "building blocks" for growth, are easily conceivable. Nevertheless, optimal growth requires an adequate intake of micronutrients, whose deficiency may contribute to growth retardation.

Longitudinal bone growth is the result of a complex interplay between several endocrine, paracrine and autocrine factors that act both directly on chondrocytes in the growth plate and indirectly by modulating other factors of the growth-promoting molecular network. Growth hormone (GH), insulin-like growth factor I (IGF-I), sex steroid hormones, thyroid hormones, insulin, leptin and glucocorticoids are key endocrine regulators of growth that can be influenced by nutritional status. The target organ of all these signals is the growth plate, a thin layer of cartilage entrapped between epiphyseal and metaphyseal bone, where all the factors either promoting or inhibiting growth interact with each other, regulating the longitudinal growth of long bones, and ultimately linear growth.

Abnormal growth patterns occur in different clinical contexts characterized by altered nutritional status. Therefore, a better understanding of the mechanisms connecting nutrition to growth may lead to the development of strategies for optimizing nutritional status and allowing for the recovery of a normal growth pattern.

2. Endocrinological Regulators

2.1. GH Axis

GH and its main effector, IGF-I, are recognized to be the main regulators of linear growth, by acting mainly but not exclusively in the growth plate.

GH regulation is sensitive to different nutritional cues, such as glucose. GH secretion is inhibited by glucose load [10], an effect that may be mediated by ghrelin [11], whereas hypoglycemia stimulates GH release [12].

GH has a lipolytic action [13] and influences the distribution of adipose tissue. On the other hand, GH secretion is affected by lipids. Animal models of exposure to high-fat diet showed impairment of GH synthesis and decreased circulating GH levels, likely through the activation of endoplasmic reticulum stress [14]. Other nutrients, which presumably influence the GH axis, include vitamins and microelements [15].

Short fasting stimulates GH secretion, coherently with the lipolytic and hyperglycemic properties of GH [15]. By contrast, prolonged fasting induces peripheral GH resistance [16]. Data from animal studies have shown that inadequate caloric intake inhibits longitudinal bone growth. In male rabbits undergoing 48 h fasting, a significant reduction in the number of both proliferative and hypertrophic chondrocytes was observed [17]. Despite increased GH levels, the hepatic expression of IGF-I was significantly down-regulated and circulating IGF-I was significantly reduced compared with fed controls. These results suggest that the inhibition of longitudinal bone growth and the associated structural changes observed in the growth plate during fasting may be secondary to the low levels of circulating IGF-I. The reduced expression of IGF-I in liver despite increased GH levels, indicates a status of GH resistance induced by fasting.

GH resistance has been described in different forms of undernutrition, such as decreased total energy intake, isolated protein calorie malnutrition and isolated micronutrient deficiencies [16].

An adequate availability of nutrients is required for the anabolic actions of IGF-I, including amino acid uptake into skeletal muscle, peripheral glucose uptake, increased protein synthesis and reduced proteolysis [18]. In case of starvation, the consequent decrease

in IGF-I is associated with protein catabolism, which increases amino acids availability for gluconeogenesis, thus favoring adequate levels of glucose for vital organs such as the brain.

Dysregulation of IGF-I levels occurs in both under and over-nutrition [18], with serum concentration decreasing in response to malnutrition [19]. Lower IGF-I levels during dietary restriction may depend on the state of hepatic GH resistance, driven by alterations in the GH receptor signaling [18]. The degree of dietary restriction has a different effect on GH receptors. A receptor defect would be responsible for reduced IGF-I levels in more severe forms, whereas a post-receptor defect may be involved in less severe forms of malnutrition.

IGF-I is sensitive to both protein and total energy intake. An adequate intake of both protein and energy is required to normalize IGF-I levels [20]. Dietary essential amino acid intake is important for IGF-I restoration after fasting [21]. Notably, other macronutrients, such as fat, influence IGF-I levels [22], but at a lower degree than protein or total energy.

In the growth plates of food-restricted mice, decreased IGF-I levels and lower GHR expression have been found [23] and may explain the reduced response to GH administration observed in malnourished animals and children.

Further components of the GH/IGF-I axis are the IGF-binding proteins (IGFBPs), whose main role is to transport IGFs, thereby regulating their availability for peripheral tissues. IGFBPs represent additional mechanisms by which nutrition influences IGF-I concentrations. Indeed, in case of malnutrition, a significant increase in IGFBP-1 and IGFBP-2 levels occurs, thus increasing IGF-I clearance and reducing its bioavailability [24]. IGFBP-3 levels parallel those of IGF-I in malnutrition and may be used as an additional marker of nutritional status [25,26].

2.2. FGF21

Fibroblast growth factors (FGFs) are a family of proteins that regulate different biological processes, including growth and development. FGF21 is an endocrine factor primarily produced by the liver and adipocytes that acts as a signal of protein restriction. FGF21 regulates metabolism and growth during periods of reduced protein intake and contributes to the adaptation to fasting by stimulation of gluconeogenesis, fatty acid oxidation, and ketogenesis [27–29]. In humans, both fasting and protein deprivation are associated with increased FGF21 levels [29–31].

FGF21 may mediate GH resistance induced by malnutrition, thus contributing to the consequent impaired skeletal growth [27]. The chronic exposure to FGF21 is associated with reduced expression of hepatic GH receptors, inhibition of GH signaling and disruption of GH action in the growth plate [32]. In animals, increased expression of FGF21 during chronic food restriction is associated with reduced bone growth [33]. Notably, growth failure induced by undernutrition is attenuated in FGF21-knockout mice compared to controls [27]. Elevated FGF21 levels are associated with impaired linear growth in very preterm infants, and in primary human chondrocytes FGF21 inhibits GH action on chondrocytes [34]. Furthermore, plasma levels of FGF21 are inversely related to linear growth in infancy [35].

2.3. Insulin

Insulin is a peptide hormone that binds to membrane-bound receptors in target cells to orchestrate an integrated anabolic response to nutrient availability. Beyond its fundamental metabolic actions, insulin is a potent mitogen, exerting its growth-promoting effects mainly by binding to the IGF-I receptor. Insulin induces chondrocyte differentiation and maturation, and the administration of insulin in hypophysectomized rats stimulates tibial growth [36].

Abnormal insulin secretion is associated with alterations of growth. Pancreatic agenesis is associated with intrauterine growth restriction [37], which also occurs in patients with insulin receptor gene mutations [38]. The impairment of growth observed in children with poorly controlled type 1 diabetes depends, in part, on low insulin levels.

The insulin growth-promoting action is exerted directly or, indirectly, through the regulation of IGF-I release [39]. Insulin signaling induces IGF-I independent actions on chondrocytes, stimulating them to proliferate, differentiate and achieve their final size [40].

2.4. Leptin

Leptin is a hormone mainly but not exclusively secreted by white adipose cells. It regulates sense of satiety and metabolism but also acts as a mediator of nutritional effects on growth [41]. Leptin stimulates GH secretion by acting on the hypothalamus [41,42] and, interestingly, exerts a direct peripheral growth-promoting effect in the growth plate by stimulating chondrocyte proliferation and differentiation [43,44]. Consistently, the administration of leptin to Ob/Ob mice reverses metabolic abnormalities and increases femoral length [45,46]. Furthermore, this animal model of leptin deficiency is characterized by reduced GH circulating levels [47], a finding also observed in patients with leptin receptor mutations [47]. Notably, leptin administration to rats with intrauterine growth retardation accelerates the elongation of bones [48]. In humans, however, the growth-promoting effect of leptin is less clear, as leptin mutation has been described in a family of tall subjects [49].

Specific hypothalamic areas are the target of hormones such as leptin and insulin, which provide information regarding nutrient availability, and connect nutritional status to linear growth and the onset of puberty. Melanocortin-3-receptor (MC3R) is a melanocortin receptor, mainly expressed in the brain, whose lack in animals impairs linear growth. Genetic variants of MC3R are associated with adult height in humans [50]. In humans, MC3R deficiency is associated with delayed puberty, impaired growth, reduced adult height and decreased IGF-I levels [51]. MC3R may thus represent a pivotal mediator between nutritional status and linear growth.

2.5. Thyroid Hormone

Thyroid hormone secretion is deeply influenced by nutrition, requiring iodine as a key component, and being also affected by other micronutrients such as selenium, zinc, iron, and vitamin A [52].

The thyroid hormone plays a well-recognized role in regulating growth and skeletal development from the late fetal life to the onset of puberty, as confirmed by growth alterations occurring in case of either excess or deficiency [1].

Thyroid hormone influences endochondral ossification, by regulating chondrocyte maturation as well as cartilage matrix synthesis, mineralization, and degradation both directly and indirectly through GH-mediated effects [24,53].

3. Nutritional Regulators

3.1. Macronutrients

Protein and amino acids are recognized as the main nutrients involved in linear growth. Proteins play a permissive role in growth, since they fulfill the metabolic demand of amino acids, required for tissue growth, and increase levels of hormones, such as insulin and IGF-I, which stimulate endochondral ossification. Amino acids are critical for normal growth and matrix formation by chondrocytes [54].

In humans, protein deficiency leads to growth failure [55]. By exposing animals to protein deficiency, tibial linear growth is quickly negatively affected. This growth-limiting effect is neutralized by increasing dietary protein concentrations [56].

By contrast, a high protein intake in infancy and early childhood leads to increased growth and higher BMI in childhood [57]. In infancy, breastfeeding has been associated with a slower growth rate [58], but results are not conclusive [59,60].

Leucine is a ubiquitous amino acid particularly present in milk and some cereals [61]. Leucine regulates insulin metabolism and exerts anabolic and anticatabolic actions. Leucine stimulates growth through the activation of the mTOR signaling pathway. This pathway integrates different environmental cues to regulate cell growth and homeostasis [62]. mTOR

is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins to form two distinct complexes, named mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 upstream signals include amino acids (especially leucine and arginine), stress, oxygen, energy, and growth factors [63]. mTORC1 favors cell growth by promoting anabolic processes such as protein and lipid synthesis and by simultaneously inhibiting autophagy. Moreover, activated mTOR stimulates angiogenesis, which allows nutrients to reach the cells [47] and influences osteoblast differentiation [64]. mTOR signaling stimulates chondrocyte differentiation [65] and affects chondrocyte autophagy in the growth plate [47]. Notably, mTORC1 signaling is active in the hypothalamus, where it integrates signals from circulating nutrients (glucose, amino acids, lipids) and hormones (leptin, insulin) to synchronize energy balance and growth. Intracerebroventricular administration of leucine and leptin promotes mTORC1 activity and reduces food intake in rats.

3.2. Micronutrients

The effects of single or micronutrient mixture supplementation on linear growth have been investigated in different studies, which have yielded conflicting results. This inconsistency may depend on the extreme variability of nutritional interventions as well as differences in control groups and study cohorts. It has to be pointed out that malnourished children have multiple nutrient deficiencies that affect the efficacy of single supplementations.

Zinc is a central component of hundreds of enzymes involved in cell growth and differentiation as well as immune function. The first evidence of zinc involvement in growth derived from the observation that human zinc deficiency secondary to acrodermatitis enteropathica, an inborn metabolic error causing reduced intestinal absorption of zinc, was associated with impaired growth and increased susceptibility to infections [66]. A growth-promoting effect of zinc supplementation has been observed in animals [67]. For instance, in rats, zinc deficiency induces structural changes of the growth plate and reduces the length of tibias and femurs [47,68]. Zinc may also influence the growth plate by reducing IGF-I secretion as well as peripheral actions of IGF-I [69].

Despite zinc supplementation effects on growth being extensively studied, results in humans are inconsistent, partially due to the considerable variability in inclusion criteria.

A small effect of zinc supplementation in stimulating growth in pre-pubertal children was reported [70], a finding consistent with results obtained in children under 5 years of age in developing countries [71]. Overall, the zinc positive effect on linear growth seems to be particularly significant after 2 years of age [72]. However, other studies reported conflicting results [73–75]. A systematic review of studies reporting data from a total of more than 27.000 children from low- and middle-income countries, under 5 years of age, showed that zinc supplementation has little or no effect on anthropometric indices [76].

The exact mechanism by which zinc influences linear growth is still unclear. It has been suggested that an adequate zinc intake is needed for chondrogenesis, collagen synthesis, osteoblast function, and calcification of bone [77].

Vitamin D influences endochondral ossification by stimulating cellular maturation through the vitamin D receptor [47]. The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily and regulates the expression of numerous genes involved in calcium/phosphate homeostasis, cellular proliferation and differentiation, and immune response, largely in a ligand-dependent manner.

VDR is largely expressed in chondrocytes. In the human fetal growth plate, vitamin D promotes chondrocyte differentiation by stimulating the expression of IGF-I and GH receptor genes [24,78].

A recent extensive meta-analysis aimed at evaluating the effects of vitamin D supplementation on several clinical outcomes in children under five years of age found little or no effect of vitamin D on linear growth [79].

Calcium homeostasis is essential for bone health and growth. In animals, calcium deficiency causes reduced bone mineralization and reduced bone strength without affecting

linear growth [80]. Vitamin D and calcium administration restore normal bone growth in children with nutritional rickets [81]. Low intake of calcium and vitamin D, likely due to inadequate milk intake after weaning, may favor stunting in African children [82]. In adolescent boys (aged 16–18), 13 months of calcium supplementation was associated with increased height [83].

Vitamin A and its derivative, retinoic acid, have no clear effects on growth [84]. Trials based on vitamin A supplementation have reported little or no benefit on linear growth [73,74]. By contrast, according to a recent extensive meta-analysis including five studies assessing the effect of vitamin A on linear growth in children, vitamin A supplementation may exert a positive effect on linear growth in children older than 2 years [77].

Iron supplementation was reported to stimulate growth only in children with iron deficiency anemia [75]. Consistently, a meta-analysis of randomized controlled trials assessing the effect of iron interventions on the growth of children younger than 5 years showed no significant effects [65]. These results were confirmed by a meta-analysis including 14 studies, performed in low- and middle-income countries on subjects with ages ranging from 34 to 167 months [77].

High dietary copper intake promotes growth in pigs [85], whereas in rats its deficiency results in low serum IGF-I levels but high IGF-I in bones [86]. Copper supplementation increases IGF-I and IGFBP-3 concentrations in culture media of chondrocytes, promoting their proliferation. Data about copper supplementation trials in infants and children are scant.

Iodine is an essential component of the thyroid hormone, through which it exerts its main effects on growth. Iodine deficiency affects people of all ages, children and adolescents being the most vulnerable. The widespread salt iodization programs have lowered the risk of iodine deficiency, which is nevertheless still present in many regions [87,88]. Data on the effect of iodine supplementation show no effect of this micronutrient on linear growth [77]. By evaluating a cohort of approximately 300 children followed up to 4 years after the assumption of iodized oil, an improvement of linear growth was observed [89].

A multiple-micronutrient approach is more effective on growth than interventions based on single micronutrients [73,90]. A thorough meta-analysis assessing the effects of different nutritional interventions on anthropometric measures (changes in height, weight, and weight-for-height z scores) showed that interventions including iron or vitamin A alone did not influence growth and zinc alone exerted only a small positive effect on weight-for-height z score but no effect on height or weight gain [74]. By contrast, interventions based on multiple micronutrients seem to be effective in stimulating linear growth. The combined administration of vitamin A and zinc in Indonesian short children (aged 48-60 months) without underlying diseases led to increased IGF-I levels and height Z-scores [91].

4. Clinical Implications

Malnutrition refers to a condition characterized by an imbalance between nutrient requirement and intake. In industrialized countries, this condition can occur in the presence of chronic or acute illnesses but, in the last decades, different forms of malnutrition have emerged in childhood and adolescence. If an excessive energy intake can lead to overweight and obesity, eating disorders, characterized by voluntary inadequate caloric intake and/or purging behaviors, lead to undernutrition.

4.1. Anorexia Nervosa

Anorexia nervosa (AN) is an increasingly widespread disorder with a progressively decreasing age at onset. It affects approximately 0.2-1% of adolescents and young women in developed countries [92], but up to 14% of adolescents are presumed to have "partial syndromes or eating disorder not otherwise specified" with signs and symptoms of malnutrition.

These patients typically have multiple endocrine disorders [92,93], mostly representing mechanisms of adaptation to chronic starvation. Patients with AN usually have acquired

GH resistance, with higher GH but low IGF-I levels. Considering that GH stimulates gluconeogenesis, its higher levels represent an adaptive mechanism favoring the maintenance of euglycemia in subjects with a reduced availability of energy substrates. Refeeding normalizes GH secretion in these patients [94].

Patients with AN show a persistent impairment of growth rate. Though nutritional interventions induce an increased linear growth, catch-up growth is often incomplete [95]. In these subjects, IGF-I may represent a biomarker of nutritional status, as its changes reflect BMI fluctuations [96].

4.2. Obesity

The opposite extreme of malnutrition is represented by an excessive food intake leading to overweight or obesity.

Obese children typically show accelerated linear growth in childhood associated with an accelerated maturation of the epiphyseal growth plate, ultimately leading to an adult height consistent with the genetic potential. Adipose tissue is a source of different hormones, which may affect the linear growth of obese children. Leptin, which is mainly produced in the adipose tissue, informs the hypothalamus about the nutritional status (by binding MC3R), thus activating the kisspeptin system and the GnRH pulse generator, ultimately regulating puberty onset and progression, and GHRH-releasing neurons, ultimately increasing pituitary GH secretion [97].

Obesity influences the IGF system. Obese subjects usually have reduced GH levels [98,99], which normalize after weight loss [15], and normal or increased IGF-I levels [100,101].

Ghrelin, which acts as an endogenous GH-releasing factor, is a peptide mainly produced in the stomach and acts as a ligand of the GH secretagogue receptor (GHSR). Ghrelin stimulates food intake, rises in fasting states, decreases after eating and has been proposed as a possible regulator of GH release in obesity [102]. Moreover, ghrelin is also synthesized by chondrocytes in the growth plate, thus exerting a combined central and peripheral growth-promoting action [103].

Insulin, whose levels are increased in obese children, reduces GH secretion [104] and exerts both direct and indirect growth-promoting actions [36].

5. Conclusions

Nutrition plays a key role in the regulation of growth in fetal life, infancy, childhood and adolescence. Nutrients represent the fundamental bricks for cartilage and bone development and, at the same time, regulate a complex network of hormones and growth factors, which act in an endocrine, paracrine and autocrine fashion, finely controlling growth plate physiology (Figure 1). A better understanding of the interplay between nutrients and the endocrine system will allow one to develop more appropriate and effective nutritional interventions for optimizing child growth.

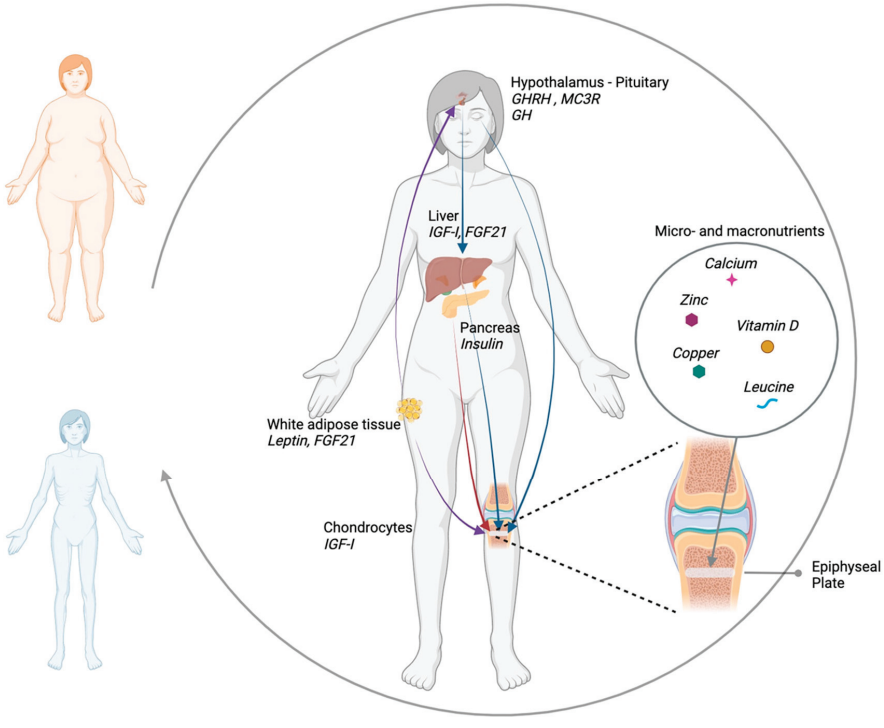

Figure 1. Schematic representation of the interplay between nutritional status and endocrine regulators of growth: growth hormone (GH) resistance induced by prolonged fasting impairs GH direct and insulin-like growth factor I (IGF-I) mediated action on chondrocytes. The increased secretion of fibroblast growth factor 21 (FGF21) by liver and adipose tissue in malnutrition contributes to GH resistance by reducing hepatic GH receptors expression and disrupting GH action in the growth plate. Insulin promotes growth by acting both directly on chondrocytes and indirectly, stimulating IGF-I production. Leptin, produced by adipose tissue, stimulates growth hormone-releasing hormone (GHRH) secretion by the hypothalamus and exerts a direct peripheral growth-promoting effect in the growth plate by stimulating chondrocyte proliferation and differentiation. Hypothalamic melanocortin 3 receptor (MC3R) integrates signals of metabolic status that affect body growth and sexual maturation. Micronutrients such as zinc, copper, calcium, vitamin D and macronutrients such as aminoacids, in particular leucine, exert a direct effect on the epiphyseal growth plate by influencing chondrocyte differentiation and proliferation.

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Article

Adherence to Dietary Recommendations of 7-Year-Old Children from a Birth Cohort in Friuli Venezia Giulia, Italy

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Abstract: Few Italian and European studies have assessed adherence to dietary recommendations in primary school children using dietary records. No Italian studies have provided an index-based nutritional adequacy assessment. We provided a comprehensive overview of dietary intake in 381 7-year-old children from NAC-II cohort study, Friuli Venezia Giulia (Italy). Energy, macro-, and micronutrient intakes were derived from 3-day dietary records. Standard (median and percentage) and index-based (Nutrient Adequacy Ratio (NAR) and Mean Adequacy Ratio (MAR)) approaches were used to evaluate adequacy to Italian dietary reference values at nutrient- and overall-diet-level. Percentage contribution of macronutrients to energy intake (%En) was unbalanced towards total fats and protein. In 25% of children, total fats intake exceeded the reference intake upper limit. In ~63% of children, protein intake was at least doubled in their child-specific population reference intake. Median intakes of sodium (1.7 g/day), saturated fatty acids (12.2 %En), and soluble carbohydrates (19.4 %En) exceeded the suggested dietary target in most (65–84%) children. Inadequacy was also observed for micronutrients, with median NARs ranging from 0.11 (vitamin D) to 0.90 (zinc). The median MAR was 0.75 (0.69–0.79), with 1 indicating optimal overall dietary intake. In conclusion, the enrolled children showed suboptimal intakes of several macro- and micronutrients, in line with Italian and European studies on primary school children. Based on the current findings, public health interventions may be targeted to specific nutrients or subpopulations.

Keywords: dietary habits; dietary intake; energy and nutrient intake; nutritional adequacy; primary school children; nutrient adequacy ratio; mean adequacy ratio; dietary reference values; food groups; dietary record

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1. Introduction

Over recent decades, environmental conditions, including nutrition, have been increasingly recognized to provide short- and long-term effects on health [1,2]. A balanced diet during pregnancy, infancy, and childhood is likely to provide a healthier development over the lifespan [3] and to prevent from the onset of the most common noncommunicable diseases, including obesity [1], type 2 diabetes [4], cardiovascular disease [5], and cancer [6], with childhood obesity itself being one of the major risk factors in the development of other noncommunicable diseases [7–9]. In Europe, the prevalence of overweight and obesity

in primary school children is still a considerable public health issue. Italy is one of the most affected countries [10], reaching 20.4% and 9.4% of prevalence, respectively [11]. In addition, in children, type 2 diabetes is becoming more common [12], even if it is still an occasional event [13]. A balanced nutritional intake including some key nutrients in childhood, such as polyunsaturated fatty acids (PUFAs), iron, iodine, and vitamin B12, is also important in children's neurodevelopment early in life, as well as in its short-term maintenance [14–18].

Childhood is a crucial period of growth and development [19]. Entering primary school, children markedly change their lifestyle. European primary school children spend 65% of school time in sedentary activities [20]. Together with modest physical activity levels outside school time [21,22], this suggests monitoring of their dietary patterns may improve our understanding of nutritional status, and the potential associations between diet and diseases [23]. Information on nutrient intakes at the population level may also provide support to organize targeted nutritional programs [24]. To address this goal, good-quality data on the macro- and micronutrient profile of children's diets are a key element [25].

Different dietary assessment tools have been traditionally used for investigating nutritional habits in children. Among them, food frequency questionnaires (FFQ) are useful to assess the habitual food consumption over long periods in large samples but may introduce errors at individual level for the assessment of energy, macro-, and micronutrient intake. Thus, dietary records (food diaries) or 24-h recalls are usually preferred when the aim is to compare nutrient intakes with country- and age-specific dietary recommendations or estimate mean energy and nutrient intakes [26].

A few studies so far have described nutrient intakes, food sources, and/or adherence to national and international dietary recommendations in primary school children. In Europe, their dietary habits were assessed using 24-h recalls or dietary records obtained from one [27,28] or multiple waves [29–31] of existing cohort studies. In Italy, a cohort was established in 2007 to investigate energy and nutrient intakes from a FFQ in 2–10-year-old children from the Lombardy region, in the north of Italy [32]; two time points were available for the description of dietary habits of primary school children (i.e., 8 and 10 years). Other dietary assessments have been carried out in Italian primary school children using 24-h recalls or dietary records too [24,33–37]. Among all the Italian studies, four [32,34–36] compared nutrient intakes with the corresponding Italian Dietary Reference Values (DRVs) [38]. The standard approach followed for each nutrient included: 1. comparison of observed mean/median intakes and DRVs; 2. calculation of the percentage of subjects meeting the DRV requirement. To our knowledge, no Italian studies so far have provided an index-based assessment of the nutrient-specific or overall-diet-specific adequacy in primary school children. Firstly introduced in Madden et al. [39], the Nutrient Adequacy Ratio (NAR) expresses an individual's intake of a nutrient as a percentage (capped at 100%) of the corresponding recommended allowance for that nutrient, given the respondent's age and sex. Later applied to the pediatric population by Hatløy et al. [40] and more recently by Eldridge [41], NARs provided the basis for the mean adequacy ratio (MAR) index. The MAR quantifies the overall nutritional adequacy of a population based on an individual's diet using the current recommended allowance for a group of nutrients of interest [40,41].

The aim of the current paper is to provide a comprehensive overview of dietary intake in 7-year-old Italian children from the Northern Adriatic Cohort (NAC-II), Friuli Venezia Giulia, in the northeast of Italy, by following different approaches:

1. Standard evaluation of adequacy to the DRVs [38];
2. Index-based evaluation of adequacy to DRVs at the following levels:
    a. Nutrient-level adequacy, through the NAR index;
    b. Overall-diet-level adequacy, through the MAR index;
3. Percentage contribution of different food sources to macro- and micronutrient intakes.

2. Materials and Methods

2.1. Study Population

Between 2007 and 2009, 900 pregnant women were enrolled in the prospective Italian NAC-II study [42], within the framework of the 'Public health impact of long-term, low-level, mixed element exposure in susceptible population strata' (PHIME) European Union project [43]. The project included a Mediterranean cohort involving 4 birth cohorts from Italy (NAC-II), Slovenia, Greece, and Croatia, with the aim of investigating the association between low-level mercury exposure from food consumption in pregnancy and child neurodevelopment at 18 months. Overall diet of mother–infant pairs was originally assessed (during pregnancy, and at 18 months of the child) to provide adjustments for potential confounding factors [43]. Within NAC-II, child's dietary habits were further assessed at 7 years (2014–2016), following an additional extended protocol [44]. Briefly, at the 7-year follow-up, parents of those children tested for the neurodevelopment outcomes at 18 months (N = 632) were contacted for further dietary and neurodevelopment evaluation. The current paper considered dietary intakes for the 381 children whose parents filled in the corresponding dietary record at 7 years of age. A comprehensive description of dietary intake at 18 months has been recently published [45].

The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of the Institute for Maternal and Child Health IRCCS Burlo Garofolo (CE/V-109-12/04/2010). All participating families were informed and consented to participate to the study.

2.2. Parental and Children's Characteristics

Parents filled in a questionnaire assessing lifestyle of the enrolled 7-year-old children. Socio-demographic characteristics of both parents, including education level, marital status, and citizenship, were obtained from a questionnaire administered at delivery [43].

Children's height and weight were measured from healthcare staff during the neuropsychological assessment at 7 years [44]. Body mass index (BMI; kg/m^2) was calculated as: (weight (kg)/height^2 (m^2)). Children were categorized as normal weight, underweight, overweight, or obese according to the World Obesity Federation [46] based on International Obesity Task Force (IOTF) BMI cut-offs for thinness, overweight, and obesity [47].

2.3. Dietary Assessment

Dietary data were collected using a 3-day dietary record (3-dDR) filled in at home by one parent instructed on how to record type and portion size of the foods consumed by the child. Common kitchen utensils were suggested as an alternative to traditional kitchen scales to measure solids and fluids (e.g., teaspoon, glass); in this case, estimated equivalents in grams were also indicated to the parents. Food fortification or supplement use was not captured within the 3-dDR as the instructions did not suggest collection of this information. Three children filled in the diary for less than 3 days and 4 children filled in the diary for more than 3 days. A researcher's telephone contact was provided whenever parents need clarification while filling in the 3-dDR.

Intakes of 39 selected macro- and micronutrients were derived after uploading individual food information from the 3-dDRs in the Microdiet V4.4.1 software (Microdiet software–Downlee Systems Ltd., High, Peak, UK), which contains the Italian "Food Composition Database for Epidemiological Studies in Italy" [48], integrated with information from nutritional labels when needed. Full details of the methodology were published elsewhere [49].

For each nutrient, the Microdiet software provided total intake over the observation period; we calculated mean daily intakes by dividing total intake by the number of collection days. Total energy intake was estimated by summing the mean daily intake of the single macronutrients each multiplied by the corresponding energy conversion factor.

Single food items from the 3-dDRs were classified into 18 food groups obtained after modification of food grouping schemes provided in previous publications of our

group [45,50]. To disentangle the main food sources, percentage contribution of the 18 food groups on energy and nutrients was calculated for each child.

All procedures were conducted by a trained food technologist and three nutritionists, who were fully familiar with the management of food composition data, nutritional assessment, food preparation methods, and nutritional labels.

2.4. Nutritional Adequacy

Individual nutrient intakes were compared with the DRVs proposed by the Italian Society for Human Nutrition [38], when available. The DRVs include adequate intake (AI), reference intake (RI) range for macronutrients, average requirement (AR), population reference intake (PRI), and suggested dietary target (SDT) for the corresponding nutrient.

For protein intake, the Italian Society for Human Nutrition provided 3 DRVs including RI range, AR, and PRI. Given the preeminent role of AR and PRI for protein and availability of anthropometric information for most of the children (N = 350; ~92%), we calculated the AR and PRI child-specific cut-offs using the individual weights and the age-specific DRVs for 7-year-old children, as: AR(protein) (g/day)= 0.8 g/kg weight per day $\times$·kg of weight; PRI(protein) (g/day)= 0.98 g/kg weight per day $\times$ kg of weight. This integrates information on RI range, which was calculated by difference, as: RI(protein) = 100% − RI(total fats) − RI(available carbohydrates) [38].

We evaluated the adequacy of individual diets at the nutrient- as well as at the overall-diet-level using the NAR and the MAR, respectively [40]. In detail, the NAR is defined as the ratio of each child's intake to the national DRV for the appropriate age category. The MAR is the sum of all (nutrient-specific) NARs divided by the total number of NARs. As any ratio, a NAR equal to 1 indicates that the corresponding subject meets the requirement fixed for that nutrient. A MAR equal to 1 indicates that the subjects meet the requirements for all the selected nutrients.

To take into account inadequacy due to excess intake, we extended the approach proposed by Atløy in children [40] to those macro- and micronutrients for which a maximum desirable intake is available. In detail:

- For all micronutrients with one DRV indicating the minimum desirable intake (i.e., AI or AR), we truncated all NARs greater than 1 to 1 so that these nutrients could not compensate those with a NAR lower than 1 in the MAR calculation;
- For the remaining macro- and micronutrients indicating a maximum desirable intake (i.e., RI: protein, available carbohydrates, total fats, monounsaturated fatty acids (MUFAs), total PUFAs, PUFAs ω-3 and ω-6; SDT: soluble carbohydrates, saturated fatty acids (SFAs), sodium, and chloride), we followed suggestions by Hilbig [51] and redefined NARs greater than 1 (inadequate intake by excess) to be equal to: 1 minus the exceeding amount. For example, when the original NAR was equal to 1.15, our modified NAR value is equal to 0.85.

To assess the importance of the individual nutrients in the MAR calculation, we also carried out an influence analysis where the single components were removed one at a time from the MAR definition.

We additionally evaluated nutrient-specific adequacy of protein intake using child-specific AR and PRI.

2.5. Statistical Analysis

General characteristics of parents and children were presented as frequency and percentage distribution for categorical variables, and as median, 25th, and 75th centile for continuous variables with a non-normal distribution. Normality assumption was tested for each continuous variable using the Shapiro–Wilk test.

Standard evaluation of nutritional adequacy was carried out using median, 25th and 75th centile and percentage of children meeting the DRV requirements. Sex-specific median, 25th, and 75th centile were also provided and the presence of potential sex differences was investigated using the two-sample Wilcoxon rank-sum (Mann–Whitney) test. Furthermore,

we investigated the presence of potential inadequacy in individual protein intakes by comparing the observed intakes (g/day) with the corresponding AR and PRI (g/day) with the two-sample Wilcoxon rank-sum (Mann–Whitney) test. Index-based evaluation of adequacy was based on median, 25th, and 75th centile of NAR and MAR.

Statistical significance for all tests was set at 0.05. Stata (StataCorp. 2013. Stata Statistical Software: Release 13. StataCorp LP, College Station, TX, USA) was used for all statistical analysis.

3. Results

3.1. Lifestyle and Anthropometric Characteristics of the Study Population

In total, 381 children (females: 48.3%; males: 51.7%) whose parents filled in 3-dDR were included in the present study. Mother and father's socio-demographic characteristics at enrollment are reported in Supplementary Table S1. Only 6.3% of the mothers were foreign citizens. Almost 82% of the mothers and ~70% of the fathers had a high school diploma (45.1% and 47.0%, respectively) or a higher educational level (38.6% and 22.0%, respectively).

Children's lifestyle and anthropometric characteristics at 7 years of age are presented in Table 1.

Table 1. Children's lifestyle and anthropometric characteristics at 7 years of age. Northern Adriatic Cohort II (NAC-II), 2014–2016 (*N* = 381).

	Median	**25th–75th Centile**
Child weight at 7 years (kg) [1]	25.5	22.8–29.2
Child height at 7 years (cm) [1]	124.3	121.0–128.5
	N	%
Sex		
Male	197	51.7
Female	184	48.3
Weight status [1]		
Underweight	9	2.6
Normal weight	255	72.9
Overweight	67	19.1
Obese	19	5.4
Extra-curricular sport or play activities		
Never	15	3.9
1–3 days/week	303	79.5
>4 days/week	58	15.2
Not reported	5	1.3
Videogames activity		
Never	106	27.8
<1 h/day	172	45.1
1–2 h/day	64	17.6
3 h/day	5	1.3
Not reported	34	8.9
Television use		
Never	15	3.9
<1 h/day	105	27.6
1–2 h/day	233	61.2
3–4 h/day	22	5.8
Not reported	6	1.6

Table 1. *Cont.*

	N	%
Food consumption while screen-time activities		
Yes	103	27.0
No	274	71.9
Not reported	4	1.0
Caregiver (weekdays) [2]		
Mother	326	86.2
Father	146	38.7
Grandparents	135	35.8
School	164	43.4
Baby-sitter	13	3.4
Others	19	5.1

[1] Anthropometric information was available for 350 children only. [2] More than a category was available for responders; therefore, the total does not sum up to 381. In detail, 376 parents selected the "Others" option, 377 parents selected the "Father", "Grandparents", and "Baby-sitter" option, 378 parents selected the "Mother" or "School" option.

Children's median age was 7.1 (7.1–7.2) years. Approximately 72.9% of the children were normal weight, whereas 19.1% and 5.4% were overweight and obese, respectively. Most of the children (79.5%) practiced extra-curricular sport activities from 1 to 3 days per week, with a 15.2% practiced sport more than 4 days per week. Approximately half of the children (45.1%) played videogames less than 1 h per day or never (27.8%), whereas 66.9% watched TV more than 1 h per day (Table 1). During the weekend, the percentage of children playing videogames and watching TV increased in all categories (data not shown).

Mothers spent more time with children during weekdays, but a prevailing caregiver role was also identified for school (43.4%), fathers (38.7%), and grandparents (35.8%); during the weekend fathers spent more time together with the child (data not shown).

3.2. Description of Daily Dietary Nutrient Intake and Standard Comparison with the Italian Dietary Reference Values

3.2.1. Energy and Macronutrients

Overall and sex-specific descriptive statistics (median, 25th–75th centile) of the observed intakes of energy and macronutrients per day are reported in Table 2, together with the corresponding Italian DRVs. The Italian DRVs include RI range, SDT, or AI, as applicable [38].

Table 2. Distribution of energy and macronutrient intakes of 7-year-old children in the overall sample and stratified by sex. Northern Adriatic Cohort II (NAC-II), 2014–2016 (*N* = 381).

					Females (*N* = 184)			**Males (*N* = 197)**			
	Median	**25th**	**75th**	**DRVs**	**Median**	**25th**	**75th**	**Median**	**25th**	**75th**	***p*-Value**
Energy (kJ/d)	6291.8	5593.2	6982.2		6139.2	5261	6862.2	6404.4	5773.6	7113.2	0.002 *
Energy (kcal/d)	1503.0	1336.2	1668.0		1466.6	1256.8	1639.3	1530.0	1379.3	1699.3	0.002 *
Protein (g/d)	55.6	47.2	64.3		54.0	46.3	63.4	56.8	48.4	64.5	0.077
Protein (%En)	14.8	13.2	16.5	12–18 %En (RI) [1]	14.9	13.3	16.9	14.8	13.0	16.3	0.165
Total fats (g/d)	52.2	42.6	61.7		51.1	42.0	60.4	53.3	43.4	63.7	0.084
Total fats (%En)	31.3	27.4	35.1	20–35 %En (RI)	32.0	27.7	35.1	30.8	27.2	35.1	0.309
Saturated fatty acids (g/d)	20.5	16.5	24.5		20.2	14.9	24.0	20.9	17.0	25.0	0.055

Table 2. *Cont.*

					Females (*N* = 184)			Males (*N* = 197)			
	Median	**25th**	**75th**	**DRVs**	**Median**	**25th**	**75th**	**Median**	**25th**	**75th**	***p*-Value**
Saturated fatty acids (%En)	12.2	10.6	14.0	<10 %En (SDT)	12.2	10.8	14.0	12.2	10.4	14.1	0.865
Monounsaturated fatty acids (g/d)	18.0	14.5	22.1		18.0	14.0	21.7	18.1	14.8	22.1	0.307
Monounsaturated fatty acids (%En)	10.8	9.3	12.6	10–15 %En (RI) [2]	11.1	9.4	12.7	10.7	9.3	12.4	0.137
Oleic acid (g/d)	16.5	13.4	20.1		16.6	13.2	20.0	16.5	13.8	20.1	0.405
Polyunsaturated fatty acids (g/d)	5.2	4.1	6.6		5.3	4.1	6.7	5.1	4.1	6.5	0.792
Polyunsaturated fatty acids (%En)	3.1	2.5	3.9	5–10 %En (RI)	3.3	2.6	4.0	3.0	2.4	3.7	0.021 *
Arachidonic acid (mg/d)	146.3	95.6	219.7		143.2	96.4	206.5	154.2	94.5	226.9	0.503
Linoleic acid (g/d)	3.9	3.1	5.2		3.9	3.1	5.3	3.8	3.0	5.2	0.509
PUFAs ω-6 (%En)	2.4	2.0	3.2	4–8 %En (RI)	2.6	2.1	3.3	2.3	1.9	3.0	0.007 *
Alpha-linolenic acid (g/d)	0.6	0.4	0.7		0.5	0.4	0.7	0.6	0.5	0.7	0.101
EPA + DHA (mg/d)	61.0	23.7	208.3	250 mg/d (AI)	53.9	24.0	207.3	67.3	23.7	210.0	0.620
PUFAs ω-3 (%En)	0.4	0.3	0.5	0.5–2.0 %En (RI)	0.4	0.3	0.5	0.4	0.3	0.5	0.572
Cholesterol (mg/d)	185.3	143.0	224.8		186	141.9	225.5	183.0	143.3	224.4	0.852
Available carbohydrates (g/d)	197.6	163.7	223.9		189.3	153.0	215.8	204.1	172.0	230.4	<0.001 *
Available carbohydrates (%En)	51.8	48.3	56.6	45–60 %En (RI)	51.3	47.7	55.6	52.5	48.7	57.3	0.095
Soluble carbohydrates (g/d)	72.5	59.0	87.5		71.2	58.6	85.8	74.7	60.1	91.3	0.057
Soluble carbohydrates (%En)	19.4	16.4	23.0	<15 %En (SDT)	19.4	16.3	23.3	19.4	16.4	22.7	0.948
Fiber (g/1000 kcal/d)	7.0	5.7	8.7	8.4 g/1000 kcal (AI)	7.2	5.8	8.9	7.0	5.4	8.5	0.139

[1] Reference Intake calculated by difference: RI(protein) = 100%- RI(total fats)- RI(available carbohydrates); [2] Reference Intake calculated by difference: RI(MUFAs) = RI(total fats)- RI(PUFAs)- SDT(SFAs). Abbreviations: d, day; DRVs, Dietary Reference Values; RI, Reference Intake; SDT, Suggested Dietary Target; AI, Adequate Intake; %En, percentage of daily energy intake; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. The two-sample Wilcoxon rank-sum (Mann–Whitney) test was applied to detect any statistical differences between females and males; * $p < 0.05$.

The median daily energy intake of the overall sample was 1503.0 kcal (1336.2–1668.0), with a statistically significant difference by sex ($p < 0.05$): females tended to have a lower intake with a median of 1466.6 kcal (1256.8–1639.3) compared to 1530.0 kcal (1379.3–1699.3) for males.

Overall, the percentage contribution of protein, total fats, and available carbohydrates to daily energy intake (%En) was found to be in line with the recommendations. The median %En from total fats (31.3 %En; 27.4–35.1) was close to the upper limit of the RI range (20–35 %En). Conversely, the median %En from available carbohydrates (51.8 %En; 48.3–56.6) was closer to the lower limit of the recommendations (45–60%). A statistically significant difference was observed for available carbohydrates (g/day) between females and males ($p < 0.05$) (females: 189.3; 153.0–215.8 vs. males: 204.1; 172.0–230.4). Based on the %En of total fats and available carbohydrates, the median %En of protein (14.8 %En; 13.2–16.5) was at the middle point of the RI range (12–18 %En). Furthermore, the median %En from total PUFAs, PUFAs ω-6, and PUFAs ω-3 were below the lower limit of the RI

range. Within the PUFAs dietary profile, median intake of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (mg/day) was below the AI. Higher %En from total PUFAs and PUFAs ω-6 were observed in females than in males ($p < 0.05$).

In median, soluble carbohydrates and SFAs intakes exceeded the SDT (<15 %En and <10 %En, respectively) in the overall sample: 19.4 %En (16.4–23.0) for soluble carbohydrates and 12.2 %En (10.6–14.0) for SFAs.

The median fiber intake (7.0 g/1000 kcal per day; 5.7–8.7) did not reach the AI (8.4 g/1000 kcal per day).

3.2.2. Protein Intake: Comparison with Average Requirement and Population Reference Intake

Providing a focus on protein intake, 100% of the enrolled children reached their AR; 99.7% of the children reached their PRI too, with only one child having an intake between AR and PRI. However, 63% of the children showed an observed protein intake 2–4 times higher than their PRI.

Figure 1 shows a comparison between individual-level distributions of observed (i.e., from the 3-dDR) and required (i.e., expressed as their age-specific AR and PRI) intakes for our 7-year-old children. In the absence of sex-specific differences in observed intakes, this analysis was carried out on the overall sample of children. The distribution of the observed protein intake modestly overlapped with that of the corresponding required intakes (*p*-values from the two-sample Wilcoxon rank-sum (Mann–Whitney) test for observed vs. AR intake and observed vs. PRI intake <0.0001). Corresponding summary statistics expressed as median (25th–75th centile) were equal to: observed intake: 56.2 g/day (47.7–64.3) vs. AR intake: 20.4 g/day (18.3–23.3), and PRI intake: 25.0 g/day (22.4–28.6).

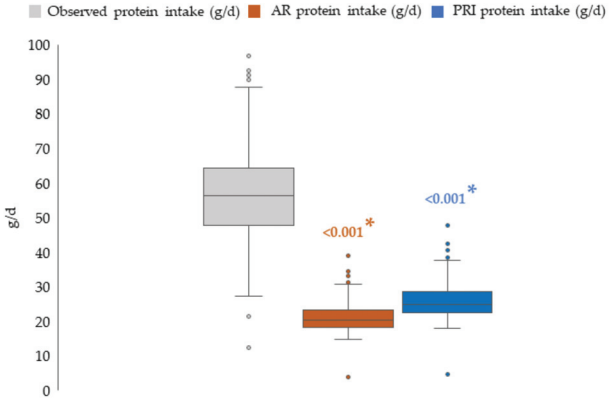


Figure 1. Box-and-whiskers plots comparing the observed protein intake of children and their protein dietary reference values. NAC-II, 2014-2016 ($N = 381$). Each child's ($N = 350$) protein requirement was estimated using Average Requirement (AR = 0.8 g/kg of weight per day) and Population Reference Intake (PRI = 0.98 g/kg of weight per day) for 7-year-old children. The bottom and top edge of each box represent the 25th and 75th centile (interquartile range); the line within each box represents the median; the ends of the bottom and top whiskers represent the minimum and maximum values and the circles represent outliers. Abbreviation: d, day. The two-sample Wilcoxon rank-sum (Mann–Whitney) test was applied to detect any statistical differences between observed and estimated intakes (AR and PRI). * $p < 0.05$.

3.2.3. Micronutrients

Overall and sex-specific descriptive statistics (median, 25th–75th centile) of the observed intakes of micronutrients per day are reported in Table 3, together with the corresponding Italian DRVs. The Italian DRVs include AI, SDT, AR, or PRI, as applicable [38].

Table 3. Distribution of micronutrient intakes of 7-year-old children in the overall sample and stratified by sex. Northern Adriatic Cohort II (NAC-II), 2014–2016 (*N* = 381).

					Females (*N* = 184) (*N* = 197)			Males (*N* = 197)			
	Median	**25th**	**75th**	**DRVs**	**Median**	**25th**	**75th**	**Median**	**25th**	**75th**	***p*-Value**
Sodium (g/d)	1.7	1.3	2.1	1.1 g/d (AI); 1.5 g/d (SDT)	1.7	1.3	2.1	1.8	1.4	2.1	0.207
Potassium (g/d)	1.8	1.4	2.1	3 g/d (AI)	1.7	1.4	2.1	1.8	1.5	2.1	0.719
Calcium (mg/d)	537.8	409.6	706.3	900 mg/d (AR); 1100 mg/d (PRI)	515.9	392.4	657.0	567.7	420.1	721.7	0.034 *
Magnesium (mg/d)	83.5	65.5	105.8	130 mg/d (AR); 150 mg/d (PRI)	81.8	61.7	104.2	85.3	67.0	106.0	0.193
Phosphorus (mg/d)	819.0	693.8	966.9	730 mg/d (AR); 875 mg/d (PRI)	778.7	668.5	941.4	846.2	715.7	975.8	0.020 *
Iron (mg/d)	5.9	4.8	7.2	5 mg/d (AR); 13 mg/d (PRI)	5.6	4.6	7.1	6.1	5.0	7.5	0.066
Zinc (mg/d)	6.3	5.3	7.4	7 mg/d (AR); 8 mg/d (PRI)	6.0	5.1	7.3	6.6	5.6	7.4	0.004 *
Selenium (µg/d)	18.5	13.0	28.2	30 µg/d (AR); 34 µg/d (PRI)	17.4	13.2	27.4	19.1	12.9	28.7	0.336
Copper (mg/d)	0.4	0.2	0.5	0.4 mg/d (AR); 0.6 mg/d (PRI)	0.4	0.2	0.5	0.4	0.3	0.5	0.288
Chloride (g/d)	1.3	1.0	1.7	1.7 g/d (AI); 2.3 g/d (SDT)	1.2	1.0	1.7	1.3	1.0	1.7	0.331
Manganese (mg/d)	0.4	0.2	0.6	1.2 mg/d (AI)	0.4	0.2	0.6	0.4	0.2	0.6	0.650
Iodine (µg/d)	75.0	51.8	104.6	100 µg/d (AI)	69.3	47.2	104.8	80.0	55.1	104.6	0.077
Vitamin B1 (mg/d)	0.7	0.6	0.9	0.6 mg/d (AR); 0.8 mg/d (PRI)	0.7	0.6	0.8	0.7	0.6	0.9	0.127
Vitamin B2 (mg/d)	1.1	0.8	1.3	0.7 mg/d (AR); 0.8 mg/d (PRI)	1.0	0.8	1.3	1.1	0.9	1.3	0.014 *
Niacin (mg/d)	9.6	7.7	12.2	9 mg/d (AR); 12 mg/d (PRI)	9.6	7.6	12.1	9.5	7.7	12.3	0.890
Pantothenic acid (mg/d)	2.1	1.6	2.6	3.5 mg/d (AI)	2.2	1.7	2.7	2.1	1.6	2.6	0.979
Vitamin B6 (mg/d)	1.3	1.0	1.5	0.7 mg/d (AR); 0.9 mg/d (PRI)	1.2	1.0	1.5	1.3	1.1	1.5	0.394
Biotin (µg/d)	11.5	8.6	14.7	20 µg/d (AI)	11.5	8.4	14.8	11.5	8.7	14.6	0.929
Folate (µg/d)	160.6	127.5	199.7	210 µg/d (AR); 250 µg/d (PRI)	155.1	125.5	194.1	165.7	135.0	204.2	0.072
Vitamin B12 (µg/d)	2.4	1.8	3.3	1.3 µg/d (AR); 1.6 µg/d (PRI)	2.4	1.7	3.1	2.5	1.9	3.4	0.133
Vitamin A (µg/d) [1]	603.7	438.8	853.4	350 µg/d (AR); 500 µg/d (PRI)	589.6	421.8	856.2	617.4	450.1	853.1	0.419
Vitamin C (mg/d)	63.2	40.7	98.4	45 mg/d (AR); 60 mg/d (PRI)	61.6	41.0	97.5	63.7	40.4	98.4	0.984

Table 3. *Cont.*

					Females (*N* = 184) (*N* = 197)			Males (*N* = 197)			
	Median	**25th**	**75th**	**DRVs**	**Median**	**25th**	**75th**	**Median**	**25th**	**75th**	***p*-Value**
Vitamin D (μg/d)	1.1	0.7	1.5	10 μg/d (AR); 15 μg/d (PRI)	1.0	0.7	1.3	1.1	0.8	1.6	0.142
Vitamin E (mg/d) [2]	5.1	4.0	6.6	8 mg/d (AI)	5.2	4.1	6.6	5.1	4.0	6.6	0.496

[1] Expressed as retinol equivalents; [2] Expressed as alpha-tocopherol equivalents. The two-sample Wilcoxon rank-sum (Mann–Whitney) test was applied to detect any statistical differences between females and males; * $p < 0.05$. Abbreviation: d, day.

Median intakes of potassium, magnesium, manganese, iodine, pantothenic acid, biotin, and vitamin E were below the corresponding AI. Median sodium intake (1.7 g/day; 1.3–2.1) reached the AI (1.1 g/day), but it exceeded the SDT (1.5 g/day).

Moreover, median intakes of calcium, chloride, magnesium, zinc, selenium, folate, and vitamin D were below the AR and consequently the PRI. Median intakes of phosphorus, iron, copper, vitamin B1, and niacin reached the corresponding AR, but not the PRI Finally, median intakes of vitamin B2, vitamin B6, vitamin B12, vitamin A, and vitamin C reached both their AR and PRI.

Significantly higher intakes were observed in males as compared to females for calcium, phosphorus, zinc, and vitamin B2 ($p < 0.05$, Table 3).

3.3. Index-Based Evaluation of Diet Adequacy to Dietary Reference Values

3.3.1. Nutrient-Level Adequacy

The proportion of children with a nutrient intake above, below, or within the recommendations, together with the corresponding median NARs, were presented in Figures 2–5.

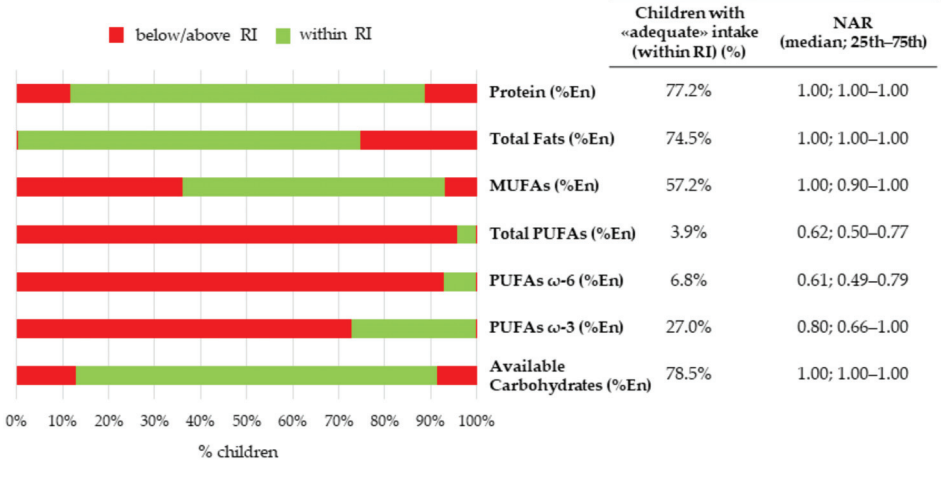
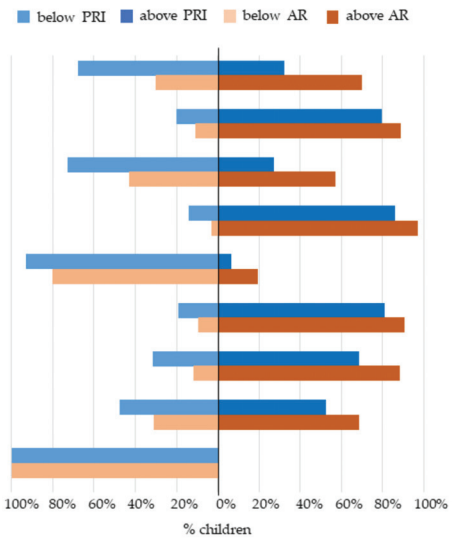
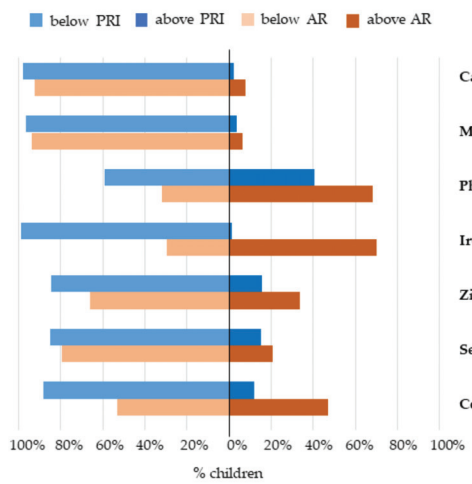


Figure 2. Nutritional adequacy of macronutrients relative to the reference intake. NAC-II, 2014–2016 (*N* = 381). NAR was based on the RI range. Children having intakes equal to the cut-off values were considered to be adequate for that specific nutrient.



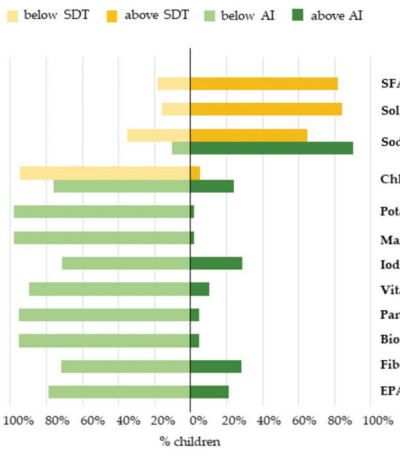
	Children with «adequate» intake (above PRI) (%)	Children with «adequate» intake (above AR) (%)	NAR (median; 25th–75th)
Vitamin B1 (mg/d)	32.3%	69.8%	1.00; 0.95–1.00
Vitamin B2 (mg/d)	79.8%	89.0%	1.00; 1.00–1.00
Niacin (mg/d)	27.3%	57.2%	1.00; 0.85–1.00
Vitamin B6 (mg/d)	86.1%	97.1%	1.00; 1.00–1.00
Folate (µg/d)	6.8%	19.7%	0.76; 0.61–0.95
Vitamin B12 (µg/d)	81.1%	90.6%	1.00; 1.00–1.00
Vitamin A (µg/d)	68.5%	88.2%	1.00; 1.00–1.00
Vitamin C (mg/d)	52.5%	68.8%	1.00; 0.90–1.00
Vitamin D (µg/d)	0.0%	0.0%	0.11; 0.07–0.15

Figure 3. Nutritional adequacy of micronutrients (i.e., vitamins) relative to the average requirement and population reference intake. NAC-II, 2014–2016 (*N* = 381). NAR was based on the AR. Children having intakes equal to the cut-off value were considered to be adequate for that specific nutrient.



	Children with «adequate» intake (above PRI) (%)	Children with «adequate» intake (above AR) (%)	NAR (median; 25th–75th)
Calcium (mg/d)	2.1%	7.6%	0.60; 0.46–0.78
Magnesium (mg/d)	3.4%	6.3%	0.64; 0.50–0.81
Phosphorus (mg/d)	40.7%	68.2%	1.00; 0.95–1.00
Iron (mg/d)	1.3%	70.3%	1.00; 0.97–1.00
Zinc (mg/d)	15.5%	33.9%	0.90; 0.75–1.00
Selenium (µg/d)	15.0%	20.7%	0.62; 0.43–0.94
Copper (mg/d)	11.8%	47.0%	0.96; 0.60–1.00

Figure 4. Nutritional adequacy of micronutrients (i.e., minerals) relative to the average requirement and population reference intake. NAC-II, 2014–2016 (*N* = 381). NAR was based on the AR. Children having intakes equal to the cut-off value were considered to be adequate for that specific nutrient.



	Children with «adequate» intake (below SDT) (%)	Children with «adequate» intake (above AI) (%)	NAR (median; 25th–75th)
SFAs (%En)	18.4%	-	0.78; 0.60–0.94
Soluble Carbohydrates (%En)	15.8%	-	0.71; 0.47–0.91
Sodium (g/d)	35.0%	89.8%	0.80; 0.58–1.00
Chloride (g/d)	94.8%	23.9%	0.75; 0.58–0.95
Potassium (g/d)	-	2.1%	0.59; 0.48–0.70
Manganese (mg/d)	-	2.1%	0.33; 0.17–0.47
Iodine (µg/d)	-	28.9%	0.75; 0.52–1.00
Vitamin E (mg/d)	-	10.2%	0.64; 0.50–0.83
Pantothenic acid (mg/d)	-	4.7%	0.61; 0.47–0.75
Biotin (µg/d)	-	5.0%	0.58; 0.43–0.73
Fiber (g/1000kcal/d)	-	28.1%	0.84; 0.68–1.00
EPA+DHA (mg/d)	-	21.3%	0.24; 0.09–0.83

Figure 5. Nutritional adequacy of macro- and micronutrients, relative to the adequate intake and suggested dietary target. NAC-II, 2014–2016 (*N* = 381). NAR was based on the SDT and AI Children having intakes equal to the AI cut-off value were considered to be adequate for that specific nutrient, while children having intake equal to the SDT cut-off value were considered to be inadequate.

The RI lower limits for total fats (%En), protein (%En), and available carbohydrates (%En) were reached by 74.5%, 77.2%, and 78.5% of children, respectively. This was confirmed by median NARs being all equal to 1.00 (1.00–1.00)—i.e., the ideal cut-off for nutrient adequacy—for all the previous nutrients (Figure 2).

Approximately 96%, 93%, and 73% of children did not reach the RI lower limit for total PUFAs (%En), PUFA ω-6 (%En), and PUFA ω-3 (%En), respectively. The corresponding median NARs were far from 1 and equal to 0.62 (0.50–0.77), 0.61 (0.49–0.79), and 0.80 (0.66–1.00), respectively, thus suggesting a substantial inadequacy (Figure 2).

In addition, ~80% of children had intakes of vitamin B2, vitamin B6, vitamin B12, and vitamin A above the AR; the corresponding median NAR was equal to 1.00, with the 25th centile already reaching 1. Almost 70% of children had intakes of vitamin B1 and vitamin C above the AR, with median NARs of 1.00, but the 25th centile reached 0.95 and 0.90, respectively. Less than 20% of children had an intake above the AR for folate, with a median NAR of 0.76 (0.61–0.95). No child had an intake above the AR for vitamin D, with a median NAR as low as 0.11 (0.07–0.15) (Figure 3).

Furthermore, almost 70% of children had an intake of iron and phosphorus above the AR, with median NARs of 1.00 (0.97–1.00) and 1.00 (0.95–1.00), respectively. However, only 1% of children had an iron intake above the PRI cut-off. Regarding selenium, zinc, and copper, 20.7%, 33.9%, and 47.0% of children had an intake above the corresponding nutrient-specific AR, respectively; the median NARs were 0.62 (0.43–0.94), 0.90 (0.75–1.00), and 0.96 (0.60–1.00), respectively, thus indicating that the observed intakes for zinc and copper were closer to the AR than selenium. The AR for calcium and magnesium was reached by less than 10% of the children (NARs equal to 0.60 and 0.64, respectively) (Figure 4).

Finally, for fiber, iodine, chloride, and EPA + DHA intake the AI was similarly reached by 20–30% of the children, but the median NARs varied from 0.24 (0.09–0.83, EPA + DHA) to 0.84 (0.68–1.00, fiber). Less than 10% of children had an intake of potassium, manganese, pantothenic acid, and biotin above the AI; the corresponding median NARs varied from 0.33 (0.50–0.81, manganese) to 0.64 (0.50–0.83, vitamin E) (Figure 5).

Overall, 90% of the children had a sodium intake above the AI; however, only 35% had an intake not exceeding the SDT cut-off value, as reflected by a median NAR smaller than 1 (NAR: 0.80, 0.58–1.00). Even if most children exceeded the SDT for SFAs and soluble

carbohydrates (81.6% and 84.2%, respectively), the corresponding median NARs were 0.78 (0.60–0.94) and 0.71 (0.47–0.91), thus witnessing a modest distance of observed intakes from the SDT (Figure 5). However, when analyzing individual intakes with more stringent cut-offs, no child from our sample had a soluble carbohydrates intake <5 %En, 4 children only were <10 %En, vs. 60 (15.8%) who were <15 %En, which corresponds to the Italian SDT. Finally, 57 children (15.0%) had a soluble carbohydrates intake exceeding the recommended 25% of energy intake [38] (data not shown).

3.3.2. Overall-Diet-Level Adequacy

In our population, no children reached the optimal MAR value of 1.00, targeting adequacy on all the available nutrients. Overall, the median MAR was 0.75 (0.69–0.79). In the influence analysis, median values of the MAR ranged from 0.74 to 0.76, after removal of one component at a time. No statistically significant differences were found in median MAR values between females and males.

3.4. Sources of Nutrient Intakes: Food Groups

Figure 6 shows the percentage contribution of food groups to energy, protein, total fats, available carbohydrates, soluble carbohydrates, and fiber. The full list of food groups and their contribution to intake of fatty acids, cholesterol, and micronutrients are presented in Supplementary Tables S2 and S3.

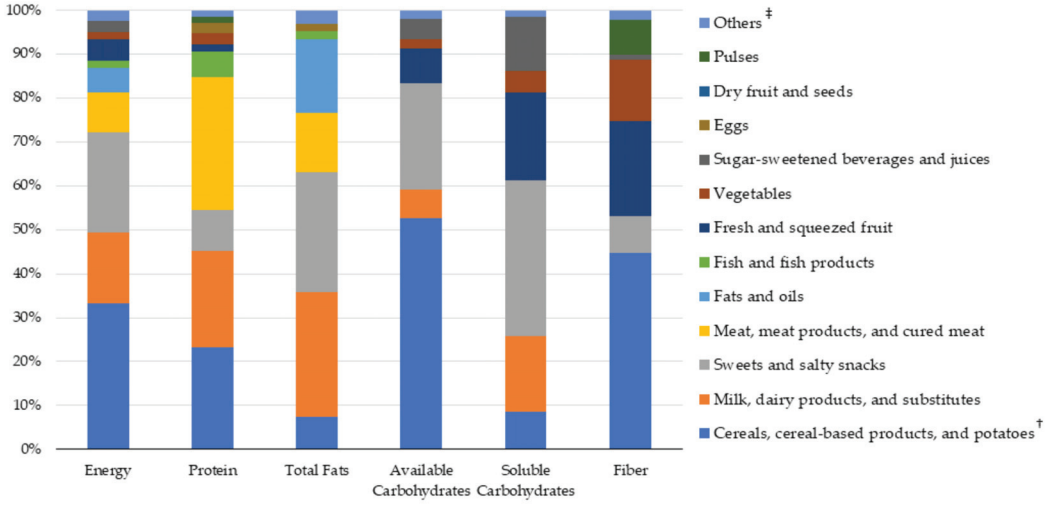


Figure 6. Percentage contribution of the different food groups to total intake of energy and macronutrients. NAC-II, 2014–2016 (N = 381). ‡ For each variable, the category "Others" included all the remaining food groups. † "Cereals and cereal-based products" and "Potatoes" were clustered together, as well as "Meat and meat products" and "Cured meat". The full list of food groups and their contribution to intake of fatty acids, cholesterol, and micronutrients are presented in Supplementary Tables S2 and S3.

The main sources of energy intake were "Cereals, cereal-based products and potatoes" (33.2%), "Sweet and salty snacks" (22.6%), and "Milk, dairy products, and substitutes" (16.1%). "Sweet and salty snacks" were the main sources of soluble carbohydrates (35.4%), followed by "Fresh and squeezed fruit" (20.1%), "Milk, dairy products, and substitutes" (17.2%), and "Sugar-sweetened beverages and juices" (12.4%). The major contributors of protein intake were "Meat, meat products, and cured meat" (30.3%), "Cereals, cereal-based products and potatoes" (23.2%), and "Milk, dairy products, and substitutes" (22.0%). "Fish

and fish products" contributed to protein intake only for ~6.0%. "Milk, dairy products, and substitutes" (28.5%) and "Sweet and salty snacks" (27.1%) were the main sources of total fats, followed by "Fats and oils" (16.7%) and "Meat, meat products, and cured meat" (13.6%). Finally, the major sources of available carbohydrates and fiber were similar and included "Cereals, cereal-based products and potatoes" (52.6% and 44.7%), "Sweet and salty snacks" (24.2% and 8.4%), "Fresh and squeezed fruit" (8.0% and 21.6%), and "Vegetables" (2.0% and 13.9%). "Pulses" contributed to fiber intake only for the 7.8%.

4. Discussion

The current study evaluated nutritional adequacy in 381 7-year-old children from Friuli Venezia Giulia, Italy, who were enrolled within a cohort study aimed at evaluating the effects of mercury on infant neurodevelopment [42]. Results revealed an inadequate intake of key nutrients, as highlighted by standard analyses and the NAR indexes, and suboptimal adequacy of the overall dietary profile, as expressed by the MAR index. In the standard comparison with DRVs, distribution of macronutrient intakes in percentage of energy was unbalanced in favor of protein and fats, with protein intake exceeding the recommendation from 2 to 4 times. Similarly, inadequacy by excess intake was found for most (range: 65.0–84.2%) of the children for soluble carbohydrates, SFAs, and sodium. Within a range of median values between 0.11 and 0.90, the NAR-based analysis further confirmed and allowed to quantify inadequacy by defect for some micronutrients, including vitamin D and folate; it also downgraded evidence on zinc inadequacy, previously emerged in standard DRV-based analysis. A median MAR value of 0.75, with no child reaching the optimal adequacy value of 1, suggested a suboptimal adequacy of the overall diet in the study population.

Considering available carbohydrates, total fats, and protein, most of the children from our sample met the Italian DRVs and showed a NAR index equal to 1. Although apparently reassuring, this hides a substantial unbalance of the overall diet towards total fats and protein. Indeed, in our sample, no child was below the RI lower limit for total fats, and 1 out of 4 children (25.2%) exceeded the upper RI limit. In addition, by calculating child-specific PRI cut-offs for protein, ~63% of the children at least doubled their recommended PRI and ~11% at least tripled it. From a different perspective, the median protein intake of our sample is 55.6 g/d, which is comparable to the daily protein requirement of an adult woman of 60 kg of weight (54 g/day) [38]. The described unbalance towards protein and total fats has been already documented in most of the other Italian [34,35] and European [27,29–31] studies on primary school children, except for one older Italian study [32] where available carbohydrates of 8-year-old children reached 60% of total energy intake. In addition, our analysis on food groups suggested that at least 60% of protein daily intake was from animal sources, indicating a low plant-based protein intake, as previously observed in children from the same age in Italy, Spain, and Belgium [37,52,53]. Western dietary pattern, which is high in animal sources, has been previously associated with an increased risk of metabolic syndrome [54].

Still in line with the Italian and European data [24,34,35,55], we observed: 1. an excess intake of SFAs, with 82% of children being above the SDT; and 2. intakes of total PUFAs below the RI lower limit in 96% of the children.

We similarly observed an excess contribution to energy intake from soluble carbohydrates, when using the SDT as the reference cut-off [24,34,35,55]. In addition, 15% of the enrolled children derived at least 25% of their total energy intake from soluble carbohydrates, against recommendations of the Italian Society of Human Nutrition [38], who considered intakes >25% to be at risk for adverse effects on health. Furthermore, no child from our sample had a soluble carbohydrates intake <5 %En, 4 children were < 10 %En vs. 60 (15.8%) < 15 %En, which corresponds to the Italian SDT. This is far from the World Health Organization recommendation to reduce the intake of free sugars to <5 %En, due to their effects on body fat deposition, overweight and obesity, cardiovascular risk, and dental caries [56].

Even if underestimation of sodium is likely to occur in dietary records, our median sodium intake (1.7 g/day) was in line with the one reported by Rosi et al. [35] (1.8 g/day), whereas Verduci et al. [34] reported a lower median intake (1.2 g/day); in the UK-based Avon Longitudinal Study of Parents and Children (ALSPAC) study sex-specific medians were higher (2.1 and 2.3 g/day) and sodium was estimated without considering added salt [30]. In addtion, 65% of our enrolled children had a sodium intake above the SDT [38], without any other possible comparisons except for Verduci et al. [34], where 9% of children were above the SDT, in line with their lower median intake. Major food sources included "Cereal and cereal-based products" (34.4%), followed by "Herbs, spices and added salt" (14.1%), probably due to an increased frequency of consumption of bread substitutes [57] and ready-to-eat products [58,59], which are rich in salt [48]. The "Sweets and salty snacks" food group provided a nonnegligible contribution (8.9%) to sodium intake, with sweets accounting for 92.9% of the food group contribution. This suggests sodium is present in sweets too. Although in "Milk, dairy products, and substitutes" and "Cured meat" salt is traditionally used as a preservative [60], in sweets it is commonly used as a flavor enhancer [60].

Except for iron, copper, and phosphorus, intake of other minerals was generally inadequate (i.e., median intake lower than the corresponding AR) in our sample. Similar conclusions were reached for iron, phosphorus, sodium, calcium, potassium, and zinc, in one or more of the available Italian studies [34,35]. However, generally, higher mean/median intakes were shown in the comparison with previous European studies [30,31,61], as well as with the very detailed but older Italian INRAN-SCAI study [24]. Downgrading evidence on zinc inadequacy from standard DRV-based analysis, the NAR-based approach revealed a modest deviation of zinc intake from the DRVs in most children (median NAR = 0.90). This indicates that dietary inadequacy was not severe in our sample, as also hypothesized for zinc deficiency in serum of European children [62].

Seven out of 12 available vitamins showed a median adequate intake, with five of them (vitamin B2, vitamin B6, vitamin B12, vitamin A, and vitamin C) even reaching the PRI. However, in our analysis we detected a major contribution of food groups of animal origin to vitamin B2 (>60%), vitamin B6 (>43%), and vitamin B12 (100%), as well as to energy (>30%). Among others, the worst degree of inadequacy was observed for folate and vitamin D. Folate median intake was below the AR and 80% of children did not reach it. In Italy and Europe, mean/median intake of folate in primary school children was similarly low [30,31,35,61]. In our sample, the main source of the folate was "cereals and cereal-based products", where folate intakes may be underestimated due to cooking losses. An inadequate intake of vitamin D was also observed in our sample: no child met the AR, and the median intake of children was 11%, as compared to the AR cut-off value (median NAR = 0.11). This is alarming, but in line with dietary data of other Italian [24,34,35] and European studies [30,31]. Evidence of serum deficiency of vitamin D was also reported in a pediatric population in Italy [63,64], suggesting an increased sun exposure and dietary intake has to be reached.

We did not observe substantial variation in nutrient intake between males and females. Most of the significant differences were found for macronutrients, with the higher available carbohydrates intake in males likely reflected in their higher energy intake, as also found in Verduci et al. [32]. This is in line with previous results from the ALSPAC [30] and from our NAC-II cohort in children at 18 months [45], where, however, soluble carbohydrates were also significantly different between males and females. Only four micronutrients showed a significantly different intake in males and females, but those differences were small and likely to be not nutritionally meaningful.

The major strength of the present work stands in its comprehensive description of dietary intake following different approaches. To our knowledge, we were the first group in Italy to propose the use of NAR and MAR indexes for a quantitative evaluation of dietary adequacy at the nutrient- and overall-diet-levels. We extended the MAR index in two directions: 1. including additional macro- and micronutrients; 2. considering

nutrient inadequacy by excess intake together with deficiency in the calculation of the corresponding NARs. We also carried out an influence analysis to assess the importance of single nutrients in the calculation of the MAR index, with reassuring results. We finally summarized information on percentage contribution of selected food groups to macro- and micronutrients, to provide an updated benchmark information for future Italian studies on primary school children. This comprehensive approach has been possible because we collected information based a 3-dDR, which provided a precise quantification of daily food intake [26]. In addition, we referred to the "Food Composition Database for Epidemiological Studies in Italy" [48] to derive intakes of a complete list of 86 macro- and micronutrients. Among the 37 nutrients compared with DRVs, 24 did not show missing values in the BDA. We were only unable to compare four nutrients with the available Italian DRVs [38]: chrome, fluorine, and molybdenum were not provided by the BDA and vitamin K was fraught with so many missing values (86% of the total BDA items) to likely not providing a reliable estimate of its intake.

The current study also has limitations. The cohort was enrolled in the Friuli Venezia Giulia region with a different aim, so generalizability of results to the Italian population of 7-year-old children of the same or next time span is questionable. However, percentages of males and females, prevalence of overweight, as well as percentages of mothers and fathers with a high school diploma were similar to those reported in the national survey "OKkio alla SALUTE" on 8–9-year-old children in 2016, for the Friuli Venezia Giulia region [65] and at the national level [66]. Prevalence of obesity in our sample was in line with data from Friuli Venezia Giulia (5.0%, [65]), but lower than the national-level data (9.3%, [66]). This likely reflects the high frequency of practicing sports and the modest screen time detected in our sample; however, a proper comparison with "OKkio alla SALUTE" was not possible due to differences in questionnaires. Although a dietary record is the gold standard dietary assessment method [26], its use may still lead to biases. Dietary records were filled in by caregivers, who may not be fully aware of child food consumption, especially when the child has lunch at school and/or more than one caregiver is in charge of him/her. Dietary records may be incomplete or inaccurately completed. In case of missing information, standard recipes and standard portion sizes were used. In our 3-dDRs, added salt was not accurately reported by all subjects, and no information on its iodization has been provided. Similarly, water consumption was sporadically reported, leading to possible underestimation of minerals, especially of calcium. An additional source of underestimation of nutrients—common to other studies—included missing data in the food composition tables. Moreover, the use of nutritional labels for the conversion of complex commercial products (~4% of the total food items) may have led to inaccurate estimates of a few macronutrients and/or underestimation of micronutrient intakes. We were also unable to properly estimate child-specific energy requirement due to lack of a dedicated tool to assess physical activity level. In our application, we have to acknowledge that the lack of standardized cut-offs for NAR and MAR have limited our ability to distinguish between modest and severe nutritional inadequacy. Finally, we cannot exclude that inadequacy observed in the present study simply reflected a limited dietary variety [67] or a low adherence to the Mediterranean diet [68,69]. Dietary pattern analysis may provide additional insight into children's overall dietary behavior and its potential relation with nutritional adequacy.

5. Conclusions

In line with previous Italian and European studies on primary school children, the nutritional assessment of a sample of 7-year-old children enrolled in the NAC-II cohort from Friuli Venezia Giulia, Italy, has revealed an unbalanced macronutrient profile towards protein and fats and a suboptimal intake of several macro- and micronutrients. For the first time in an Italian study, our paper has explored the use of two indexes integrating the standard evaluation of nutritional adherence to DRVs. These indexes may provide the basis for targeted public health interventions. They indeed may allow to identify critical

nutrients whose intakes have to be modified or subsets of subjects to be targeted within the general population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14030515/s1, Table S1: Parents general information at delivery. Northern Adriatic Cohort II (NAC-II), 2014–2016 (N = 381); Table S2: Percentage contribution of food groups to total intake of fatty acids and cholesterol. NAC-II, 2014–2016 (N = 381); Table S3: Percentage contribution of food groups to total intake of micronutrients. NAC-II, 2014–2016 (N = 381).

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nutrients

MDPI

Review

The Effects of Nutritional Interventions on the Cognitive Development of Preschool-Age Children: A Systematic Review

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Abstract: The developing human brain requires all essential nutrients to form and to maintain its structure. Infant and child cognitive development is dependent on adequate nutrition. Children who do not receive sufficient nutrition are at high risk of exhibiting impaired cognitive skills. This systematic review aimed to examine the effects of nutritional interventions on cognitive outcomes of preschool-age children. PubMed, PsycInfo, Academic Search Complete, and Cochrane Library electronic databases were searched to identify Randomized Controlled Trials (RCTs) published after the year 2000. Studies assessing the effects of food-based, single, and multiple micronutrient interventions on the cognition of nourished and undernourished children aged 2–6 years were deemed eligible. A total of 12 trials were identified. Eight out of the twelve studies found significant positive effects on cognitive outcomes. Iron and multiple-micronutrients supplementation yield improvements in the cognitive abilities of undernourished preschool-age children. Increased fish consumption was found to have a beneficial effect in the cognitive outcomes of nourished children. On the other hand, B-vitamin, iodized salt, and guava powder interventions failed to display significant results. Findings of this review highlight the importance of adequate nutrition during preschool years, and the crucial role sufficient nutrition plays in cognitive development.

Keywords: child development; cognition; preschool; nutrition

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1. Introduction

1.1. Nutrients and Cognitive Development

Malnutrition is characterized by an imbalance between a person's nutrient requirements and their nutrient consumption, and includes conditions of overnutrition and undernutrition [1–3]. Undernutrition is caused by an inadequate intake of energy, protein, or vitamins and minerals [2], and is a present-day global problem hindering the development of young children [4–9]. For young children, undernutrition can cause emaciation, stunting, and wasting, or various micronutrient deficiencies [1–4]. Worldwide, 149 million children are stunted and 45 million are wasted [4]. Inadequate protein and energy intake in childhood is directly associated with reduced growth, and is indicative of several psychosocial problems later in life [3,10]. Undernourished children also exhibit impaired development and decreased functional capacity [10]. Pediatric undernutrition is characterized by a lack of adequate weight gain, low weight per height, or low weight per length, and is a direct contributor to impaired cognitive skills [11–13].

The human brain requires all essential nutrients, including protein, fats, carbohydrates, vitamins, minerals, and water, to form and maintain its structure. Therefore, adequate nutrition is essential for brain development and function [14–16]. However, micronutrients, such as iron, zinc, choline, iodine, folate, B12, and long-chain polyunsaturated fatty

acids (LC-PUFAs) have been identified to be particularly relevant to cognitive development [14]. Iron is essential for the development of neurological pathways in the brain that influence brain function [15,17–19]. During the first two years of life, children experience rapid growth, which increases their iron requirement and places them at a higher risk for iron deficiency anemia [20]. Iron deficiency or iron deficiency anemia can negatively impact overall intelligence and cognitive development, especially if it occurs in early childhood [15,19,21]. Zinc is an essential trace mineral present in the brain that contributes to cerebral structure and function [22]. Zinc deficiency during infancy is associated with motor development delays [23] and detrimental effects on attention and short-term memory [15]. Long-term zinc deficiency is associated with stunting [24,25]. Choline is essential for the structural integrity of cell membranes and myelination [9,26,27]. Animal studies have shown choline deficiency to adversely impact memory [15,28,29]. Yet, the effects of choline on cognition in humans are still not fully understood. Iodine is an essential mineral for thyroid hormone synthesis and is required for brain development [15]. Iodine deficiency can have detrimental effects on cognitive function, and is the primary cause of intellectual disability around the world [15,30]. Folate is a water-soluble vitamin needed for DNA and RNA synthesis and the formation of the nervous system [15,31,32]. Maternal folate deficiency during the early stages of pregnancy is associated with an increased incidence of congenital malformations, including spina bifida and anencephaly [15,33]. Vitamin B12 is a cofactor in numerous catalytic reactions required for neurotransmitter synthesis and functioning [31,34]. Studies have linked B12 deficiency to cerebral atrophy and neurological disorders [34,35]. Vitamin A plays a critical-essential role in visual function [14,15]. LC-PUFAs, specifically docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are required for brain growth and development [15]. Inadequate intake of LC-PFAs is associated with impaired neurodevelopment, visual recognition, and memory [36–38].

1.2. Nutritional Interventions during the Preschool Years and Cognitive Outcomes

The first 1000 days of life are a crucial brain development period in which adequate nutrition is vital for optimal growth and cognitive development [39–41]. This has been identified as a sensitive time in which children are most vulnerable to behavioral and cognitive deficits [39]. A systematic review focusing on the first 1000 days of life identified the important role macronutrients, such as protein and LC-PUFAs, play in optimizing brain development [7]. Specifically, protein-energy malnutrition in early life can impede adequate brain growth, resulting in smaller brains [7]. Another review showed maintenance of adequate iron and zinc status contributes to adequate growth in early life, as significant positive effects were seen on child weight-for-age *z*-score (WAZ) and weight-for-height *z*-score [42]. Furthermore, maternal or child supplementation with choline has also been shown to support normal brain development [43]. Since the identification of the first 1000 days of life as a crucial cognitive development period, policy makers have placed strong emphasis on implementing nutritional policies that promote the healthy brain development of infants and toddlers [7]. However, public policy often does not extend to preschool-age children, even though the second 1000 days of life also represent a critical time in children's cognitive and behavioral growth [44]. Children experience the most dynamic developmental changes during the preschool years, and acquire important skills that contribute to school readiness [44]. In particular, working memory and attention control undergo rapid progress, having an extensive impact on children's academic achievement in later years [44,45]. Cognitive development reached in preschool years often predicts later achievements in life [45–47]. However, few articles have explored the effects of nutritional interventions on the cognitive outcomes of preschool-aged children. Children who do not receive adequate nutrition and psychosocial stimulation are likely to underperform in school and to have poor levels of cognition and education, which are linked to low-income earnings later in life [46,48,49]. This systematic review aimed to synthesize and evaluate the impact of nutritional interventions on the cognitive outcomes of preschool-aged children. The effects of food-based, single, and multiple micronutrient supplementation interventions

were considered, in order to explore the correlation between nutrition interventions and cognitive performance.

2. Methods

This systematic search of scientific literature was conducted in May 2021, following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [50]. The search strategy was developed in collaboration with an agriculture and forest resources librarian and with the guidance of the Population, Intervention, Comparators, Outcome (PICO) framework [51,52] (Table A1). Figure 1 illustrates the PRISMA flow diagram portraying the stream of evidence in different phases of this review.

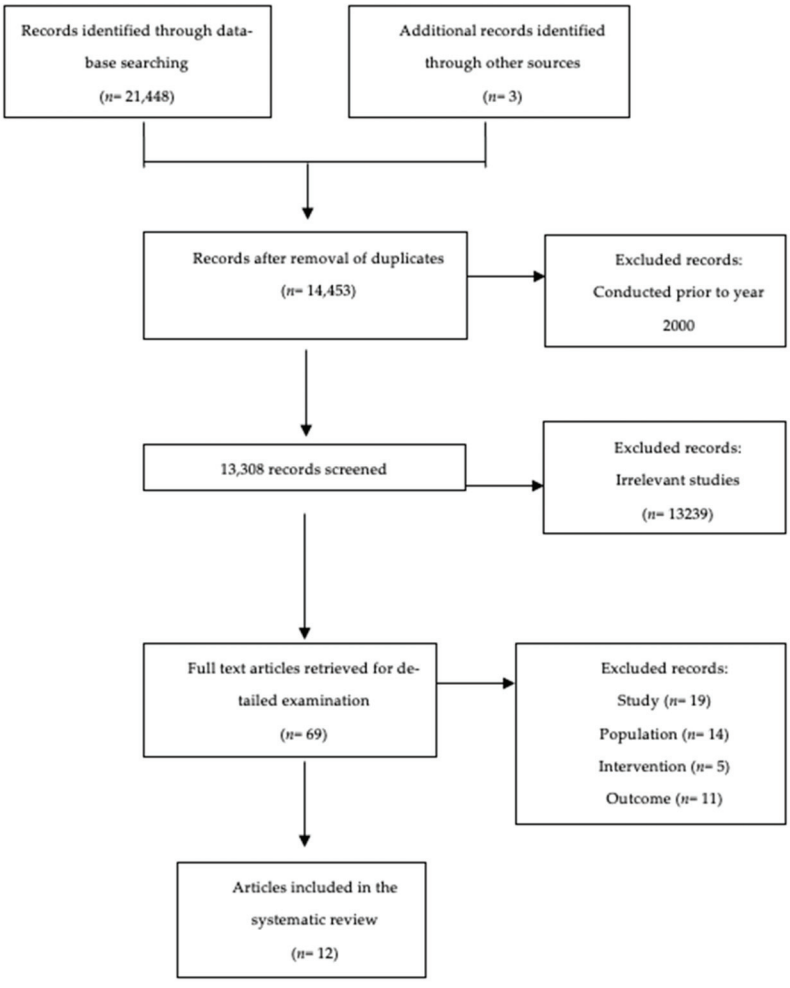

Figure 1. Study Selection Flow Diagram.

2.1. Search Strategy

The authors used PubMed, PsycInfo, Academic Search Complete, and Cochrane Library electronic databases. RCTs performed between 2000 and 2021 were retrieved. The following search terms were used: (nutrient* OR nutrition* OR micronutrient* OR macronutrient* OR diet* OR "meal diversity" OR "food intake") AND ("child development"

OR cognition OR focus OR brain OR attentiveness OR attention OR memory OR verbal OR vocabulary OR learning OR literacy OR neuro* OR problem-solving OR reasoning OR "school performance" OR school) AND (child* OR preschool* OR "school age" OR school-age). Search terms were adjusted for each database to optimize the record retrieval process (Table A2) with the guidance of the Cochrane highly sensitive search strategies for identifying randomized trials [53].

2.2. Data Extraction

One author worked autonomously to determine the eligibility of titles and abstracts retrieved by the initial computerized search. Two other authors worked independently to evaluate qualified full-text records. Eligibility conflicts between reviewers were solved through discussions and with the assistance of a fourth reviewer. A backward search was conducted, in which reference pages of eligible articles were reviewed to ensure no pertinent studies were ignored in this review. Inclusion criteria consisted of: RCTs conducted after the year 2000, and that assessed cognitive outcomes of subjects aged 2–6 years consequent to food fortification, supplementation, or food-based interventions. Trials featuring secondary interventions including psychosocial stimulation and anthelmintic treatment combined with nutritional interventions were deemed eligible if cognitive function was a designated primary outcome. No language, geographical, or study duration restrictions were imposed. Exclusion criteria comprised: cross-sectional studies, non-randomized controlled trials, small sample size RCTs (<60 subjects), trials conducted in disease-specific population, studies focusing on children older than 6 years or younger than 2 years of age, trials providing dietary interventions targeted to the first 1000 days of life, and those that only provided parent nutrition education as the nutritional intervention. Table 1 includes a detailed description of the inclusion and exclusion criteria.

Table 1. Inclusion and Exclusion Criteria.

Criteria	Study Design	Population	Intervention	Outcome
Include	RCTs conducted after the year 2000	Preschool Children (2–6 years of age)	Nutritional intervention including food-based, single, and multiple micronutrient supplementation intervention/s	Cognitive outcomes measured using cognitive assessment tests
		Healthy children and children suffering from undernutrition, anemia, parasitic infections, or HIV	Nutritional intervention/s provided after the first 1000days and in children <6 years of age	Cognition was measured after the first 1000 days or in children <6 years of age
	All other study designs and animal studies RCTs with a sample size <60 subjects	Newborns, infants, primary school-aged children, adolescents, adults, elderly	Nutritional intervention/s not provided to preschool-aged children	Cognitive outcomes not measured in preschool-aged children
Exclude		Children with specific diseases, such as cystic fibrosis, attention deficit hyperactivity disorder (ADHD), epilepsy, phenylketonuria, autism, and gluten-related neurological disorders		

2.3. Risk of Bias

Retrieved studies were assessed by two reviewers independently using the Quality Criteria Checklist (QCC) for Primary Research from the American Dietetic Association [54]. The risk of bias tool includes four questions on relevance and ten questions on validity to appraise the appropriateness of study designs and the quality of how the studies were conducted [54]. The items assessed by the QCC include the research question, subject selection, comparability of groups, withdrawals, blinding, intervention/exposure, outcomes, analysis, conclusion support, and the likelihood of bias. Each item was classified as "Yes", "No", or "Unclear." Studies were classified as negative (−) if six or more validity questions were answered as "No." A positive (+) sign was assigned if the first four items and most

answers were "Yes." If the answer to any of the first four items was "No" and other items indicated strengths, the study was classified as neutral (Ø).

3. Results

3.1. Selection of Studies

The comprehensive database search resulted in 14,453 records once duplicates were removed. After reviewing titles and abstracts, 13,239 irrelevant studies were removed. A total of 69 full-text articles were further assessed. This systematic review identified 12 RCTs that met the inclusion criteria, of which three articles were identified through a backward search of the reference list of included publications. All selected studies were published in English.

Due to the high heterogeneity among studies, a meta-analysis was not deemed appropriate for this review. Specifically, variations in the way cognitive outcomes were defined and measured, as well as differences in the type and length of nutritional interventions, created major interpretative challenges.

3.2. Description of Studies

Table 2 summarizes the characteristics of RCTs contained in this review. In summary, 50% of trials were conducted in developed countries [55–60] and 50% in low and middle-income countries (LMICs) [61–66]. Four experimental studies were conducted in rural areas with low socioeconomic status [61,62,64,66], and six studies in urban areas with high socioeconomic status [55–60]. The earliest trial was completed in 2004 [58] and the latest in 2020 [65]. The shortest intervention was implemented for two months [58], and the longest trial lasted ten months [61].

Table 2. Overview of the twelve RCTs exploring the effects of nutritional interventions on the cognitive development of preschool age children.

Reference, Year, Country	Sample Size	Age	Subject Characteristics at Baseline	Intervention Group/s	Control Group/s	Duration	Cognitive Tests	Cognitive Domain Assessed	Major Cognitive Outcomes
Rauh-Pfeiffer et al. [55], 2014, Germany	250	4–6 y	Urban area, high socioeconomic status, healthy children with low but not insuficient total folate catabolite concentrations (<34 nmol/mmol creatinine)	Children received flavorless powder containing folic acid (220 µg), riboflavin (1.1 mg), pyridoxine (0.73 mg), cobalamin (1.2 µg) and calcium lactate pentahydrate (130 mg)	Children received flavorless powder in sachets matching the intervention product in taste and appearance containing only 130 mg of calcium	3 months	WPPSI-III & (K-ABC)	Verbal I.Q., short-term memory, and processing speed	No significant difference between groups
Aboud et al. [61], 2017, Ethiopia	1602	4–6 y	Rural, low socioeconomic status, two control and four intervention districts had high UIC levels at baseline	Children had access to iodized salt for 8 to 10 months. Children received iodized salt via assistance from regular salt distributors	Children had access to non-iodized salt for 4 months, and 4 to 6 months of iodized salt. Iodized salt was introduced by market forces	10 months	WPPSI	Verbal and nonverbal reasoning, and school readiness	No significant difference between groups
Demmelmair et al. [56], 2019, Germany	205	4–6 y	Urban area, high socioeconomic status, healthy children	Children received three meals weekly containing 50 g Atlantic salmon per meal	Children received three meals weekly containing 50 g of meat per meal	4 months	WPPSI-III & 9-HPT	Fine-motor skills, verbal reasoning, vocabulary, word and matrix reasoning, picture concepts, processing speed, coding, and symbol search	Intervention children displayed superior outcomes in WPPSI-III FIQ and PIQ. No significant changes were found in the WPPSI-III I.Q. scale scores between groups
Choudhury et al. [62], 2021, India	352	3–5 y	Rural, low socioeconomic status, ICDS beneficiaries only, and children with hemoglobin concentration >7 g/dL	Children received 25 g of guava with a supplementary meal (guava group) or 25 g of banana with a meal (banana group)	Children did not receive any fruits with the meal (cucumber was given with meal if caregivers of participants wished)	8 months	MSEL	Visual reception, expressive language development, and fine-motor coordination	No significant difference between groups

Table 2. *Cont.*

Reference, Year, Country	Sample Size	Age	Subject Characteristics at Baseline	Intervention Group/s	Control Group/s	Duration	Cognitive Tests	Cognitive Domain Assessed	Major Cognitive Outcomes
Øyen et al. [57], 2018, Norway	232	4–6 y	Urban area, high socioeconomic status, healthy children	Children received three lunch meals per week with fatty fish (herring/mackerel), with a mean (SD) of 15.2 (14.2) mg/g EPA + DHA	Children received three lunch meals per week with meat (chicken/lamb/beef) with mean (SD) of 0.21 (0.15) mg/g EPA + DHA	4 months	WPPSI-III & 9-HPT	Fine-motor skills, verbal reasoning, vocabulary, word and matrix reasoning, picture concepts, processing speed, coding, and symbol search	Intervention children improved speed of processing and fine-motor coordination in a sub-analysis adjusting for dietary compliance. No significant difference was found between in main analysis of total I.Q. scores (WPPSI-III)
Schneider et al. [63], 2018, Indonesia	192	3–5 y	Urban, an upper middle-income country, children with a below-average level of stimulation at home, normal cognitive development, and weight for height within 2 SD from the median z-score	Children consumed milk powder (477.7kcal) fortified with zinc (8), iron (11.4), magnesium (141), thiamin (1), niacin (11), pyridoxine (1.7), biotin (0.0177), Vitamin C (97.3), AHA (556.6) mg/100 g, and performed psychosocial stimulation 3 times a week	Children consumed 72 g of unfortified skimmed milk powder diluted in 180 mL of warm water (467.8 kcal) and did not receive psychosocial stimulation	6 months	WPPSI-IV, CBCL 1.5–5 & PICCOLO	Cognitive functioning, cognitive development, memory, language, psychomotor skills, problem-solving, and attention	Children in the intervention group displayed increased cognitive performance and full-scale I.Q. composite score (WPPSI-IV), and reduction in attention problems (CBCL 1.5–5)
Metallinos-Katsaras et al. [58], 2004, Greece	124	3–4 y	Urban, high-income country, children with birth weight $\geq$ 2500 g, I.Q. $\geq$ 1 s.d. below the age-adjusted mean, blood Pb $\leq$ 200 ppb, weight and head circumference for the age $\geq$ 10th percentile	Children received 15 mg of iron and a multivitamins supplement (MV) five days per week at their respective day care center	Children received only the multivitamins supplement (MV) five days per week at their respective day care center	2 months	Simple reaction time test, CPT & O.L. tasks	Speed of information processing, speed of discrimination, the accuracy of discrimination, and rate of conceptual learning	Iron-deficient children who received iron supplementation showed 14% increase in discrimination speed and 8% improvement in the accuracy domain. No effects in cognitive functioning were seen in good iron status children
Ogunlade et al. [64], 2011, South Africa	151	3–6.5 y	Urban, low socioeconomic status, children with Hb $\leq$ 12.5 g/dl, all children received anthelmintic	Children consumed 35 g of stiff maize-meal porridge with added micronutrient powder (8 g) containing amylase-rich light malted barley flour 5 days per week	Children consumed 28 g of soft maize-meal with added placebo powder (8 g) 5 days per week	2.7 months	MPI, KABC-II, Atlantis & NVI	Learning abilities, sequential and simultaneous processing, and intellectual functioning	Intervention children showed significantly higher conceptual thinking abilities, higher MPI and NVI scores
Ryan and Nelson [59], 2008, USA	175	4 y	High-income country, healthy children consuming <6 oz of fish per week, English speakers, between 10th and 95th percentiles for weight and height, and currently not taking LC-PUFA supplements or consuming LC-PUFA fortified foods	Children received 400 mg of DHA supplementation as two 200-mg bubblegum-flavored softgel chewable	Children received capsules or placebo of high-oleic sunflower oil supplied as 2 soft capsules	4 months	PPVT & kCPT	Memory, attention, vocabulary, processing speed, response time, listening skills, and verbal ability	There was no significant difference between groups in Leiter-R Test of Sustained Attention, (PPVT), Day-Night Stroop Test, and (kCPT). Regression analysis showed a significant positive association between levels of DHA in capillary whole blood and improved listening comprehension and vocabulary (PPVT)
Black et al. [65], 2021, India	321	3–5 y	Rural, low socioeconomic status, children living in a district with prevalence of anemia >70%, >50% of children consumed <50% of the recommended intake of several essential micronutrients, high-quality and low-quality preschools were included	Children received 300g of cooked food fortified with MNP (13 mg iron, 5 mg zinc, 20 µg folic acid, 150 µg vitamin A, 20 mg vitamin C, 0.5 µg vitamin B-12, and 0.5 mg riboflavin	Children received 300 g of cooked food containing 0.5 mg riboflavin (no effects on outcome measures)	8 months	MSEL & BSID-III	Fine motor skills, gross motor skills, visual reception, receptive language, expressive language, and social-emotional behaviors	For children attending low-quality preschools, MNP fortification improved expressive language and marginally improved inhibitory control and social-emotional development in comparison to children attending control low-quality preschools. MNP fortification did not impact any area of cognitive development in children attending high-quality preschools
Kvestad et al. [60], 2018, Norway	232	4–6 y	High-income country, healthy children with no food allergies	Children received lunch meals containing 50–80 g of fatty fish (herring/mackerel) three times per week	Children received lunch meals containing 50–80 g of meat (chicken/lamb/beef) three times per week	4 months	WPPSI-III	Information, vocabulary, block design, word and matrix reasoning, picture concepts, coding, and symbol search	No significant difference between groups

Table 2. *Cont.*

Reference, Year, Country	Sample Size	Age	Subject Characteristics at Baseline	Intervention Group/s	Control Group/s	Duration	Cognitive Tests	Cognitive Domain Assessed	Major Cognitive Outcomes
Roberts et al. [66], 2020, Guinea-Bissau	1059	1.3–7 y	Rural, low socioeconomic status, children living in one of the 10 rural villages in the Oio and Cacheu regions of Guinea-Bissau, children with severe acute malnutrition or relevant food allergies were excluded from the study	One group of children received NEWSUP (≈310 kcal) for breakfast as a raw paste containing 98% of recommended daily micronutrients for children under 4 y, the second group received FBF (≈310 kcal) for breakfast served as a corn soy blend with cooked porridge, fortified oil, sugar, and salt containing an average of 16% recommended daily micronutrients	Children received white rice cooked in water, soybean oil, and salt (≈310 kcal) containing an average of 1% of recommended daily micronutrients	5.7 months	Working Memory Task Test	Working memory	Intervention children younger than 4y receiving NEWSUP displayed increased working memory compared to control children

Randomized Controlled Trials (RCTs); Wechsler Pre-school and Primary Scale of Intelligence (WPPSI); Kaufman Assessment Battery for Children (KABC); Intelligence Quotient (I.Q.); Urine Iodine Concentration (UIC); 9-Hole Peg Test (9-HPT); Full Scale I.Q. (FIQ); Performance Intelligence Quotient (PIQ); Integrated Child Development Services (ICDS); Mullen Scales of Early Learning (MSEL); Standard Deviation (SD); Eicosapentaenoic acid (EPA); Docosahexaenoic acid (DHA); Calories (kcal); Child Behavior Checklist (CBCL); Parenting Interactions with Children: Checklist of Observations Linked to Outcomes (PICCOLO); Multivitamin (MV); Cognitive Performance Test (CPT); Oddity Learning (O.L.); Multidimensional Prognostic; Index (MPI); Nonverbal Index (NVI); Long-chain polyunsaturated fatty acids (LC-PUFAs); Polyunsaturated Fatty Acids (PUFAs); Peabody Picture Vocabulary Test (PPVT); Conners Kiddie; Continuous Performance Test (kCPT); Bayley Scales of Infant Development (BSID); Multiple-micronutrient (MMN); Point-of-use multiple micronutrient powder (MNP). y: years old.

3.3. Study Quality

Six studies had an overall low risk of bias [55,56,59,60,65,66] and six had a moderate risk of bias [57,58,61–64] as indicated in Table 3. Five studies did not record methods of handling withdrawal [57,58,61–63] and two studies [57,64] reported moderate differences between study groups at baseline.

Table 3. Quality Criteria Checklist (QCC; risk of bias) assessment of each study included in the review [55–66].

First Author, Year of Publication (Reference)	Rauh-Pfeiffer, 2014 [55]	Demmelmair, 2019 [56]	Ogunlade, 2011 [64]	Choudhury, 2021 [62]	Black, 2021 [65]	Kvestad, 2018 [60]	Roberts, 2020 [66]	Katsaras, 2004 [58]	Aboud, 2017 [61]	Øyen, 2018 [57]	Schneider, 2018 [63]	Ryan, 2008 [59]
Primary Research QCC												
1. Was the research question clearly stated?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2. Was the selection of study subjects/patients free from bias?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3. Were study groups comparable?	Y	Y	N	Y	Y	Y	Y	Y	N	Y	Y	Y
4. Was method of handling withdrawals described?	Y	Y	Y	N	Y	Y	Y	N	N	N	N	Y
5. Was blinding used to prevent introduction of bias?	Y	N	Y	Y	Y	Y	N	Y	N	Y	N	Y
6. Were intervention/exposure factor or procedure and any comparison(s) described in detail?	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
7. Were outcomes clearly defined and the measurements valid and reliable?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
8. Was the statistical analysis appropriate for the study design and type of outcome indicators?	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y
9. Were conclusions supported by results with biases and limitations taken into consideration?	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
10. Is bias due to study's funding or sponsorship unlikely?	Y	Y	Y	Y	Y	Y	Y	?	Y	Y	N	Y
OVERALL QUALITY	(+)	(+)	(Ø)	(Ø)	(+)	(+)	(+)	(Ø)	(Ø)	(Ø)	(Ø)	(+)

Plus/positive (+); neutral (Ø).

3.4. Study Participants

The study populations were comprised mostly of preschool-age healthy children. However, children with insufficient folate levels, children at risk of undernutrition and micronutrient deficiencies, children with anemia, and children receiving anthelmintic medication were also included.

3.5. Nutritional Interventions

Supplement-based interventions were adopted in five out of twelve studies. These interventions included guava supplementation [62], DHA tablets [59], iron supplementation [58], B vitamin sachets [55], and iodized salt [61]. Multiple-micronutrient (MMN) food fortification interventions were implemented in three studies. For MMN interventions, the fortification was added to maize-porridge [64], rice, or wheat [65], and provided as a raw paste [66]. A total of three studies included food-based nutritional interventions in which subjects consumed fatty fish with daily meals [56,57,60]. Finally, one study conducted a dietary intervention in the form of a fortified milk powder [63], combined with cognitive stimulation.

3.6. Cognitive Tests

A diverse range of standardized cognitive tests were used to assess outcomes in multiple cognitive domains, including learning abilities, verbal reasoning, intellectual functioning, information processing speed, vocabulary, word reasoning, speed and accuracy of discrimination, fine and gross motor skills, coding, symbol search, and working memory. Half of the studies administered the Wechsler Pre-school and Primary Scale of Intelligence (WPPSI) test [55–57,60,61,63], two experimental studies administered the 9-Hole Peg Test (9-HPT) [56,57], and two studies administered the Mullen Scale of Early Learning (MSEL) [59,62]. Table 2 includes a detailed description of cognitive tests used in each trial, cognitive domains assessed, and effects on cognitive outcomes of preschool-aged children.

3.7. Major Cognitive Outcomes

3.7.1. Single Nutrient Supplementation

Five RCTs measured the effect of supplement-based interventions on children's cognition. Three trials failed to find a significant impact on cognition: the B vitamin [55], iodized salt [59], and guava [62] supplementation interventions. Guava is a fruit high in several vitamins and minerals and is also rich in lycopene, a carotenoid phytonutrient known for its antioxidant effect [67]. Guava was used as an intervention due to its high vitamin C content and its effects in facilitating nonheme iron absorption [68]. Although Guava supplementation yielded significant improvements in the iron status of children, no significant effects were seen in cognitive function [68]. Out of the two trials that found significant results, one of them was the iron intervention [58], which found that for children with iron deficiency anemia, iron supplementation increased accuracy and the speed of discrimination on the continuous performance task; however, for children with adequate iron status at baseline, iron supplementation did not affect performance on the continuous processing task. The DHA supplementation intervention [59] did not find any significant differences in cognitive function scores between the intervention and placebo groups; however, higher blood DHA levels were significantly associated with higher scores on the Peabody Picture Vocabulary Test, which measures listening comprehension and vocabulary.

3.7.2. Multiple-Micronutrient Supplementation

Three RCTs measured the impact of multiple-micronutrient food fortification on children's cognitive development. In the 11-week intervention consisting of eight grams of a point of use multiple micronutrient powder added to maize-meal porridge at breakfast for children 36–79 months of age [64], children in the intervention group significantly increased their scores on the simultaneous scale and the non-verbal index of the Kaufman Assessment Battery for Children compared to the control group. Conversely, in another longer trial where the children were somewhat younger [65], the cognitive benefits of a point of use multiple micronutrient powder were dependent upon whether the child was attending a low-quality or a high-quality preschool. Here, the quality of preschools was assessed by combining two validated scales, the Early Childhood Environment Rating Scale-Revised and the Home Observation for the Measurement of the Environment (HOME) adjusted for teachers rather than parents. Preschool quality was determined by two psychologists who

independently assessed preschools as low or high-quality based on playing space, learning opportunities available to children, organization of environment, teacher-child interaction levels, and involvement levels of preschool-age children to activities and practices offered. Children from low-quality preschools who received the micronutrient powder fortification displayed improvements in expressive language and some, but to a lesser degree, improvement in inhibitory control and social-emotional development, whereas children attending high-quality pre-schools showed no improvements in cognitive outcomes. In the New Multicomponent Supplementary Food (NEWSUP) study [66], children 15–48 months of age were given a unique supplementary food that contained not just multiple micronutrients, but also plant polyphenols, omega-3 fatty acids, and protein for 23 weeks. Among the children younger than four years of age, the intervention group significantly increased working memory compared to the control group. However, among children 4–7 years of age who were administered the same intervention, no improvements in working memory were seen.

3.7.3. Food-Based Interventions

Three RCTs evaluated the impact of food-based interventions on children's cognition. All trials involved the provision of meals containing fatty fish. Among children aged 4-6 years enrolled in kindergartens in Germany, children who consumed Atlantic salmon three times per week for 16 weeks saw modest improvements in two indicators of non-verbal fluid intelligence that were greater than the improvements seen in the control group who received beef in place of salmon [56]. The salmon intervention did not improve total IQ scores, but the slight improvement in raw scores over the beef group in two subcomponents suggests that provision of fatty fish may offer benefits to certain specific aspects of cognitive function. In the FINS-KIDS trial, a similarly designed study that used the same measurements of cognitive function as the aforementioned study (the WPPSI-III and the 9-HPT), provision of herring and mackerel to 4–6-year-old children in Norway increased total raw scores on the WPPSI-III when analyses were adjusted for dietary compliance [57]. When looking at individual sub-tests of the WPPSI-III, the fatty fish group showed improved performance on three of the sub-tests: the symbol search test, which is one of the sub-tests that showed improved scores in the Atlantic salmon trial, and the vocabulary and block design sub-tests. The FINS-KIDS trial also examined whether there was any association between changes in total hair mercury concentrations after the fatty fish intervention and cognition [60] and found that while the herring/mackerel intervention did increase mercury levels, the values remained below a level of concern and were not associated with cognitive function.

3.7.4. Effects of Nutritional Intervention Combined with Psychosocial Stimulation

One RCT [63] measured the impact of dietary intervention combined with psychosocial stimulation on children's cognitive development. This trial was conducted in Indonesia and included children 3–5 years of age who had a below-average level of stimulation in the home. Children receiving a fortified milk powder supplement who performed psychosocial stimulation activities three times per week [63] demonstrated a larger increase in the full-scale IQ composite score component of the WPPSI-IV compared to the control group; however, none of the IQ sub scores were different between the intervention and control group. Parents of children in the experimental group additionally reported a larger reduction in attention problems compared to that reported for children in the control group.

4. Discussion

This review suggests nutritional interventions significantly improve cognitive outcomes of undernourished preschool-age children. Trials conducted in LMICs demonstrated that nutrient-deprived children who received dietary interventions consistently showed improvements in cognition. However, caution should be taken when interpreting findings due to the clinical heterogeneity of eligible studies. Metallinos-Katsaras et al. [58] suggests

preschool children with anemia who receive iron supplementation can process information faster while making fewer mistakes. These findings are consistent with another systematic review [69]. According to Roberts et al. [66], nutrient-deprived children in the intervention group of the NEWSUP study showed improved working memory. Ogunlade et al. [64] stated that undernourished children who consumed point-of-use fortification displayed superior information analysis and problem-solving skills and improved mental processing abilities. Black et al. [65] noted that children attending low-quality preschools who received a multiple micronutrient powder with their meals improved expressive language. Considering the detrimental effects of nutrient deficiencies on children's cognitive, social, and emotional skills, multiple micronutrient supplementation is a promising intervention to re-establish nutrient balance and to increase the developmental potential of undernourished preschool-age children. Although the first 1000 days of life are well-established as the most critical period for brain development and growth in a child's life, this review suggests the second 1000 days to also be a critical period in cognitive development. Thus, preschool-age children at risk for nutrient deficiencies who receive dietary intervention can further develop their cognitive abilities. Although promising outcomes can be achieved from nutrient supplementation interventions, operational, monetary, and sustainability challenges might hinder supplement-based trial design and implementation. Additionally, multiple-micronutrient fortification intervention trials discussed in this review yielded positive effects on cognition in the short-term, and little research exists regarding the long-term efficacy of such interventions.

This review indicates well-nourished children benefit from increased fish consumption. Demmelmair et al. [56] found that healthy children in the intervention group who consumed fish increased their full-scale IQ scores from baseline, and Øyen et al. [57] found that children displayed improved processing speed abilities with fish consumption. The benefits of increased fish consumption on the cognitive development of preschool-age children identified in this review are consistent with the findings of another review [70]. On the other hand, three studies that implemented single nutrient supplement-based interventions, B vitamin sachets [55], iodized salt [61], and guava [62] showed no significant benefits associated with supplementation on cognitive outcomes of preschool-aged children. Of the studies included in this review, only 25% measured the effect of real food interventions on children's cognition, and of these, all were conducted in high-income countries and solely interested in the impact of fatty fish and DHA on children's cognition.

To our knowledge, there were no trials designed to explore the benefits of increased vegetable and fruit consumption on the cognitive performance of preschool-age children. Furthermore, no study measured the effect of improved dietary diversity on preschoolers' cognitive development. Dietary diversity is adequate when a child's diet contains five or more of the eight recommended food groups, including breast milk, grains, roots and tubers, legumes and nuts, dairy products (milk, yogurt, cheese), flesh foods (meat, fish, poultry, liver, or other organs), eggs, vitamin A-rich fruits and vegetables, and other fruits and vegetables [53,71]. Dietary diversity is the preferred approach to improving the nutrition of a population, as it is the most sustainable and desirable approach [71]. Therefore, future trials must include interventions intended to explore the effects of increased dietary diversity on the cognitive development of young children.

Additionally, further evidence is needed to determine whether nutritional interventions combined with psychosocial stimulation result in superior cognitive outcomes. For example, Schneider et al. [63] suggested that nutritional supplementation with low-level cognitive stimulation yields improved cognitive functioning in preschool-age children. However, considering as many as 200 million children in the world lack access to sufficient nutrition and adequate cognitive stimulation [5], trials that combine psychosocial stimulations and nutrient interventions on the cognitive development of preschool-age children are needed to provide more definite evidence on the impact of a combined intervention approach on cognitive outcomes.

Further research is also required to investigate the long-term effects of multiple micronutrient supplementation in cognitive outcomes of nutrient-deprived children, as short-term benefits have been consistently demonstrated. In addition, more research is needed to explore the effects of nutritional interventions combined with psychosocial stimulation on cognitive outcomes of preschool-aged children. This evidence is essential, because cognitive development reached in preschool years often determines school readiness and predicts later life achievements [5].

5. Conclusions

In conclusion, the findings of this review show that nutritional interventions have a positive effect on the cognitive development of undernourished preschool-age children. Nutrient-deficient children who receive micronutrient supplementation consistently display significant advances in cognitive outcomes. Furthermore, nourished children who increase fish consumption display improvements in cognitive abilities. This review highlights the importance of adequate nutrient intake during the second 1000 days of a child's life, and the crucial role sufficient nutrition plays in cognitive development.

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Appendix A

Table A1. PICO framework: keywords.

Population	Intervention	Comparison	Outcome
Preschool children (2–6 years of age)	Multiple-micronutrient (MMN) food fortification, supplement-based, or food-based nutritional interventions	Placebo or control group	Cognitive outcomes using cognitive assessment tests

Table A2. Search Strategy used for each electronic database.

Database	Searched Terms
PubMed	(nutrient OR nutrition OR micronutrient OR macronutrient OR diet OR dietary OR "food intake" OR "meal diversity") AND ("child development" OR cognition OR focus OR brain OR attentiveness OR attention OR memory OR verbal OR vocabulary OR learning OR literacy OR neuro* OR problem-solving OR reasoning OR "school performance" OR "school achievement" OR "academic achievement" OR "educational measurement" OR "academic success" OR "academic performance") AND (randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized [tiab] OR placebo [tiab] OR clinical trials OR randomly [tiab] OR trial [ti]) AND (child OR children OR preschool OR "pre-school")

Table A2. *Cont.*

Database	Searched Terms
CENTRAL	(nutrient* OR nutrition* OR micronutrient* OR macronutrient* OR diet* OR "food intake" OR "meal diversity") AND ("child development" OR cognition OR focus OR brain OR attentiveness OR attention OR memory OR verbal OR vocabulary OR learning OR literacy OR neuro* OR problem-solving OR reasoning OR "school performance" OR "school achievement" OR "academic achievement" OR "educational measurement") AND (child OR children OR preschool OR "pre-school")
PsycInfo and Academic Search Complete	(nutrient* OR nutrition* OR micronutrient* OR macronutrient* OR diet* OR "food intake" OR "meal diversity") AND ("child development" OR cognition OR focus OR brain OR attentiveness OR attention OR memory OR verbal OR vocabulary OR learning OR literacy OR neuro* OR problem-solving OR reasoning OR "school performance" OR "school achievement" OR "academic achievement" OR "educational measurement") AND (random* OR control* OR trial* OR placebo* OR "double-blind") AND (child OR children OR preschool OR "pre-school")

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Article

Maternal Knowledge, Attitude and Practices toward Free Sugar and the Associations with Free Sugar Intake in Children

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Abstract: Research addressing factors related to free sugar (FS) consumption among children in Saudi Arabia is lacking. We aimed to evaluate maternal knowledge, attitude, and practices toward FS and the associations with children's intake of FS. This cross-sectional study included 424 Saudi children aged 6–12 years and their mothers. Data related to maternal knowledge, attitude, and practices were collected using an online survey. Data concerning children's habitual intake of FS were collected through phone interviews using a validated food frequency questionnaire. Limited knowledge on FS was observed among mothers of children [median 7.00 [interquartile range 6.00–8.00] out of 11.0. Maternal knowledge was not correlated with maternal attitude or practices toward FS. Maternal knowledge towards FS did not predict children's intake of FS, whereas maternal attitude and practices toward limiting the consumption of FS predicted lower intake of FS among Saudi children, particularly the FS consumed from solid food sources (B: −5.73 [95% confidence interval (CI): −9.79 to −1.66]) and (B: −6.85 [95% CI: −11.9 to −1.80]), respectively. Despite the limited knowledge pertaining to FS among mothers in Saudi Arabia, they were making efforts to limit their children's consumption of FS.

Keywords: maternal; knowledge; attitude; practices; children; free sugar intake; Saudi Arabia

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1. Introduction

Excessive intake of free sugar (FS) among children has been reported in several settings [1–3]. High intake of FS has been of concern due to its negative impact on health [4,5]. Existing evidence suggests that excessive intake of sugary food replaces the consumption of important foods in the diet, such as fruits and vegetables, leading to lower intake of crucial nutrients and lower quality of diet [6–8]. Insufficient nutrient intake among children for a prolonged period of time may result in growth impairment in addition to many other long-term health consequences [9].

The theory of knowledge, attitude, and practice is important to explain health-related behaviors. The theory consists of three components: acquiring knowledge, generating a belief, and forming a behavior [10]. However, the palatability and convenience of sugary foods could also influence practices related to the consumption of FS foods [11–14]. In addition, the cost per calorie for energy-dense nutrient-poor foods is generally less, which may influence food-related decisions among individuals and result in excessive intake of FS [15,16].

National interventions have been implemented to reduce the consumption of FS among Saudis, e.g., sugary drink tax policy and food labeling regulations to limit FS consumption among the population of Saudi Arabia [17,18]. However, the problem of high consumption of FS remains despite efforts that have been made [3].

Limited knowledge of the FS recommendation is one important factor explaining the high consumption of FS [19,20]. For example, a female parent, a female child, and a higher education level were found to be linked to high recognition level of FS [19]. Understanding the link between maternal knowledge, attitude, and practices toward FS is crucial to design effective interventions aiming to reduce the consumption of FS among Saudi children.

During childhood, parents play a significant role in shaping the dietary behaviors of children. The impact of maternal feeding practices on children's dietary intake has been previously established [21,22]. Maternal adherence to a healthy lifestyle, including a high-quality diet, and the utilization of healthy feeding practices have been shown to be effective in promoting positive dietary behaviors in children [23,24]. However, research addressing factors related to the consumption of FS among children in Saudi Arabia is lacking. Hence, the present study aimed to evaluate maternal knowledge, attitude, and practices related to FS and the associations with children's intake of FS.

2. Materials and Methods

In this cross-sectional study, a minimum of 365 healthy children aged between 6–12 years and their mothers were needed based on an effect size (expected correlation coefficient between maternal knowledge and children intake of FS) of 0.20, 99.9 confidence level (two-sided test), and 90% power [25]. Exclusion criteria include children with allergies, chronic diseases, non-Saudi, sibling of a child who is already part of this study, and children who reside outside of Saudi Arabia within the past three months. Initially, we recruited 539 children and their mothers online through different social media channels (Instagram, Telegram, Twitter, WhatsApp, and Facebook). An invitation for participation was accompanied by a short online survey that collected demographic data [age of mother and child, child's sex, maternal education level, and family income per month] and assessed maternal knowledge, attitude, and practices toward FS. A phone interview was scheduled with each mother within one week of collecting the initial data. The online survey and the phone interview were all administered in Arabic. The ethical approval for this study was granted from the ethical review board of the College of Applied Medical Sciences, Taibah University (certificate no. 2020/55/202/CLN). Informed consent was obtained online from all mothers involved in the study before collecting children's data.

2.1. Assessment of Children's Intake of Free Sugar

Data concerning children's intake of FS were collected from mothers during the phone interviews. FS intake among children was assessed using a previously validated food frequency questionnaire (FFQ) that was specifically designed to assess the habitual intake of FS among children in Saudi Arabia [26]. The FFQ included 12 food groups and 40 food items. Frequency of consumption was recorded as follows: daily (6 times or more, 4–5 times, 2–3 times- once); weekly (5–6 times, 2–4 times, once); monthly (1–3 times, less than once). A template table presenting the response options (frequency of consumption) along with a guide to estimate portion sizes of food consumed was sent to mothers during the phone interview via WhatsApp/text message to improve the accuracy of responses to the FFQ. The questionnaire provided estimates concerning quantities of FS intake per day coming from liquid food sources, solid food sources, and total FS intake.

2.2. Assessment of Maternal Knowledge Related to Free Sugar

Maternal knowledge related to FS was assessed using 11 items as follows:

(1) Do you think eating too much FS is bad for your child's health?

Response options were "Yes" and "No". Mothers who responded "Yes" were awarded a score of one.

(2) Free sugar is:
   - (2.1.) Sugar added to coffee and tea;
   - (2.2.) Sugar added to food during processing or cooking;
   - (2.3.) Sugar used to prepare sweets;
   - (2.4.) Sugar exist in fruits and milk.

(3) The following food contains a large amount of free sugar:
   - (3.1.) Diet Pepsi;
   - (3.2.) Cookies;

(3.3.) Milk;
(3.4.) Fruit drinks;
(3.5.) Toast bread and buns;
(3.6.) Strawberry flavored Greek yogurt.

Mothers were instructed to select multiple responses as applicable for items included in sections two and three. Mothers who selected responses 2.1, 2.2, 2.3, 3.2, 3.4, and 3.6 were awarded a score of one for each question. A zero score per question was awarded to mothers who selected items 2.4, 3.1, 3.3, and 3.5, and a score of one was awarded for each of these items if the items were not selected. The total knowledge score was calculated (total score ranged between zero and 11), wherein a score of 11 indicated the highest level of knowledge on added sugar.

2.3. Assessment of Maternal Attitude to Limit Children's Intake of Free Sugar

Maternal attitude towards limiting children's FS intake was assessed using three items as follows:

(1) Are you trying to limit the purchase of foods that are high in free sugar?
(2) Are you trying to limit your child's intake of foods that are high in free sugar?
(3) Are you trying to provide healthy food options for your child to replace foods that are high in free sugar?

Responses to all three items were "Yes" or "No". Mothers who responded "Yes" were awarded a score of one per item, whereas a score of zero was awarded if mothers responded as "No" to any of the items. The total attitude score was later calculated (total scores ranged between zero and three).

2.4. Assessment of Maternal Practices to Limit Children's Intake of Free Sugar

Maternal practices related to limiting children's intake of FS were assessed using positive behaviors, and consisted of three items as follows:

(1) How often do you read the nutrition fact label of your child's favorite products to determine the amount of free sugar intake?

Responses to this item were "Always" (awarded two scores); "Sometimes" (awarded one score); "Never" (recorded as a zero score).

(2) Are you discussing with your child the importance of replacing foods that are high in free sugar with healthy food options?

Responses to this item were "Yes" (awarded one score) and "No" (recorded as a zero score).

(3) Mother successfully limits/controls her child's free sugar intake (assessed using the FFQ).

A score of one was awarded if the mother successfully limits/controls her child's intake of FS to meet the recommendation of the World Health Organization (WHO) and the Ministry of Health in Saudi Arabia (MOH) (<25 g of FS per day) [27,28], whereas a score of zero was recorded if the mother did not limit/control her child's intake of FS to meet the WHO/MOH recommendation ($\geq$25 g of FS per day).

Scoring of maternal practices toward limiting children's consumption of FS ranged between zero and three.

2.5. Statistical Analyses

Data for continuous variables included in this study are presented as median [interquartile range (IQR)] and mean $\pm$ standard deviation (SD), while data for categorical variables are presented as frequency and percentage (%). The Shapiro–Wilk test was used to assess the normality of all continuous variables. Data of maternal knowledge, attitude, and practices toward FS were skewed; thus, non-parametric tests were used to analyze data presented in this study. Spearman's correlation test was used to assess the correlations between maternal knowledge, attitude, and practices toward FS. The Mann–Whitney and

Kruskal Wallis tests were used to explore differences in mean scores of maternal knowledge, attitude, and practices toward FS within the categorical variables. Pairwise comparisons were performed to further investigate the significant associations found in the Kruskal Wallis test. Multiple linear regression analyses were performed to investigate if maternal knowledge, attitude, and practices toward FS predict children's intake of FS after adjusting for children's age and sex. Tests used in this study were two-tailed with a significance level of 95%. We corrected for multiple testing using the Bonferroni adjustment method when the significance of the pairwise comparisons were assessed. The SPSS (version 20, SPSS, Inc., Chicago, IL, USA) was used for data analysis.

3. Results

3.1. Characteristics of the Study Sample

Children with allergies and chronic diseases were excluded (n = 36, 6.68%). A total of 79 mothers (14.7%) were excluded due to missing dietary data (no phone interview was conducted) for one of the following reasons: (1) mother did not provide her contact information; (2) mother did not respond to text messages and reminders to schedule the phone interview to collect data concerning child's FS intake. The final analysis of this study included data of 424 children and their mothers. Detailed data of the characteristics of children and their mothers are presented in Table 1.

Table 1. Characteristics of children and their mothers (n = 424).

Variable	*n*	%
Region of residency		
Western region	242	57.1
Central region	56	13.2
Eastern region	53	12.5
Other regions	73	17.2
Age		
6–7 years	144	34.0
8–9 years	126	29.7
10–12 years	154	36.3
Sex		
Boys	210	49.5
Girls	214	50.5
Order of child		
Older child	139	32.8
Middle child	135	31.8
Younger child	128	30.2
Only child	22	5.20
Maternal age		
≤30 years	76	17.9
31–40 years	239	56.4
>40 years	109	25.7
Maternal education level		
≤High school	105	24.8
University degree	270	63.7
Postgraduate degree	49	11.6

Table 1. *Cont.*

Variable	*n*	%
Maternal employment status		
Employed	173	40.8
Unemployed	251	59.2
Family income per month in SR [1]		
<4000	29	6.80
4000–10,000	166	39.2
>10,000	229	54.0

[1] SR: Saudi Riyal ($1 = SR 3.75).

3.2. Maternal Knowledge, Attitude, and Practices toward Free Sugar

Items included to assess maternal knowledge, attitude, and practices toward FS are provided in Table 2. The median of knowledge was 7.00 (6.00–8.00) out of 11.0. The median score of maternal attitude to limit children's intake of FS was 2.00 (1.00–3.00) out of a total score of 3.00. The median score of maternal practices to limit children's intake of FS was 2.00 (2.00–2.00) out of a total score of 4.00.

Table 2. Maternal knowledge, attitude, and practices toward free sugar (*n* = 424).

	Item	*n*	%
	Maternal knowledge toward free sugar		
1	Do you think eating too much free sugar is bad for your child's health?		
	Yes [1]	402	94.8
	No	22	5.20
2	What is free sugar?		
2.1	Sugar added to coffee and tea		
	Yes [1]	147	34.7
	No	277	65.3
2.2	Sugar added to food during processing or cooking		
	Yes [1]	302	71.2
	No	122	28.8
2.3	Sugar used to prepare sweets		
	Yes [1]	194	45.8
	No	230	54.2
2.4	Sugar exist in fruits and milk		
	Yes	29	6.80
	No [1]	395	93.2
3	The following food contains a large amount of free sugar:		
3.1	Diet Pepsi		
	Yes	254	59.9
	No [1]	170	40.1
3.2	Cookies		
	Yes [1]	344	81.1
	No	80	18.9
3.3	Plain milk		
	Yes	16	3.80
	No [1]	408	96.2
3.4	Fruit drinks		
	Yes [1]	367	86.6
	No	57	13.4

Table 2. *Cont.*

	Item	*n*	%
3.5	Toast bread and buns		
	Yes	172	40.6
	No [1]	252	59.4
3.6	Strawberry flavored Greek yogurt		
	Yes [1]	78	18.4
	No	346	81.6
	Maternal attitude to limit children's intake of free sugar		
1	Are you trying to limit the purchase of foods that are high in free sugar?		
	Yes [1]	174	41.0
	No	250	59.0
2	Are you trying to limit your child's intake of foods high in free sugar?		
	Yes [1]	289	68.2
	No	135	31.8
3	Are you trying to provide healthy food options for your child to replace foods high in free sugar?		
	Yes [1]	394	92.9
	No	30	7.10
	Maternal practices to limit children's intake of free sugar		
1	How often do you read the nutrition fact label of your child's favorite products to determine the amount of free sugar intake?		
	Always	64	15.1
	Sometimes	283	66.7
	Never	77	18.2
2	Are you discussing with your child the importance of replacing foods high in free sugar with healthy food options?		
	Yes [1]	396	93.4
	No	28	6.60
3	Mother successfully limit/control her child free sugar intake		
	Child's intake of free sugar < 25 g per day [1]	7	1.70
	Child's intake of free sugar ≥ 25 g per day	417	98.3

[1] Response awarded a score of one.

Spearman's correlation test showed a negligible correlation between maternal knowledge and attitude toward FS ($r_s = 0.20$; $p < 0.001$) and between maternal knowledge and practices toward FS ($r_s = 0.18$; $p < 0.001$). A low positive correlation was observed between maternal attitude and practices toward limiting FS intake ($r_s = 0.35$; $p < 0.001$).

3.3. Maternal Knowledge, Attitude, and Practices toward Free Sugar and the Associations with Sociodemographic Characteristics

Detailed data concerning the associations between maternal knowledge, attitude, and practices related to FS and children's characteristics are presented in Table 3. The mean maternal knowledge score towards FS was significantly different across the different regions in Saudi Arabia ($p = 0.015$). After using the Bonferroni adjustment method to correct for multiple testing, the pairwise comparisons showed a significantly higher mean of knowledge score among mothers in the Eastern region as compared to mothers in the Western region (7.83 ± 1.66 vs. 7.07 ± 1.55, respectively, $p = 0.018$). The mean maternal knowledge score toward FS was similar for all other sociodemographic variables. The mean maternal attitude score related to limiting FS intake was similar for all sociodemographic variables. The mean maternal practices score related to limiting FS intake was significantly different across regions in Saudi Arabia ($p = 0.005$). After using the Bonferroni adjustment method to correct for multiple testing, the pairwise comparisons showed significantly higher mean scores of practices among mothers in the Eastern region as compared to mothers in the Western region (2.17 ± 0.64 vs. 1.83 ± 0.73, respectively, $p = 0.005$). In

addition, unemployed mothers had a higher mean maternal practices score toward limiting the consumption of FS compared to employed mothers (2.02 ± 0.64 vs. 1.77 ± 0.72, respectively, $p < 0.001$). The mean score of maternal practices related to limiting FS intake was similar for all other characteristics of children included in this study.

Table 3. Associations between maternal knowledge, attitude, and practices related to free sugar and children's characteristics ($n = 424$).

Characteristics	Maternal Knowledge Related to Free Sugar [1] Score out of 11	Maternal Attitude to Limit Free Sugar Intake [1] Score out of 3	Maternal Practices to Limit Free Sugar Intake [1] Score out of 4
Region of residency			
Western region	7.07 ± 1.55 7.00 [6.00–8.00]	1.98 ± 0.89 2.00 [1.00–3.00]	1.83 ± 0.73 2.00 [1.00–2.00]
Central region	7.43 ± 1.35 7.50 [7.00–8.00]	2.07 ± 0.81 2.00 [1.25–3.00]	1.91 ± 0.61 2.00 [2.00–2.00]
Eastern region	7.83 ± 1.66 8.00 [7.00–9.00]	1.98 ± 0.75 2.00 [2.00–2.00]	2.17 ± 0.64 2.00 [2.00–3.00]
Other regions	7.11 ± 1.48 7.00 [6.00–8.00]	2.16 ± 0.80 2.00 [2.00–3.00]	2.03 ± 0.55 2.00 [2.00–2.00]
p-value	0.015 [3]	0.405	0.005 [3]
Age			
6–7 years	7.19 ± 1.56 7.00 [6.00–8.00]	2.00 ± 0.78 2.00 [2.00–3.00]	1.92 ± 0.67 2.00 [2.00–2.00]
8–9 years	7.42 ± 1.54 7.00 [7.00–8.00]	2.04 ± 0.88 2.00 [1.00–3.00]	1.94 ± 0.70 2.00 [2.00–2.00]
10–12 years	7.09 ± 1.52 7.00 [6.00–8.00]	2.03 ± 0.89 2.00 [1.00–3.00]	1.90 ± 0.69 2.00 [2.00–2.00]
p-value	0.497	0.122	0.114
Sex			
Boys	7.09 ± 1.57 7.00 [6.00–8.00]	2.01 ± 0.87 2.00 [1.00–3.00]	1.89 ± 0.66 2.00 [2.00–2.00]
Girls	7.35 ± 1.51 7.00 [6.00–8.00]	2.03 ± 0.82 2.00 [2.00–3.00]	1.95 ± 0.71 2.00 [2.00–2.00]
p-value	0.077	0.876	0.347
Order of child			
Older child	7.15 ± 1.56 7.00 [6.00–8.00]	2.12 ± 0.80 2.00 [2.00–3.00]	1.94 ± 0.66 2.00 [2.00–2.00]
Middle child	7.27 ± 1.50 7.00 [6.00–8.00]	2.09 ± 0.85 2.00 [2.00–3.00]	1.99 ± 0.71 2.00 [2.00–2.00]
Younger child	7.13 ± 1.50 7.00 [6.00–8.00]	1.87 ± 0.89 2.00 [1.00–3.00]	1.84 ± 0.70 2.00 [2.00–2.00]
Only child	7.86 ± 1.81 8.00 [6.00–9.00]	1.91 ± 0.75 2.00 [1.00–2.25]	1.86 ± 0.56 2.00 [2.00–2.00]
p-value	0.236	0.074	0.322
Maternal age			
≤30 years	7.41 ± 1.38 7.00 [7.00–8.00]	2.01 ± 0.77 2.00 [2.00–3.00]	1.96 ± 0.74 2.00 [2.00–2.00]
31–40 years	7.17 ± 1.60 7.00 [6.00–8.00]	2.09 ± 0.84 2.00 [2.00–3.00]	1.97 ± 0.65 2.00 [2.00–2.00]
>40 years	7.19 ± 1.52 7.00 [6.00–8.00]	1.88 ± 0.90 2.00 [1.00–3.00]	1.79 ± 0.71 2.00 [1.00–2.00]
p-value	0.497	0.122	0.114

Table 3. *Cont.*

Characteristics	Maternal Knowledge Related to Free Sugar [1] Score out of 11	Maternal Attitude to Limit Free Sugar Intake [1] Score out of 3	Maternal Practices to Limit Free Sugar Intake [1] Score out of 4
Maternal education level			
≤High school	7.18 ± 1.49 7.00 [6.00–8.00]	1.98 ± 0.75 02.00 [2.00–2.−0]	1.98 ± 0.59 2.00 [2.00–2.00]
University	7.24 ± 1.49 7.00 [6.00–8.00]	2.00 ± 0.86 2.00 [1.00–3.00]	1.90 ± 0.70 2.00 [2.00–2.00]
Postgraduate	3.16 ± 1.94 7.00 [5.50–9.00]	2.20 ± 0.93 2.00 [2.00–3.00]	1.92 ± 0.79 2.00 [2.00–2.00]
p-value	0.985	0.124	0.565
Maternal employment ststus			
Employed	3.18 ± 1.62 7.00 [6.00–9.00]	1.98 ± 0.91 2.00 [1.00–3.00]	1.77 ± 0.72 2.00 [1.00–2.00]
Unemployed	7.25 ± 1.49 7.00 [6.00–8.00]	2.05 ± 0.80 2.00 [2.00–3.00]	2.02 ± 0.64 2.00 [2.00–2.00]
p-value	0.625	0.528	<0.001 [3]
Family income per month in SR [2]			
<4000	7.00 ± 1.79 7.00 [6.00–8.00]	1.83 ± 0.85 2.00 [1.00–2.00]	1.97 ± 0.73 2.00 [2.00–2.00]
4000–10,000	7.39 ± 1.45 7.00 [6.00–8.00]	2.05 ± 0.81 2.00 [2.00–3.00]	1.95 ± 0.67 2.00 [2.00–2.00]
>10,000	7.12 ± 1.57 7.00 [6.00–8.00]	2.02 ± 0.87 2.00 [1.00–3.00]	1.90 ± 0.69 2.00 [2.00–2.00]
p-value	0.293	0.428	0.813

[1] The numbers presented are mean ± SD and median [IQR]. [2] SR: Saudi Riyal [$1 = SR 3.75]. [3] *p*-value was considered statistically significant at the 95% confidence level.

3.4. Maternal Knowledge, Attitude, and Practices Related to Free Sugar and the Associations with Children's Intake of Free Sugar

Multiple linear regression analysis was performed to investigate the association between maternal knowledge, attitude, and practices related to FS and children's intake of FS adjusting for children's age and sex (Table 4). Maternal knowledge toward FS was not found to be associated with children's intake of FS (from liquid food sources, solid food sources, and total FS from all food sources). Positive maternal attitude related to limiting FS intake predicted lower intake of FS from solid food sources in children (B: −5.73, SE: 2.07 [95% confidence interval (CI): −9.79 to −1.66], R-square = 0.02) and total FS from all food sources (B: −7.60, SE: 3.01 [95% CI: −13.5 to −1.68], R-square = 0.03), but no association with children's intake of FS from liquid food sources was observed. Positive maternal practices related to limiting FS intake predicted lower intake of FS from solid food sources in children (B: −6.85, SE: 2.57 [95% CI: −11.9 to −1.80], R-square = 0.02) and total FS from all food sources (B: −7.92, SE: 3.74 [95% CI: −15.3 to −0.56], R-square = 0.02). Maternal practices related to FS were not associated with children's intake of FS coming from liquid food sources.

Table 4. Multiple linear regression analysis of associations between maternal knowledge, attitude, and practices related to free sugar and children's intake of free sugar [1].

	Beta Estimate	Standard Error	95% Confidence Interval	*p*-Value	R-Square
Maternal knowledge related to free sugar					
Free sugar intake from liquid food sources, g/day	−0.72	0.89	−2.47 to 1.03	0.418	0.02
Free sugar intake from solid food sources, g/day	−0.70	1.16	−2.97 to 1.57	0.543	0.00
Total free sugar intake, g/day	−1.53	1.68	−4.83 to 1.76	0.361	0.01
Maternal attitude to limit children's intake of free sugar					
Free sugar intake from liquid food sources, g/day	−2.03	1.60	−5.18 to 1.12	0.206	0.02
Free sugar intake from solid food sources, g/day	−5.73	2.07	−9.79 to −1.66	0.006 [2]	0.02
Total free sugar intake, g/day	−7.60	3.01	−13.5 to −1.68	0.012 [2]	0.03
Maternal practices to limit children's intake of free sugar					
Free sugar intake from liquid food sources, g/day	−0.97	1.99	−4.89 to 2.95	0.627	0.02
Free sugar intake from solid food sources, g/day	−6.85	2.57	−11.9 to −1.80	0.008 [2]	0.02
Total free sugar intake, g/day	−7.92	3.74	−15.3 to −0.56	0.035 [2]	0.02

[1] All models were adjusted for children's age and sex. [2] *p*-value was considered statistically significant at the 95% confidence level.

4. Discussion

Limited knowledge about FS was observed among the mothers included in this study. Maternal knowledge was not correlated with maternal attitude or practices related to limiting the intake of FS. Maternal knowledge towards FS did not predict children's intake of FS, whereas maternal attitude and practices to limit the consumption of FS predicted lower intake of FS among Saudi children, specifically FS coming from solid food sources.

The limited knowledge related to FS reported among mothers in our study has also been reported in other settings. A study conducted in China showed limited maternal knowledge related to FS [19]. In Ireland, a study also shows limited knowledge related to FS among adults where 65% of participants did not know the WHO recommendation for FS [20]. In fact, knowledge regarding FS might be influenced by many factors. The current food labeling practices in the Saudi market are inadequate [29], but notable progress has been observed [30,31]. For example, the Saudi government has implemented a policy for establishing front-of-pack nutrition labeling requirements and the FS nutritional labeling requirement [18,32,33]. Clear information provided in nutrition labels can help in increasing knowledge regarding FS content in prepackaged foods. However, improving knowledge of how to read nutrition labels is needed, as limited interpretation skills of nutrition labels have been reported previously in Saudi Arabia [34]. It is noteworthy that obtaining information from creditable sources is very important, especially with the wide use of social media applications, where inaccurate or even incorrect information is posted. Previous research in Ghana conducted among young adults reported that nutrition-related information is mostly obtained from online resources, whereas the least used source was healthcare professionals, e.g., nutritionists [35]. Thus, social media has been used effectively by national and international health organizations to deliver health-related messages to the public to raise awareness and improve knowledge.

In this study, the univariate analysis revealed that unemployed mothers have better practices related to limiting children's intake of FS. This finding could be explained by the greater time availability for stay-at-home mothers. It has been noted that time constraints are the most common reasons for negative nutrition-related practices, e.g., not reading food labels [34]. Therefore, unemployed mothers who have more time on their hands can be more inclined to better nutrition-related practices, including reading food labels,

discussing with children the importance of replacing less healthy foods with healthful food options, and limiting/controlling their children's intake of FS.

Despite their limited knowledge of FS, mothers were making efforts to limit children's intake of FS. Yet, the majority of children exceeded the recommendation of the WHO/MOH. The excessive consumption of FS could be due to the affordability, palatability, and convenience of sugary foods [13,14,16]. In fact, it is evident that the sweet taste of food is preferred, particularly among children [11,12]. Our data also showed that maternal attitude and practices related to limiting FS only predicted children's intake of FS from solid food sources and total FS, but not FS coming from liquid food sources. The lack of association between maternal attitude and practices to limit children's intake of FS from liquid food sources could be due to efforts that have been made by the Saudi government to limit the intake of sugary drinks [17]. In fact, the sugary drink tax policy implemented in 2017 might result in limiting the consumption of FS from liquid food sources. Liquid food sources of FS were found to be the greatest contributor to total energy intake among many populations [1,36]. However, data regarding top food sources of FS among children in Saudi Arabia are lacking. Previous research showed high consumption of sweet snacks and candies among children and university students [37,38].

Maternal attitude and practices toward FS can influence children's intake of FS despite their level of knowledge. Efforts made by mothers to limit children's intake of FS can help in eliminating many food sources that contain high quantities of FS and replace them with healthy food options. Previous nutrition education interventions conducted among preschoolers and their parents were found to be effective in reducing children's consumption of FS and increasing the density of important nutrients, including protein, fiber, potassium, iron, and zinc [39]. However, replacing sugary foods with healthy food options, e.g., fruits and vegetables, might be more costly [15,16].

This is the first study in the region to explore maternal knowledge, attitude, and practices related to FS in relation to children's intake of FS. The FS intake was assessed using a validated FFQ that can estimate the habitual intake of FS among Saudi children. The study is limited by the convenience sampling method used. Thus, the findings of this study might be generalizable only to mothers who commonly use social media channels.

5. Conclusions

Despite the limited knowledge observed pertaining to FS among mothers in Saudi Arabia, these mothers were making efforts to limit their children's consumption of FS. Future research should be directed towards exploring other potential predictors of FS intake among Saudi children. In addition, longitudinal associations between knowledge, attitude, and practices related to FS intake should be further explored among individuals from different age groups in Saudi Arabia. Nutrition education interventions to improve maternal knowledge on FS and its food sources are urgently needed.

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Institutional Review Board Statement: The ethical approval for this study was granted from the ethical review board of the College of Applied Medical Sciences, Taibah University (certificate no. 2020/55/202/CLN).

Informed Consent Statement: Informed consent was obtained from all mothers involved in the study.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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Associations between Food Preferences, Food Approach, and Food Avoidance in a Polish Adolescents' COVID-19 Experience (PLACE-19) Study Population

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Abstract: Food preferences are among the strongest predictors of the food choices of adolescents. These are associated with appetitive traits (food approach and avoidance) to some extent. However, no research has been conducted so far analyzing the association between food preferences and appetitive traits of adolescents. The aim of this study was to evaluate the associations between food preferences and appetitive traits in adolescents (aged 15–20 years) within the Polish Adolescents' COVID-19 Experience (PLACE-19) Study population. The PLACE-19 Study was carried out in a population-based sample of 2448 secondary school students sampled across the country (random quota sampling). Food preferences (including the preference for vegetables, fruit, meat/fish, dairy, snacks, and starches) of the adolescents were assessed using the validated Food Preference Questionnaire (FPQ) while their appetitive traits (hunger, food responsiveness, emotional overeating, enjoyment of food, satiety responsiveness, emotional undereating, food fussiness, slowness in eating) were assessed using the validated Adult Eating Behavior Questionnaire (AEBQ). The k-means clustering was performed to identify the homogenous clusters of respondents based on their preferences, and linear regression was performed to determine the relationship between food preferences and appetitive traits with a model adjusted for sex and age. Based on their preferences, three homogenous clusters of respondents were defined: low-preferring respondents (low preference for all food categories), respondents preferring snacking foods (low preference for all food categories, except for fruit and snacks), and high-preferring respondents (high preference for all food categories). The low-preferring respondents showed the lowest values for all appetitive traits ($p = 0.0008$), as well as the lowest total score ($p = 0.0001$), except for food fussiness, for which they showed the highest value ($p = 0.0008$). All preference scores were positively associated with traits such as hunger, food responsiveness, enjoyment of food, and emotional under-eating, while negatively associated with food fussiness (all $p < 0.05$). The largest amount of variance was observed for preference for dairy (14.6%; $R^2 = 0.146$, $p = 0.008$) and snacks with respect to enjoyment of food (16.2%; $R^2 = 0.162$, $p = 0.008$), for vegetable with respect to food fussiness (22%; $R^2 = 0.220$, $p = 0.008$), and for meat/fish with respect to enjoyment of food (19.9%; $R^2 = 0.199$, $p = 0.008$) and food fussiness combined (19.1%; $R^2 = 0.191$, $p = 0.008$). These results support the association of food preferences with both food approach traits and food avoidance traits.

Keywords: food preferences; food approach; food avoidance; appetitive traits; Food Preference Questionnaire (FPQ); Adult Eating Behaviour Questionnaire (AEBQ); adolescents; national study; population-based study; PLACE-19 Study

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1. Introduction

As indicated by the World Health Organization (WHO), adolescence is a transitional period between childhood and adulthood, which allows preventing nutrition-related chronic diseases in the future, while addressing specific nutritional issues and correcting those that originated in the past [1]. However, in this period, individuals prioritize personal preferences over family eating habits, as they have progressively more control over their own diet [2]. It has been stated that self-reported food preferences are among the strongest predictors of the food choices of adolescents [3]. Thus, understanding food preferences is necessary to promote healthy eating patterns in this population [4]. Several studies conducted among youths have confirmed the role of food preferences as a powerful determinant of diet and consequently health. Their results indicate that food preferences influence weight status [5] and nutritional risk factors of diet-related diseases [6].

Food preferences, associated with the acceptability of food products, are defined as evaluative attitudes expressed by people toward foods including how they qualitatively evaluate them and how much they like or dislike specific products [7]. These preferences are established from early childhood [8]. The development of food preferences is influenced by multiple factors, including personal ones (e.g., innate preferences, applied feeding practices), as well as those associated with parents (e.g., maternal diet during pregnancy and breastfeeding), community (e.g., restaurants, food retailers), and macroenvironment (e.g., food marketing, media) [6]. The possibility of modifying the developed food preferences is limited, as they remain stable until adulthood [9]. However, during the lifetime of an individual, acuity and discriminating ability of senses change, which also lead to some changes in taste and smell perceptions and as a result modify food preferences [10].

The other key determinants of food choices are appetitive traits [11], which are also associated with food preferences to some extent. Appetitive traits are defined as persistent predispositions toward food [12]. They include food approach traits (hunger, food responsiveness, emotional over-eating, enjoyment of food) and food avoidance traits (satiety responsiveness, emotional under-eating, food fussiness, slowness in eating) [13]. A study conducted on children aged 3–4 years indicated that preference for fruit or vegetables is positively associated with enjoyment of food and negatively associated with satiety responsiveness, slowness in eating, and food fussiness [14]. Similarly, another study conducted on children aged 2–5 years showed that preference for fruit or vegetables is positively associated with enjoyment of food and negatively associated with food fussiness [15]. However, it should be mentioned that in both studies [14,15], parents reported the preferences of their children and the preferences were not self-reported.

As previous studies analyzed only children, but not adolescents, the associations in older age groups are still unknown. Moreover, even in the case of children, the authors of the studies emphasize that further research should be performed to describe the causal mechanisms driving the associations between appetitive traits and food preferences [14], as well as to evaluate when, how, and why some appetitive traits are associated with food preferences [15].

Taking into account the COVID-19 pandemic, it must be noted that the present global situation may change food priorities [16]. Appetitive traits also appear to be influenced by the pandemic, as it was reported that in this period students experience moderate-to-severe anxiety symptoms due to increased hunger, emotional over-eating, and food and satiety responsiveness, as well as decreased enjoyment of food [17]. Thus, the associations of food preferences and appetitive traits may also be modified, and so they should be studied in the period of the COVID-19 pandemic. Moreover, as it is predicted that 1.58–8.76 million COVID-19 deaths can be observed over 5 years until the end of 2024 [18], we must be prepared for the prolonged pandemic and more studies should be conducted during this period [19].

Considering the above, the aim of the present study was to verify the association between food preferences and appetitive traits in adolescents aged 15–20 years within the Polish Adolescents' COVID-19 Experience (PLACE-19) Study population.

2. Materials and Methods

2.1. Ethical Statement

The PLACE-19 Study was carried out at the Institute of Human Nutrition Sciences, Warsaw University of Life Sciences (WULS-SGGW). It was conducted based on the agreement of the Ethics Committee of the Institute of Human Nutrition Sciences of the Warsaw University of Life Sciences. The participants, as well as their parents/legal guardians, provided informed consent for participation, and all the procedures were in agreement with the Declaration of Helsinki. The PLACE-19 Study included two phases to assess hygienic and personal protective behaviors [20–22], as well as nutritional behaviors [23–25].

2.2. Studied Population

To assess nutritional behaviors within the second phase of the PLACE-19 Study, the studied population was recruited in the period from 29 April 2020 to 23 May 2020. The studied group was population-based and it was sampled in whole Poland, while using a random quota sampling method. As described in the previous studies [23–25], the procedure included stratified random sampling of counties within voivodeships (being the Polish basic administrative units), followed by random sampling of schools within counties, in order to gather population-based sample including proportional share of adolescents from all regions. While arranging, the local Boards of Education participated, if needed, but the participation of school was for its headmaster voluntary, as well as individual participation was for each student voluntary.

The inclusion criteria were formulated as follows:

- Students of the sampled school,
- Aged 15–20 years,
- Providing informed consent to participate in the PLACE-19 Study, as well as informed consent of parents/legal guardians.
- The exclusion criteria were formulated as follows:
- Any missing or unreliable data in the questionnaire,
- For the participants of the second phase of the PLACE-19 Study (assessment of nutritional behaviors): not participating in the first phase of the PLACE-19 Study (assessment of hygienic and personal protective behaviors).

The second phase of the PLACE-19 Study was finally carried out in a sample of 2448 secondary school students which were included based on the presented inclusion and exclusion criteria, as described in the previous studies [23–25].

2.3. Applied Questionnaires

As in the period from April to May 2020, on the basis of the decision of Polish Ministry of Education [26] education in all secondary schools in Poland was provided within a remote learning system, the study was conducted while using a method of Computer-Assisted Web Interview (CAWI). The questionnaire did not collect any sensitive or personal data which would allow to recognize a respondent, either for researchers, or for school headmasters and teachers.

The food preferences were assessed while using Food Preference Questionnaire (FPQ) by Smith et al. [27]. The FPQ is a validated self-report tool to be applied in case of children and adolescents which was developed by Smith et al. [27] based on the previously validated tool for children to be proxy-reported by parents [28]. The FPQ includes a list of 62 various food items to be defined on how much on average the respondent like the specific item with the possible answers as follows: (1) dislike a lot, (2) dislike a little, (3) neither like nor dislike, (4) like a little, (5) like a lot (for any food item they have ever tried, independently from the actual consumption), as well as (6) not applicable (for any food item they don't know, or don't remember ever having tried) [29]. The FPQ allows to assess the preferences of vegetables (questionnaire includes 18 food items in this food category), fruit (7 items), meat/fish (12 items), dairy (10 items), snacks (9 items), and starches (6 items), which may

be obtained by summing the single food preference item scores within each food category and dividing this sum by the number of items [29].

The appetitive traits were assessed while using Adult Eating Behavior Questionnaire (AEBQ) by Hunot et al. [13]. The AEBQ is a validated self-report tool to be applied in case of adolescents and adults which was developed to assess food approach and food avoidance. The AEBQ includes a list of 35 items to be defined while using a 5-point Likert scale (from 'strongly disagree' to 'strongly agree'). The AEBQ allows to assess following food approach sub-scales: hunger (5 items), food responsiveness (4 items), emotional over-eating (5 items), enjoyment of food (3 items) and following food avoidance sub-scales: satiety responsiveness (4 items), emotional under-eating (5 items), food fussiness (5 items), slowness in eating (4 items). The scores for each scale are obtained by attributing points to each item–depending on a question either from 1 to 5 points, or from 5 to 1 point, and by calculating mean score for each subscale.

2.4. Statistical Analysis

The normality of distribution was verified by using Shapiro-Wilk test. Due to non-parametric distribution, the Kruskal–Wallis analysis of variance (ANOVA) with post-hoc Tukey test was applied to compare groups and Spearman correlation for analysis of associations within heatmap of correlation matrix. Based on the assumption that the individual food product preferences do not present equally in the entire population and that specific consumer groups typically differ within the population, presenting various profiles, the clustering of respondents based on their preferences was conducted. For clustering, k-means algorithm was applied with Euclidean distance, as generally recommended [30], to identify homogenous clusters of respondents based on their preferences, while the optimal number of clusters was verified using the Elbow method. For analysis of associations, the linear regression between food preferences and appetitive traits was performed with model adjusted for sex and age, as this approach was confirmed by the previous studies as adequate [14]. The standardized β-coefficients were presented in order to allow the comparison of results between the scores. As an additional analysis, the multiple linear regression was applied and F-statistics was presented for the variables indicated as significant based on the previous analysis. Due to the multiple analysis conducted, Bonferroni correction was applied.

The statistical significance was defined for the level of $p \leq 0.05$. The statistical analysis was performed while using Statistica version 13.3 (StatSoft Inc., Tulsa, OK, USA) and JASP version 0.14.0.0 (JASP Team, 2020).

3. Results

The characteristics of the population studied within the second phase of the PLACE-19 Study is presented in Table 1. The sex and age were indicated as the most important variables and in the further analysis they were included to the model.

Table 1. The characteristics of the population studied within the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2448).

Variable		Number of Participants	% of the Studied Population
Sex	Female	1552	63.4
	Male	896	36.6
Age (years)	15	297	12.1
	16	755	30.8
	17	799	32.6
	18	445	18.2
	19	139	5.7
	20	13	0.5

The food preferences assessed by FPQ within the population of the second phase of the PLACE-19 Study are presented in Table 2. The highest preferences in the studied group were defined for the categories of fruit, snacks and starches (median values higher than 4 points attributed to 'like a little' category) and the lowest—for the category of meat/fish (median value higher than 3 points attributed to 'neither like nor dislike' category).

Table 2. The food preferences assessed by Food Preference Questionnaire (FPQ) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2448) (distribution different than normal for all the variables verified using Shapiro-Wilk test—$p \leq 0.05$).

Food Category	Mean ± SD	Median	25th Quartile	75th Quartile
Vegetable	3.7 ± 0.7	3.8	3.2	4.3
Fruit	4.3 ± 0.8	4.6	4.0	5.0
Meat/fish	3.4 ± 0.8	3.5	2.9	4.0
Dairy	3.7 ± 0.7	3.8	3.2	4.2
Snacks	4.2 ± 0.8	4.3	3.8	4.8
Starches	4.0 ± 0.8	4.2	3.7	4.7

The food preferences for sub-groups stratified based on the clustering of the preferences assessed by FPQ are presented in Table 3 and the normalized mean values for the preferences within the sub-groups stratified based on the clustering of the preferences assessed by FPQ within the population of the second phase of the PLACE-19 Study are presented in Figure 1. While the k-means algorithm was applied, 3 homogenous clusters of respondents were defined, based on their preferences—low-preferring respondents (low preference for all food categories), respondents preferring snacking foods (low preference for all food categories, except for fruit and snacks, as for them median values were higher than 4 points attributed to 'like a little' category), and high-preferring respondents (high preference for all food categories, as median values were higher than 4 points attributed to 'like a little' category).

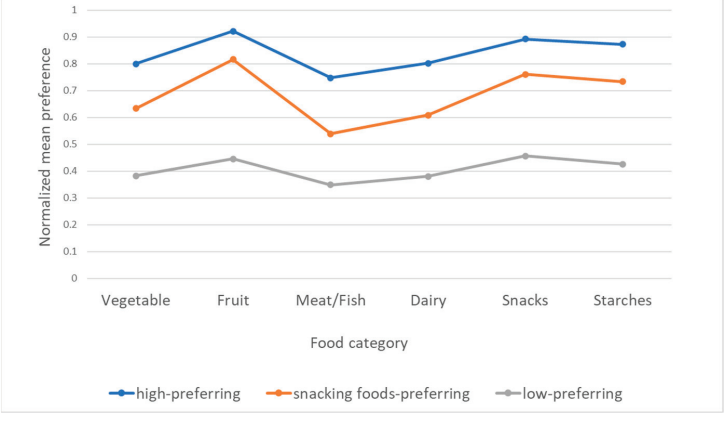

Figure 1. Normalized mean values for the preference within the sub-groups stratified based on the clustering of the preferences assessed by Food Preference Questionnaire (FPQ) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2419).

Table 3. The food preferences for sub-groups stratified based on the clustering of the preferences assessed by Food Preference Questionnaire (FPQ) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2419) (distribution different than normal for all the variables, except for dairy for cluster 1, verified using Shapiro-Wilk test—$p \leq 0.05$).

Food Category	Cluster 1 (Low-Preferring) (n = 270)		Cluster 2 (Snacking Foods-Preferring) (n = 1109)		Cluster 3 (High-Preferring) (n = 1040)	
Vegetable	2.5 ± 0.6	2.5 (2.1–2.9)	3.5 ± 0.6	3.6 (3.2–3.9)	4.2 ± 0.5	4.2 (3.9–4.6)
Fruit	2.8 ± 0.8	3.0 (2.1–3.0)	4.3 ± 0.6	4.3 (4.0–4.8)	4.7 ± 0.4	4.9 (4.6–5.0)
Meat/fish	2.4 ± 0.7	2.3 (1.9–2.9)	3.2 ± 0.7	3.3 (2.8–3.6)	4.0 ± 0.6	4.0 (3.6–4.4)
Dairy	2.5 ± 0.6	2.6 (2.1–2.9)	3.4 ± 0.5	3.4 (3.1–3.8)	4.2 ± 0.5	4.2 (3.9–4.6)
Snacks	2.8 ± 0.7	2.9 (2.4–3.1)	4.1 ± 0.6	4.1 (3.8–4.6)	4.6 ± 0.5	4.8 (4.3–5.0)
Starches	2.7 ± 0.7	2.8 (2.2–3.0)	3.9 ± 0.6	4.0 (3.7–4.3)	4.5 ± 0.5	4.5 (4.2–4.8)

The appetitive traits assessed by AEBQ for sub-groups stratified based on the clustering of the preferences assessed by FPQ within the population of the second phase of the PLACE-19 Study are presented in Table 4. All appetitive traits differed between respondents from various clusters. The low-preferring respondents (cluster 1) were characterized by the lowest values for all appetitive traits (p = 0.0008), as well as for the total score (p = 0.0001), except for food fussiness, as for it this cluster was characterized by the highest value (p = 0.0008). The snacking-preferring respondents (cluster 2) and high-preferring ones (cluster 3) were characterized by comparable traits of hunger and emotional overeating, while all other appetitive traits differed significantly (p = 0.0008).

Table 4. The appetitive traits assessed by Adult Eating Behavior Questionnaire (AEBQ) for sub-groups stratified based on the clustering of the preferences assessed by Food Preference Questionnaire (FPQ) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2419) (distribution different than normal for all the variables verified using Shapiro-Wilk test—$p \leq 0.05$).

Appetitive Traits	Cluster 1 (Low-Preferring) (n = 270)		Cluster 2 (Snacking Foods-Preferring) (n = 1109)		Cluster 3 (High-Preferring) (n = 1040)		p
Food approach subscales							
Hunger (H)	2.3 ± 0.5	2.0 (2.0–2.4) [a]	2.5 ± 0.6	2.4 (2.0–2.8) [b]	2.5 ± 0.6	2.4 (2.0–2.8) [b]	0.0008
Food responsiveness (FR)	2.3 ± 0.6	2.0 (2.0–2.5) [a]	2.8 ± 0.6	2.8 (2.5–3.3) [b]	3.0 ± 0.7	3.0 (2.5–3.3) [c]	0.0008
Emotional over-eating (EOE)	2.6 ± 0.3	2.6 (2.4–2.6) [a]	2.8 ± 0.6	2.6 (2.4–3.0) [b]	2.8 ± 0.6	2.6 (2.4–3.0) [b]	0.0008
Enjoyment of food (EF)	2.5 ± 0.8	2.0 (2.0–3.0) [a]	3.6 ± 0.8	3.7 (3.0–4.0) [b]	3.9 ± 0.8	4.0 (3.3–4.3) [c]	0.0008
Food avoidance subscales							
Satiety responsiveness (SR)	2.5 ± 0.8	2.0 (2.0–3.0) [a]	3.0 ± 0.7	3.0 (2.5–3.5) [b]	2.8 ± 0.7	2.8 (2.3–3.3) [c]	0.0008
Emotional under-eating (EUE)	2.5 ± 0.8	2.0 (2.0–2.8) [a]	2.9 ± 0.8	2.6 (2.0–3.4) [b]	2.8 ± 0.8	2.6 (2.0–3.2) [c]	0.0008
Food fussiness (FF)	3.2 ± 0.5	3.2 (3.2–3.2) [a]	2.8 ± 0.8	2.8 (2.2–3.4) [b]	2.4 ± 0.7	2.2 (1.8–2.8) [c]	0.0008
Slowness in eating (SE)	2.8 ± 0.6	2.5 (2.5–3.0) [a]	3.0 ± 0.8	3.0 (2.5–3.5) [b]	2.9 ± 0.8	2.8 (2.3–3.5) [c]	0.0008
Total	2.6 ± 0.4	2.3 (2.3–2.9) [a]	2.9 ± 0.3	2.9 (2.7–3.1) [b]	2.8 ± 0.3	2.8 (2.6–3.0) [c]	0.0001

[a,b,c]—values marked with different letters in rows differ significantly.

Analysis of association between appetitive traits and vegetable preference assessed by FPQ, in model adjusted for sex and age, within the population of the second phase of the PLACE-19 Study is presented in Table 5. Vegetable preference score was positively associated with hunger, food responsiveness, emotional over-eating, enjoyment of food, emotional under-eating, and slowness in eating, while negatively associated with food fussiness. Food fussiness explained the largest amount of variance (22%, R^2 = 0.220,

$p = 0.008$) for the studied population. The appetitive traits that were significantly associated with the vegetable preference were included to the regression model with sex and age as additional demographic variables ($R^2 = 0.26$, $F(82,406) = 106.91$, $p = 0.001$).

Analysis of association between appetitive traits and fruit preference assessed by FPQ, in model adjusted for sex and age, within the population of the second phase of the PLACE-19 Study is presented in Table 6. Fruit preference score was positively associated with hunger, food responsiveness, enjoyment of food, satiety responsiveness, and emotional under-eating, while negatively associated with food fussiness. Enjoyment of food and food fussiness explained the largest amount of variance (7.6%, $R^2 = 0.076$, $p = 0.008$ and 7.7%, $R^2 = 0.077$, $p = 0.008$, respectively) for the studied population. The appetitive traits that were significantly associated with the fruit preference were included to the regression model with sex and age as additional demographic variables ($R^2 = 0.14$, $F(82,321) = 46.85$, $p = 0.001$).

Table 5. Analysis of association between appetitive traits and vegetable preference assessed by Food Preference Questionnaire (FPQ) (model adjusted for sex and age) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study ($n = 2415$).

Appetitive Traits	Unstandardized Coefficients		Standardized Coefficients β	*p*	R	R^2
	β	SE				
Hunger (H)	0.108	0.025	0.087	0.008	0.132	0.017
Food responsiveness (FR)	0.201	0.022	0.185	0.008	0.210	0.044
Emotional over-eating (EOE)	0.118	0.026	0.092	0.008	0.135	0.018
Enjoyment of food (EF)	0.258	0.015	0.321	0.008	0.336	0.113
Satiety responsiveness (SR)	0.014	0.021	0.014	1.000	0.101	0.010
Emotional under-eating (EUE)	0.060	0.018	0.070	0.008	0.121	0.015
Food fussiness (FF)	−0.427	0.017	−0.459	0.008	0.469	0.220
Slowness in eating (SE)	0.058	0.019	0.062	0.024	0.117	0.014

SE—standard error.

Table 6. Analysis of association between appetitive traits and fruit preference assessed by Food Preference Questionnaire (FPQ) (model adjusted for sex and age) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study ($n = 2330$).

Appetitive Traits	Unstandardized Coefficients		Standardized Coefficients β	*p*	R	R^2
	β	SE				
Hunger (H)	0.082	0.023	0.075	0.008	0.096	0.009
Food responsiveness (FR)	0.183	0.020	0.189	0.008	0.197	0.039
Emotional over-eating (EOE)	0.031	0.024	0.027	1.000	0.065	0.004
Enjoyment of food (EF)	0.197	0.015	0.269	0.008	0.275	0.076
Satiety responsiveness (SR)	0.102	0.019	0.112	0.008	0.125	0.016
Emotional under-eating (EUE)	0.056	0.016	0.073	0.008	0.093	0.009
Food fussiness (FF)	−0.226	0.017	−0.272	0.008	0.278	0.077
Slowness in eating (SE)	0.027	0.017	0.033	0.960	0.068	0.005

SE—standard error.

Analysis of association between appetitive traits and meat/fish preference assessed by FPQ, in model adjusted for sex and age, within the population of the second phase of the PLACE-19 Study is presented in Table 7. Meat/fish preference score was positively associated with hunger, food responsiveness, enjoyment of food, and emotional under-eating, while negatively associated with food fussiness. Enjoyment of food and food fussiness explained the largest amount of variance (19.9%, $R^2 = 0.199$, $p = 0.008$ and 19.1%, $R^2 = 0.191$, $p = 0.008$, respectively) for the studied population. The appetitive traits that

were significantly associated with the meat/fish preference were included to the regression model with sex and age as additional demographic variables (R^2 = 0.27, F(62,418) = 145.62, p = 0.001).

Analysis of association between appetitive traits and dairy preference assessed by FPQ, in model adjusted for sex and age, within the population of the second phase of the PLACE-19 Study is presented in Table 8. Dairy preference score was positively associated with hunger, food responsiveness, emotional over-eating, enjoyment of food, and emotional under-eating, while negatively associated with food fussiness. Enjoyment of food explained the largest amount of variance (14.6%, R^2 = 0.146, p = 0.008) for the studied population. The appetitive traits that were significantly associated with the dairy preference were included to the regression model with sex and age as additional demographic variables (R^2 = 0.17, F(82,408) = 64.33, p = 0.001).

Table 7. Analysis of association between appetitive traits and meat/fish preference assessed by Food Preference Questionnaire (FPQ) (model adjusted for sex and age) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2425).

Appetitive Traits	Unstandardized Coefficients		Standardized Coefficients β	p	R	R^2
	β	SE				
Hunger (H)	0.126	0.028	0.088	0.008	0.278	0.077
Food responsiveness (FR)	0.250	0.024	0.201	0.008	0.332	0.110
Emotional over-eating (EOE)	0.038	0.029	0.026	1.000	0.265	0.070
Enjoyment of food (EF)	0.331	0.017	0.361	0.008	0.446	0.199
Satiety responsiveness (SR)	0.032	0.024	0.027	1.000	0.265	0.070
Emotional under-eating (EUE)	0.058	0.020	0.059	0.032	0.270	0.073
Food fussiness (FF)	−0.373	0.020	−0.349	0.008	0.437	0.191
Slowness in eating (SE)	0.040	0.021	0.038	0.472	0.267	0.071

SE—standard error.

Table 8. Analysis of association between appetitive traits and dairy preference assessed by Food Preference Questionnaire (FPQ) (model adjusted for sex and age) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2417).

Appetitive Traits	Unstandardized Coefficients		Standardized Coefficients β	p	R	R^2
	β	SE				
Hunger (H)	0.190	0.025	0.152	0.008	0.209	0.044
Food responsiveness (FR)	0.278	0.021	0.254	0.008	0.291	0.085
Emotional over-eating (EOE)	0.098	0.026	0.076	0.008	0.162	0.026
Enjoyment of food (EF)	0.287	0.015	0.354	0.008	0.382	0.146
Satiety responsiveness (SR)	0.036	0.021	0.035	0.720	0.148	0.022
Emotional under-eating (EUE)	0.059	0.018	0.069	0.008	0.158	0.025
Food fussiness (FF)	−0.249	0.018	−0.264	0.008	0.300	0.090
Slowness in eating (SE)	0.031	0.019	0.033	0.832	0.147	0.022

SE—standard error.

Analysis of association between appetitive traits and snacks preference assessed by FPQ, in model adjusted for sex and age, within the population of the second phase of the PLACE-19 Study is presented in Table 9. Snacks preference score was positively associated with hunger, food responsiveness, emotional over-eating, enjoyment of food, satiety responsiveness, and emotional under-eating, while negatively associated with food fussiness. Enjoyment of food explained the largest amount of variance (16.2%, R^2 = 0.162, p = 0.008) for the studied population. The appetitive traits that were significantly associated

with the snacks preference were included to the regression model with sex and age as additional demographic variables (R^2 = 0.18, F(82,332) = 66.23, p = 0.001).

Analysis of association between appetitive traits and starches preference assessed by FPQ, in model adjusted for sex and age, within the population of the second phase of the PLACE-19 Study is presented in Table 10. Starches preference score was positively associated with hunger, food responsiveness, emotional over-eating, enjoyment of food, satiety responsiveness, and emotional under-eating, while negatively associated with food fussiness. Enjoyment of food explained the largest amount of variance (8.9%, R^2 = 0.089, p = 0.008) for the studied population. The appetitive traits that were significantly associated with the starches preference were included to the regression model with sex and age as additional demographic variables (R^2 = 0.12, F(92,358) = 36.36, p = 0.001).

Table 9. Analysis of association between appetitive traits and snacks preference assessed by Food Preference Questionnaire (FPQ) (model adjusted for sex and age) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2341).

Appetitive Traits	Unstandardized Coefficients		Standardized Coefficients β	p	R	R^2
	β	SE				
Hunger (H)	0.182	0.023	0.164	0.008	0.172	0.030
Food responsiveness (FR)	0.312	0.019	0.318	0.008	0.322	0.103
Emotional over-eating (EOE)	0.084	0.024	0.074	0.008	0.092	0.009
Enjoyment of food (EF)	0.296	0.014	0.399	0.008	0.403	0.162
Satiety responsiveness (SR)	0.070	0.020	0.075	0.008	0.093	0.009
Emotional under-eating (EUE)	0.034	0.016	0.044	0.328	0.070	0.005
Food fussiness (FF)	−0.075	0.017	−0.089	0.008	0.105	0.011
Slowness in eating (SE)	0.012	0.018	0.014	1.000	0.058	0.003

SE—standard error.

Table 10. Analysis of association between appetitive traits and starches preference assessed by Food Preference Questionnaire (FPQ) (model adjusted for sex and age) within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2368).

Appetitive Traits	Unstandardized Coefficients		Standardized Coefficients β	p	R	R^2
	β	SE				
Hunger (H)	0.112	0.025	0.093	0.008	0.099	0.010
Food responsiveness (FR)	0.199	0.021	0.189	0.008	0.191	0.036
Emotional over-eating (EOE)	0.122	0.025	0.099	0.008	0.103	0.011
Enjoyment of food (EF)	0.233	0.015	0.297	0.008	0.298	0.089
Satiety responsiveness (SR)	0.076	0.021	0.077	0.008	0.082	0.007
Emotional under-eating (EUE)	0.080	0.017	0.098	0.008	0.100	0.010
Food fussiness (FF)	−0.191	0.018	−0.213	0.008	0.215	0.046
Slowness in eating (SE)	0.022	0.019	0.025	1.000	0.041	0.002

Models adjusted for covariates including sex and age; SE—standard error.

Heatmap of correlation matrix presenting association between food preferences assessed by FPQ and appetitive traits assessed by AEBQ, within the population of the second phase of the PLACE-19 Study is presented in Figure 2. This unadjusted analysis confirmed the weakest associations or negative associations for food fussiness, while for other appetitive traits the associations were more significant.

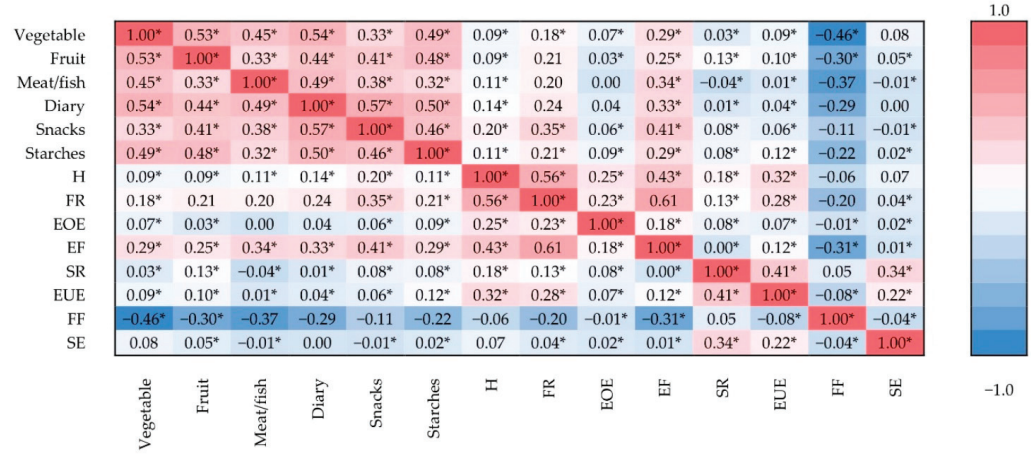

Figure 2. Heatmap of correlation matrix presenting association between food preferences assessed by Food Preference Questionnaire (FPQ) and appetitive traits assessed by Adult Eating Behavior Questionnaire (AEBQ), conducted while using Spearman correlation (due to nonparametric distribution), within the population of the second phase of the Polish Adolescents' COVID-19 Experience (PLACE-19) Study (n = 2419); H—Hunger; FR—Food responsiveness; EOE—Emotional over-eating; EF—Enjoyment of food; SR—Satiety responsiveness; EUE—Emotional under-eating; FF—Food fussiness; SE—Slowness in eating; * significant association ($p \leq 0.05$).

4. Discussion

The study assessed the preference for vegetables, fruit, meat/fish, dairy, snacks, and starches in a group of Polish adolescents. The preference scores for all food categories were found to be positively associated with hunger, food responsiveness, enjoyment of food, and emotional under-eating, as well as negatively associated with food fussiness. The associations between appetitive traits and general food preferences may suggest that these features are predetermined. As indicated by Fildes et al. [14], it appears that appetitive traits associated with an increased risk of excessive body mass may have additional lifelong consequences and also influence dietary diversity and nutrient intake.

As mentioned above, hunger, food responsiveness, and enjoyment of food are food approach traits, while emotional under-eating and food fussiness are food avoidance traits [31]. Taking this into account, it may be indicated that the association between food preferences and appetitive traits is not influenced by the type of appetitive traits (approach or avoidance), but other factors may more likely determine the associations between food preferences and food fussiness compared to other appetitive traits.

Food fussiness is an appetitive trait described as refusing and not tasting new foods at first, disliking a food before tasting it, not enjoying a wide variety of foods, as well as not being interested in tasting new foods that have not been tried before [13]. This trait is observed to differ from the other food avoidance as well as other appetitive traits, as in adults it is not associated with Body Mass Index (BMI), although in general BMI is positively associated with food approach traits [32] and negatively associated with food avoidance traits [13,32]. The difference can be explained by the fact that food fussiness is qualitatively distinct as it reflects a food choice selectivity, while the other food avoidance traits reflect decreased appetite and increased sensitivity to satiety cues [33].

Previous studies conducted on children have also confirmed that food fussiness was negatively associated with food preferences. In children aged 2–5 years, it was negatively associated with preference for vegetables, fruit, extra foods (those not essential to provide the nutrients needed by the body, and hence similar to snacks as indicated in the present study), dairy, meats, cereals, and a number of liked foods, while it was positively associated

with a number of disliked foods [15]. At the same time, in children aged 3–4 years, this trait was negatively associated with the preference for vegetables and fruit, but not associated with the preference for noncore foods (energy-dense and nutrient-poor discretionary foods, and hence similar to snacks as indicated in the present study) [14]. The lack of association between food fussiness and snacks in the case of the youngest children may be, to some extent, related to the fact that this trait seems to decrease with age [34], and hence, its association with food preference may also change during the lifespan.

The positive associations observed between food preferences and hunger, food responsiveness, enjoyment of food, as well as emotional under-eating in this study are in line with the results of the previous studies by other authors. In the study on children aged 2–5 years, emotional under-eating was positively associated with the preference for vegetables, extra foods, dairy, meats, and cereals; enjoyment of food was positively associated with the preference for vegetables, fruit, dairy, meats, and cereals, but negatively associated with the preference for extra foods; and food responsiveness was positively associated with preference for extra foods, but negatively with the preference for vegetables [15]. On the other hand, in the study on children aged 3–4 years, food responsiveness was positively associated with the preference for vegetables and fruit, while enjoyment of food was positively associated with the preference for noncore foods [16].

Considering the role of appetitive traits, it seems that they may be crucial for choosing and consuming specific food products. Thus, based on the present study, it can be assumed that adolescents with higher food fussiness may reject some products, especially fruit and vegetables [35]. Similarly, adolescents with higher food responsiveness and enjoyment of food may eagerly try novel, previously unknown food products, which may lead to an increased preference and intake of fruit and vegetables [36].

Similarly, the analysis of the clusters of low-preferring respondents, respondents preferring snacking foods, and high-preferring respondents indicated the influence of appetitive traits on low-preferring respondents, as they had the lowest values for all appetitive traits, except for food fussiness, for which they showed the highest value. The number of respondents in this cluster was low (only 11%), and they may be generally defined as picky eaters, who reject or restrict both familiar and unfamiliar foods [37], while their food fussiness was significantly higher compared to the other clusters. At the same time, the other clusters showed a high preference for fruit and snacks only (respondents preferring snacking foods) or a high preference for all products in general. Taking this into account, the preference and resultant intake of fruit and vegetables may be supposed to be higher only in the high-preferring cluster, which may be considered a public health problem due to the potential benefits of fruit and vegetables [38], as well as the inadequate intake of fruit and vegetables in the case of adolescents [39].

It should be indicated that the present study assessed only the preferences and not the intake of fruit and vegetables. However, the findings of a recent systematic review and meta-analysis by Bawajeeh et al. [40], which analyzed the association between taste, preference, and food choices in adolescents, emphasized that food choices and intake are created by a complex combination of various factors, which should be studied in detail to expand the existing knowledge.

The present study not only reproduced, for a Polish cohort, specific associations between appetitive traits and food preferences defined by previous studies [14,15], but also confirmed the in-depth observations reported by their authors. Such observations are based on positive associations between food approach traits and food preferences. As food approach traits have been assumed to promote excessive body mass, their association with a higher preference for various products may be an additional factor promoting overconsumption. This is an important issue to be taken into account, as it may have lifelong consequences on the quality of diet and body mass.

5. Conclusions

This study showed that all preference scores were associated with both food approach and food avoidance traits. They were positively associated with hunger, food responsiveness, enjoyment of food, and emotional under-eating, while negatively associated with food fussiness. The largest amount of variance was observed for the preference for dairy and snacks with respect to enjoyment of food, for vegetables with respect to food fussiness, and for meat/fish with respect to enjoyment of food and food fussiness combined.

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Review

Egg Allergy in Children and Weaning Diet

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Abstract: Eggs are a fundamental food in the human diet, and together with cow's milk, they are the most common food allergen. This work highlights the main nutritional characteristics of eggs to show how their absence from a child's diet can constitute a serious deficiency. We then analyze the risk factors that facilitate the onset of egg allergy. The third part of the paper reports possible interventions to lower the appearance of food allergy that have been occurred in trials. The last part of the paper is a synthesis of this research study that has been taken from several of the latest guidelines or from position papers.

Keywords: weaning; egg; food allergy; dietetic interventions; prevention; infant; egg allergy

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1. Introduction

Hen's egg (HE) allergy is one of the most frequent food allergies in Western countries, and its rate is increasing [1]. It has thus far been estimated that HE allergy affects between 1.6% and 10.1% [2] and as many as 9.5% of children [3–5]. The onset most often occurs before the first birthday [6]. The prevalence and age of spontaneous oral tolerance acquisition in childhood vary among studies because of differences in populations, settings and the means for ascertaining the diagnosis. It has generally been found that HE allergy has a prevalence of 1.2–2.9% at 2 years of age [7–11]. Natural resolution has been estimated in up to 80% of cases at 3 years of age [12] and in 38–90% of children within 5–6 years of age [3,13–18]. Clinical HE hypersensitivity is lost by 60% of children between 6 and 12 years of age [2]. Tolerance to baked egg is more quickly achieved. Baked HE allergy is outgrown by 94% of patients at 12 years of age [17], while that to otherwise cooked egg is outgrown by 76% at 12 years of age and by 95% at age 18 [13]. The burden of HE allergy is heavy as it can trigger anaphylactic reactions or severe chronic gastroenteropathy. An elimination diet can provoke nutritional imbalance and increase the risk for poor growth. Furthermore, HE allergy reduces the quality of life by limiting the social activities of children and parents, regardless of symptom severity [19,20]. It is also associated with bullying [21], depression, anxiety, attention/deficit hyperactivity disorder [22] and higher healthcare costs [23–25]. In view of these issues, various approaches for preventing HE development in children have been proposed. The current review focuses on prevention, especially by dietary interventions in a child's first year. The paper is based on evidence from the literature, on the recommendations of scientific societies and on clinical experience.

2. Eggs as Nutrients

HE is a basic part of the pediatric diet. This makes it important to know its components in order to properly manage an egg-free diet. In addition to energy, fatty acid and protein intake, HE avoidance mainly reduces the intake of B vitamins and vitamin D [26]. HE is composed of three different parts: 63% egg white, 27.5% egg yolk and 9.5% eggshell and

membranes. The basic composition of HE may have some minor changes in its nutritional composition due to production methods. A medium HE weighs 44 g and can provide 62.5 calories while a large HE provides 77 calories. The main components consist of water (76%), proteins (12%) and lipids (9.51%). In addition, it also contains low amounts of carbohydrates (0.72%), minerals and vitamins (Table 1) [27].

Table 1. Main nutrient contents in one boiled or poached egg weighing 44 g according to the United States Department of Agriculture [27].

Protein 5.5 g
Total fat: 4.2 g, of which 1.4 g are saturated
Cholesterol: 162 mg
Sodium: 189 mg
Phosphorus: 86.7 mg
Potassium: 60.3 mg
Calcium: 24.6 mg
Magnesium 5.3 mg
Iron: 0.8 mg
Lutein and zeaxanthin: 220 µg
Folate: 15.4 µg
Selenium: 13.4 µg

Egg white is the principal HE component responsible for allergic reactions (Table 2), though alegg yolk protein may also be responsible. It is difficult to separate the white from the yolk, however, without traces of egg white protein contaminating the yolk.

Table 2. Differences in allergenic proprieties between egg yolk and egg white proteins.

Egg White Protein	Egg Yolk Protein
ovomucoid (Gal d 1)	Phosvitin
ovalbumin (Gal d 2)	α-livetin (Gal d 5)
ovotransferrin or conalbumin (Gal d 3)	apovitellenins I
egg lysozyme (Gal d 4)	apovitellenins VI (or apoprotein B)
ovomucin	

HEs contain vitamins, minerals, high-quality protein with a perfect amino acid profile, and good fats and various other lesser-known nutrients. Almost all the nutrients are contained in the yolk. However, 60% of the high-quality protein in eggs can be found in the egg white. The main elements of egg white are water and proteins (mainly ovalbumin, ovotransferrin and ovomucoid). Egg white consists primarily of about 90% water into which about 10% proteins are dissolved. Unlike the yolk, which is high in lipids, egg white contains almost no fat and less than 1% of carbohydrate [28]. Egg yolk is a homogeneous emulsion, containing proteins and lipids. Egg yolk proteins mainly consist of livetins (38%, α-, β- and γ-forms), lipovitellins (36%, α- and β-forms), low-density lipoproteins LDL (17%) and phosvitin (8–9%). Among these egg yolk proteins, special attention needs to be paid to γ -livetin (also called γ-globulin or immunoglobulin Y) and phosvitin [29]. Immunoglobulin Y is the main antibody derived from chicken egg, and it is popular for the control of enteric infections of either bacterial or viral origin. Phosvitin has the highest phosphorus content and has high antioxidant activity associated with a high metal binding capacity. Phosvitin has the capacity to bind much more Fe than what is naturally found in egg yolk. Egg yolk lipids comprise 65% neutral lipids, 30% phospholipids and 4% cholesterol [30]. Phospholipids represent the most significant component of egg lipids, and they consist of 78% phosphatidylcholine (PC), 17% phosphatidylethanolamine (PE), 2.5% sphingomyelin (SM), and 0.5% phosphatidylinositol, phosphatidylserine and lysophosphatidylcholine [31]. Egg yolk is a better source for phospholipids than oil seeds.

PC plays a vital role in the functions of nerve cells, and it is a key component of biological membranes. Therefore, PC separated from egg yolk can be used in infant formula to improve brain development. PC can also be used as a dietary supplement for choline, an important nutrient for prenatal and postnatal infants whose nervous system is developing quickly [32]. Among egg-derived phospholipids, the most critical ones are in egg yolk. Choline is an essential nutrient for humans, and a dietary deficiency of choline in humans causes fatty liver [33,34]. PE is mainly found in the white matter of the brain and in the neural tissues of the spinal cord. High amounts of PE can induce relaxation and overall improvements in cognitive function. PE also functions as a critical anticoagulant at the luminal endothelial surface of the aortic flow dividers, the ascending aorta and the outer curvature of the aortic arch [35]. SM is an essential component of central nervous system myelin sheaths. It also affects the viability of brain cells and signal transduction in T-cell activation (Table 3) [36,37].

Table 3. Functions and benefits of eggs in a child's diet (modified from [37]).

Contain high-quality proteins with all nine essential amino acids
Improves cholesterol profile increasing HDL and does not raise the risk of heart disease
A good source of omega-3s. Omega-3s play an important role in the way cell membranes work, from heart and brain health to protecting the eyes
Contain choline, a nutrient that contributes mainly to healthy brain development. Choline is required to synthesize the neurotransmitter acetylcholine and is also a component of cell membranes.
Are a great source of Vitamin D.
Have an antioxidant effect: the presence of the carotenoids lutein and zeaxanthin improves the pigment density in the retina. Vitamin A, vitamin E and selenium also help.
Help with weight management because they are relatively low in calories. The high satiety levels of eggs leads to greater feelings of satisfaction, less hunger and a lowered desire to eat later in the day.

3. Risk Factors for Egg Allergy

Infants with a family history of allergy and/or atopic diseases [38,39], but not maternal asthma, allergic rhinitis [40], atopic eczema and sensitization to HE, are at high risk for developing HE clinical allergy. Atopic eczema is frequently related to food allergies, especially to HE, cow's milk allergy and, in countries with a high consumption, peanut allergy. An IgE-mediated HE allergy has been found in 42% of eczematous children [41]. In population studies, eczematous infants were 5.8 times more likely to be affected by HE allergy than healthy children [42] and 6.18 times more likely to be sensitized against certain foods [43]. The relative risk of developing specific IgE antibodies to HE is 14 months in infants with atopic eczema. However, it should be noted that a third of infants with HE sensitization did not suffer from atopic dermatitis [44]. Atopic eczema seems to act as a canary in the coal mine, since it can be diagnosed 3.5 months before egg allergy, and the severity of the eczema is correlated with a higher probability of HE allergy [45]. The likelihood of HE allergy is higher in males with early-onset atopic eczema [46]. Eczematous infants or infants with cow's milk allergy who have a positive skin-prick test (serum specific IgE to HE) and who have never ingested HE have a higher chance of developing a reaction to HE upon the first ingestion. In such infants, the rate of clinical hypersensitivity reaction to HE on the first known exposure varies from 42% to 72% [47–49]. Palmer et al. [50] showed that 20% of infants with moderate to severe atopic eczema reacted on the first HE intake, and 36% had positive serum IgE to HE at 4 months of age. Patch tests for HE are of little help for identifying children with egg allergies [51]. These findings suggest that IgE relative to HE tests should be performed before the first HE intake in infants with atopic eczema or food allergy [52]. When IgE tests have a positive result, the initial HE introduction needs medical supervision [53]. Moreover, population studies have shown that, irrespective of suffering

from atopic eczema, most infants are sensitized to HE before weaning at 4–6 months of age [44]. In total, 4–6 out of 18–20 infants with a positive skin prick test relative to eggs before its introduction into the diet have a positive HE challenge [54]. Overall, these results indicate that sensitization to HE likely occurs following allergen exposure in utero and, after birth, through breast milk, topical application to the skin or inhalation, but not after weaning. Growing data show that allergen exposure through the skin provokes food allergy, while oral exposure induces allergens specific tolerance [55]. It has been hypothesized that the association between HE allergy and atopic eczema may be explained by the facilitated passage of allergens through inflamed skin, which promotes the activation of a Th2 dominant response [56] and leads to allergic sensitization [57]. In agreement with this hypothesis, the epicutaneous application of ovalbumin on damaged skin induces sensitization and anaphylactic reactions after gastric challenge in mice [58]. It is noteworthy that the regular use of moisturizers involves a risk of food allergy to infants irrespective of eczema [59]. Moisturizers increase transepidermal water loss and might favor sensitization by increased percutaneous penetration of food allergens or the disruption of the skin barriers leading to inflammation. In animals with filaggrin loss-of-function mutations, contact with HE protein on the undamaged skin barrier can induce local inflammation and transcutaneous sensitization [58]. However, it is unclear whether filaggrin mutations are linked with food hypersensitivity in infants without atopic eczema [60,61]. Taking into consideration that the exposure of damaged skin to HE induces allergic sensitization, the treatment of eczema is a first-line preventive measure. Accordingly, topical steroids reduce food allergy development by inducing remission of atopic eczema [62]. Additional environmental factors have been associated with HE allergy [63]. It has been hypothesized that when the immune system lacks microbial exposure in infancy, the normal maturity of the immune system against infections is not reached and allergy develops. HE allergy, therefore, more easily occurs in children treated with antibiotics during the first week of life [45]. The absence of contact with vaginal and perianal maternal flora during childbirth may select a different child microbiome that is believed to favor the Th2 phenotype and food allergy. Several studies have not observed the association of HE allergy with caesarean delivery [64–66]; however, when it was detected [44,67], it was unclear whether it was due to a publication bias [68]. Traffic-related air pollution but not second-hand tobacco smoke exposure [69] has been found to increase the risk for HE sensitization [70].

4. Dietary Interventions

Current evidence suggests that in infancy it may be possible to take advantage of a time window to introduce the main food allergens for inducing oral tolerance ("window of opportunity") [44]. An early and continuous oral intake of food protein may induce long-lasting immune tolerance [63], with systemic immune unresponsiveness to ingested allergens, through the GALT (Gut Associated Lymphoid Tissue). The ability of the GALT to ensure a response against pathogens and to suppress that against commensal bacteria and foods is favored by the intestinal microbiota during weaning [71]. The timing of HE introduction into the diet may, therefore, play a part in the development of HE allergy. On the one hand, there is no evidence that avoidance of HE during pregnancy or breast-feeding, or postponing introduction to the weaning diet, prevents clinical HE allergy [72–75]. On the other hand, there are some observations that the early and habitual consumption of HE after birth can decrease the occurrence of HE allergy. The Australian HealthNuts study showed that HE intake between 4 and 6 months of age was related to a decreased chance of HE allergy, compared to delaying HE exposure to between 10 and 12 months of age or beyond 12 months of age [76]. Furthermore, Lai et al. [77] found an increased expansion of ovalbumin-specific T regulatory cells and no HE allergy in infants eating HE between 5 and 10 months of age, in contrast to those who had been introduced to HE after 10 months. In infants with bronchiolitis, 82% of 770 participants were introduced to HE in their diet by age twelve months. The cumulative incidence of likely HE allergy by three years of age was 0.2% among children with HE ingestion before 12 months and 2.2% among children

who were introduced to HE later [78]. Discordant results have been found, however, in six randomized placebo-controlled trials conducted for assessing the efficacy of an early HE introduction into the diet when complementary feeding starts to prevent HE allergy. In infants with atopic eczema, Natsume et al. [79] administered 50 mg/day (about 1/160th of one HE) of cooked lyophilized HE or placebo from 6 to 9 months and then 250 mg/day or placebo until 12 months. At 1 year of age, open HE oral challenge confirmed HE allergy in 8% of infants in the active group and in 38% of controls (RR (95% CI): 0.22 (0.09–0.54); $p = 0.0001$). However, two factors should be considered. First, infants were treated with topical steroids to maintain remission. This may have reduced the sensitization rate [62]. Second, the food challenge was not conducted in 26 members (17%) of the study population, and ITT analysis was not performed. Perkin et al. [54] enrolled 1303 breast-fed infants aged 3 months. A group that was introduced earlier to six allergenic foods (including boiled HE) between three and six months of age was compared to infants with standard weaning. HE allergy was observed in 1.4% of infants in the early-introduction group and in 5.5% of infants in the standard-introduction group between 1 year and 3 years of age ($p = 0.009$). They also found that the intake of cooked (boiled) HE before 6 months predisposed subjects to the occurrence of food protein-induced enterocolitis syndrome (FPIES) to HE. In contrast, Bellach et al. [44] found that the introduction of pasteurized HE that is considered equivalent to raw egg [80] before 6 months of age did not decrease HE allergy onset. They fed a general population 2.5 g (about one-third of one egg) 3 times per week of pasteurized white HE or with a rice placebo from between 4 and 6 to 12 months. At 1 year, the HE oral challenge had a positive result in 2.1% of the active group and in 0.6% of the placebo group, respectively (RR (95% CI): 3.30 (0.35–31.32); $p = 5.35$). In the Solid Timing for Allergy Research (STAR) trial [50], 86 infants from 4–6 months to 8 months with moderate to severe atopic eczema had 0.9 g (about one-sixth of one egg) daily of HE protein, whole pasteurized raw HE or rice. At one year of age, HE allergy was diagnosed in 33% of treated children and in 51% of controls (RR (95% CI): 0.65 (0.38–1.1); $p = 0.11$). In the Starting Time of Egg Protein (STEP) trial [81], infants who had never eaten HE and without allergic diseases, but had mothers suffering from atopic conditions, had 0.9 g of pasteurized raw egg per day (about one half a HE per week) or rice (placebo) from 4–6 months up to 10 months of age. At 12 months, HE challenges resulted positive in 7% of children in the active group and in 10.3% of the control group (RR (95% CI) 0.75 (0.48–1.17); $p = 0.20$). In the Beating Egg Allergy Trial (BEAT) [82], infants with one or birth parents presenting atopic disorder were introduced to 350 mg of pasteurized whole HE from 4 to 8 months, after successful first weaning. At 1 year of age, 6.2% of subjects in the active group had HE allergy, compared to 10.5% in the placebo group ($p > 0.05$). Early exposure to raw HE was further linked with anaphylaxis [44,50]. It seems that a complementary feeding that includes the daily intake of a very small amount of cooked HE probably decreases the onset of HE allergy in the first year of life.

5. What about Guidelines

With a nod to recent trials, recommendations on the timing of introduction of HE in infancy have been released (Table 4). The European Food Safety Authority (EFSA) [83] suggests HE introduction between 4 and 6 months. The Australasian Society of Clinical Immunology and Allergy (ASCIA) [84] recommends that all infants should be given allergenic solid foods, including cooked HE, in the first year of life. It states that there is moderate evidence that introducing cooked HE (raw HE is not recommended) into the diet before 8 months of age in infants with a family history of allergy could reduce the risk of developing HE allergy. Both the European Academy of Allergy and Clinical Immunology (EAACI) [38] and the American Academy of Allergy, Asthma, and Immunology/Canadian Society of Allergy and Clinical Immunology (AAAAI/CSACI) guidelines [85] suggest introducing well-cooked HE but not raw egg or uncooked pasteurized HE into the infant diet from 4 to 6 months of life as part of complementary feeding to prevent HE allergy in infants. In families with infants at general and increased risk, EAACI guidelines [38]

suggest introducing about half a well-cooked, small HE twice a week as part of complementary feeding from 4 to 6 months of age. AAAA/CSACI guidelines [85] suggest the introduction of HE or egg-containing products to all infants, irrespective of their relative risk of developing allergy, between 4 and 6 months of life using only cooked forms of egg and avoiding any raw, pasteurized egg-containing products. The British Society for Allergy and Clinical Immunology (BSACI) [86] recommends starting complementary foods, including HE, alongside breastfeeding, when the infant is ready around 6 months of age (but not before 4 months) since delaying HE from the infant diet does not prevent allergy. Infants with atopic eczema should be introduced to solids, including HE, between 4 and 6 months, when they are ready. A small amount (1 teaspoon) of well-cooked HE egg should initially be given; then, the amount can be gradually increased to a full dose. The Deutschen Gesellschaft für Allergologie und Klinische Immunologie (DGAKI) guideline [87] advises the introduction of complementary foods and HE from 4 to 6 months of age. Baked or hard-boiled HE should be regularly given, while raw eggs (including scrambled and soft-boiled eggs) are not recommended.

Table 4. Guidelines on dietary interventions for preventing egg allergy.

Guideline	Year	Healthy Children	Children with Atopic Eczema	Children with Food Allergy	Family History for Food Allergy
EFSA [83]	2019	Egg introduction at 3–4 months of age compared with 6 months of age may reduce the risk of developing egg allergy (low to moderate confidence in the evidence). There were some anaphylactic reactions associated with the consumption of raw egg but not with cooked egg.	Egg introduction between 4 and 6 months of age may be associated with a lower risk of developing egg allergy at 1 year of age.	Egg introduction between 4 and 6 months of age may be associated with a lower risk of developing egg allergy at 1 year of age	Egg introduction between 4 and 6 months of age may be associated with a lower risk of developing egg allergy at 1 year of age
ASCIA [84]	2020	Between four and six months, start to introduce a variety of solid foods, while continuing breastfeeding. Introduce allergenic solid foods, including cooked egg products, in the first year of life.	As in the healthy child.	As in the healthy child.	Introducing cooked egg (raw egg is not recommended) before 8 months of age, where there is a family history of allergy, can reduce the risk of developing egg allergy.
EAACI [38]	2021	Introducing well-cooked hen's egg, but not raw egg or uncooked pasteurised egg, into the infant diet as part of complementary feeding to prevent egg allergy in infants from 4 to 6 months of life.	Families with infants at general and increased risk to start introducing about half of a well-cooked, small egg twice a week as part of complementary feeding from 4 to 6 months of age.	As in the children with eczema.	NS
AAAAI/CSACI [85]	2021	Egg should be introduced around 6 months of age, but not before 4 months.	Introduce egg or egg-containing products to all infants (only cooked forms of egg and avoid administering any raw, pasteurized egg-containing products) around 6 months of age, though not before 4 months of age.	NS	NS
BSACI [86]	2021	Exclusive breastfeeding until around 6 months of age with complementary foods from around this age.	Introduce egg as a starting weaning food after more traditional weaning foods between 4 and 6 months of age. Give well-cooked egg starting with 1 teaspoon and gradually increase to a full dose.	NS	NS

Table 4. *Cont.*

Guideline	Year	Healthy Children	Children with Atopic Eczema	Children with Food Allergy	Family History for Food Allergy
DGAKI [87]	2021	Exclusive breastfeeding for the first 4–6 months that should continue during introduction of complementary foods. Egg should be introduced at weaning as described for infants with family history.			At weaning, introduction and regular administration of heated-through egg (baked, hard-boiled). It is not recommended to introduce raw egg (including scrambled and soft-boiled eggs).

6. Conclusions

HE is a fundamental food for the development of the child. It contains not only a complete protein profile, which includes all the essential amino acids for growth, but also includes antioxidants, microelements and phospholipids, which have a key role in the maturation of the nervous system. HE allergy, which implies a HE-free diet, must therefore be carefully diagnosed to avoid generalizations and the transformation of a simple sensitization into a clinical diagnosis. In children with HE allergy, it is necessary to periodically check whether they outgrow it. When tolerance to HE is not achieved, oral immunotherapy for HE can be performed. The method of preventing HE allergy should be carefully assessed. The debate on the timing of HE introduction in weaning has led to a change in positions over the last decade. Several studies to which the guidelines have been subsequently adapted identify the opportunity of not delaying the introduction of HE at the time of weaning. HE introduction should be avoided before the fourth month of age. Moreover, there is only one study supporting the preventive effect of introducing cooked HE after the sixth month of age in infants with atopic eczema. Looking at the studies taken together, a delayed introduction has no preventive benefit and may negatively influence the growth and psychological wellbeing of children and their families. In agreement with the most recent guidelines (Table 3), we suggest that HE or HE-containing products should be a regular part of the diet from around 6 months of age, and they should not be introduced earlier than 4 months of age. This can be applied to all infants, even those without atopic eczema or when an atopic family history is lacking, regardless of the relative risk of developing HE allergy. At weaning, well-cooked HE, such as baked and boiled HE, should be given starting with small amounts, while any raw, pasteurized egg-containing products should be avoided to reduce the development of HE clinical hypersensitivity. In infants with mainly moderate to severe atopic eczema or food allergy, it is advisable to carry out the HE skin-prick test before the introduction of HE. When the HE skin-prick test is positive, performing the first HE intake under medical supervision should be considered. Finally, we must not forget that starting weaning, as suggested by EFSA [83], depends on the acquisition of neuromotor skills (such as head and trunk control which allow improved movements of jaw, lip, and tongue) and anatomical changes in the mouth to protect infants from aspiration and choking.

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Abbreviations

AAAAI	American Academy of Allergy, Asthma, and Immunology
ASCIA	Australasian Society of Clinical Immunology and Allergy
BSACI	British Society for Allergy & Clinical Immunology
CSACI	Canadian Society of Allergy and Clinical Immunology
DGAKI	Deutschen Gesellschaft für Allergologie und Klinische Immunologie
EAACI	European Academy of Allergy and Clinical Immunology
EFSA	European Food Safety Authority
FPIES	Food protein-induced enterocolitis syndrome
GALT	Gut Associated Lymphoid Tissue
HE	Hen's egg
PC	phosphatidylcholine
PE	phosphatidylethanolamine
SM	sphingomyelin
STAR	Solid Timing for Allergy Research
STEP	Starting Time of Egg Protein

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How Are Infants Suspected to Have Cow's Milk Allergy Managed? A Real World Study Report

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Abstract: The purpose of this study was to evaluate the diagnosis and management of infants presenting with symptoms attributable to cow's milk allergy (CMA) in a real life setting and to test how the Cow's Milk-related Symptom Score (CoMiSS®) can be used to support the awareness to diagnose cow's milk protein allergy in primary care practice. The CoMiSS is an awareness tool based on various symptoms such as crying, gastrointestinal symptoms, dermatological and respiratory symptoms. The study was conducted on 268 infants from four countries (Belgium, Czech Republic, Germany, UK) aged 0 to 18 months consulting for CMA related symptoms. The analysis was based on two visits of these subjects. The results show an average CoMiSS of 11 at the first visit. After a therapeutic dietary intervention, the score at the second visit, which happened 3 weeks ± 5 days after the first one, dropped to an average value of 4. A satisfaction questionnaire completed by the primary care practitioners suggested an overall high level of satisfaction with the application of the CoMiSS tool in routine practice. These data highlight a huge discrepancy in the diagnosis and management of infants suspected of CMA in the different countries. The findings suggest that the CoMISS questionnaire is an effective tool in aiding awareness of CMPA in primary health care.

Keywords: cow's milk allergy; CoMiSS; extensive hydrolysate; partial hydrolysate; amino acid formula

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1. Introduction

The risk of developing an allergy has become a significant public health issue with increasing prevalence [1]. The diagnosis of cow's milk allergy (CMA) can be challenging, since symptoms can be immediate (IgE mediated) as well as delayed (non-IgE mediated), and involve many organ systems. Gastro-intestinal (GI) symptoms attributed to non-IgE mediated CMA include, amongst others, infantile colic, food protein induced enterocolitis syndrome, food protein induced allergic proctocolitis, food allergic enteropathy, eosinophilic disorders, and food protein induced dysmotility disorders, food protein induced constipation, and food protein induced gastro-esophageal reflux (GER) [2]. Cutaneous manifestations, such as deterioration of atopic dermatitis and urticaria, respiratory symptoms and general manifestations such as failure to thrive, distress and crying are part of the spectrum of CMA [3]. CMA is the most common food allergy in childhood and its prevalence ranges from 1.9% to 4.9% [4]. The diagnosis of CMA is suspected after a thorough history and physical examination, including the evaluation of growth [5].

Up to 25 to over 50% of infants develop functional gastro-intestinal disorders (FGIDs) [6,7]. As the spectrum of manifestations of FGIDs and mainly non-IgE mediated CMA do overlap,

they may be difficult to separate leading to difficulty in distinguishing from FGIDs, GERD and CMA [8]. As a consequence, the prevalence of CMA is debated. Laboratory tests assist the diagnosis of IgE mediated CMA, but can be negative [5]. A confirmed diagnosis of non-IgE mediated CMA requires an oral food challenge (OFC) after a diagnostic elimination diet of two to four weeks [5]. An open food challenge after the elimination diet is considered an adequate diagnostic tool in clinical practice [5]. The double-blind-placebo-controlled-food challenge, considered as the "gold standard", is needed to confirm the diagnosis in clinical research [9]. A well performed challenge includes the progressive at-home reintroduction of milk, which can be safely done, especially in children with non-IgE mediated CMA with delayed reactions [9]. The oral food challenge in patients with IgE mediated reactions (Skin Prick Test (SPT) and/or sIgE positive) should be performed under medical supervision. An early diagnosis of CMA is important as a delayed diagnosis may lead to nutritional disorders and as a consequence an increased risk of impaired growth [10]. Moreover, a delayed diagnosis and incorrect management also increases parent and caregiver anxiety and economic cost as the symptoms place a burden on both the infant and their caregivers [11,12].

Infants with allergic disorders are presented to different healthcare professionals (HCPs) spanning multiple specialties (e.g., general practitioners, general pediatricians but also pediatric subspecialists in gastroenterology, allergy/immunology, dermatology) with diverse levels of expertise [1]. As a consequence, there is a great variability in dietary management approaches [1]. The purpose of this study was to assess in a real life situation the diagnossis and management of infants presenting with symptoms attributable to CMA, and to test the usefulness of the Cow's Milk-related Symptom Score (CoMiSS®) (Table 1).

Table 1. The Cow's Milk-related Symptom Score (CoMiSS®) [3].

Symptom	Score			
Crying	0	≤1 h/day		
	1	1 to 1.5 h/day		
	2	1.5 to 2 h/day		
	3	2 to 3 h/day		
	4	3 to 4 h/day		
	5	4 to 5 h/day		
	6	≥5 h/day		
Regurgitation	0	0 to 2 episodes/day		
	1	≥3 to ≤5 episodes of small volume		
	2	>5 episodes of >1 coffee spoon		
	3	>5 episodes of ±half of the feedings in< half of the feedings		
	4	continuous regurgitations of small volumes >30 min after each feeding		
	5	regurgitation of half to complete volume of a feeding in at least half of the feedings		
	6	regurgitation of the complete volume after each feeding		
Stools (Bristol scale)	4	type 1 and 2 (hard stools)		
	0	type 3 and 4 (normal stools)		
	2	type 5 (soft stool)		
	4	type 6 (liquid stool, if unrelated to infection)		
	6	type 7 (watery stools)		
Skin symptoms	0 to 6	Atopic eczema	Head-neck-trunk	Arms-legs-hands-feet
		Absent	0	0
		Mild	1	1
		Moderate	2	2
		Severe	3	3
	0 to 6	Urticaria (0: no, 6: yes)		
Respiratory symptoms	0	no respiratory symptoms		
	1	slight symptoms		
	2	mild symptoms		
	3	severe symptoms		

2. Materials and Methods

A multicentre prospective observational single cohort study was carried out by 84 HCPs between September 2016 and September 2018. Recruiting sites were located in four European countries including Belgium, Germany, Czech Republic and the United Kingdom (UK). The study was approved by the Ethics Committee and regulatory authority, where applicable. Children were enrolled by independent primary health care practitioners in Belgium and Germany. In the Czech Republic, inclusion was done by primary health care and allergologists. Due to the specific structure of the medical organization in the UK, recruitment was conducted in four specialized pediatric care centers.

Infants of both sexes, of any ethnicity, aged 0 to 18 months suspected of mild to moderate symptoms of CMA as primary clinical impression of the practitioner were consecutively enrolled. Subjects having congenital disease or malformations, significant pre-natal or post-natal diseases, subjects with minor parents or parents who could not comply with study procedures and subjects included in other clinical trials were excluded. Prior to enrolment, a written informed consent was obtained from both parents, or one parent in single-parent families. The study design included two visits: at baseline and after 3 weeks ± 5 days (Figure 1: study flow chart).

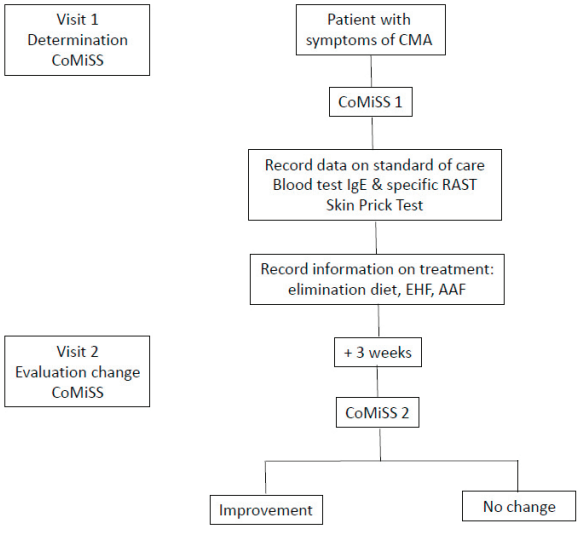

Figure 1. Study flow chart.

The CoMiSS score was determined by the HCP during both visits using the CoMiSS awareness tool form. At baseline, basic information such as date of birth, sex, weight, length and head circumference were recorded, as well as the history of CMA-related symptoms, such as duration of symptoms and diet at baseline. The dietary and medical intervention recommended by the HCP was registered. Information was also recorded regarding the requests of HCPs for investigations such as blood sampling and skin prick testing for diagnostic purposes.

At the end of the study each practitioner was asked to complete a satisfaction questionnaire about the use of CoMiSS awareness tool.

3. Results

Two hundred and sixty-eight subjects (145 boys/117 girls/6 unknown) were found eligible and enrolled and are reported as the intent-to-treat (ITT) population (Table 2. Baseline characteristics; Table 3: Age at inclusion).

Table 2. Baseline characteristics of the 268 included infants.

	Mean	SD	Median	Min	Max	N
Age (weeks)	21.9	16.3	18.4	1.4	80.6	265
Weight (kg)	6.4	2.0	6.3	3.1	13.1	251
Length (cm)	62.7	8.3	62.5	37.0	88.6	231
Duration of symptoms(weeks)	12.1	12.9	7.4	0.0	64.1	255

Legend: SD: standard deviation; Min: minimum; Max: Maximum; n: number of patients for whom information was available.

Table 3. Age distribution of included infants per country.

	Age (Weeks)					
	Mean	SD	Median	Min	Max	N
Czech Republic	24.1	14.2	22.6	1.4	65.1	84
Germany	21.4	17.7	16.4	3.7	79.4	36
Belgium	12.7	10.0	8.7	2.4	53.4	90
UK	34.1	17.7	32.1	4.0	80.6	55
All	21.9	16.3	18.4	1.4	80.6	265 °

Legend: SD: standard deviation; Min: minimum; Max: Maximum; °: age of 3 infants missing.

Out of 268 enrolled subjects, 84 were recruited from Czech Republic, 84 from Belgium, 36 from Germany and 54 from the UK. The final visit was between 16 and 26 days after baseline visit in 208 infants and is reported as the per protocol (PP) population. Among the 268 infants, 16 did not make the final visit, and in 44 the final visit was either less than 16 days or more than 26 days post-baseline visit. Preliminary analysis showed no clinically meaningful difference in the results between the ITT and the PP population; therefore data according to the ITT population analysis are reported

The mean duration of symptoms (Table 4) was 12 weeks and ranged between 7 weeks in Belgium to 24 weeks in the UK.

Table 4. Duration of symptoms before inclusion per country.

	Duration of Symptoms (Weeks)					
	Mean	SD	Median	Min	Max	N
Czech Republic	11.5	10.4	10.5	0.0	49.0	80
Germany	9.7	12.4	5.9	0.0	49.6	35
Belgium	6.9	9.1	4.0	0.0	56.1	90
UK	24.0	15.4	21.2	1.6	64.1	50
All	12.1	12.9	7.4	0.0	64.1	255 °

Legend: SD: standard deviation; Min: minimum; Max: Maximum; °: data missing for 13 infants.

At baseline visit, 31% (83/268) of the included infants were exclusively breastfed, 33% (88/268) were formula-fed, 31% (and 30% (80/268) were on mixed breastfeeding and formula feeding (Data missing of 17/268 (6%) infants). Further, 56% of the formula fed infants were fed standard infant formula, 21% partially hydrolysed formula (pHF), 11% an extensively hydrolysed formula (eHF) and 7% were fed amino acid formula (AAF) formula (Table 5: Feeding per country).

Table 5. Type of formula at inclusion visit in formula-fed or mixed breastfeeding and formula-fed infants.

	Type of Formula									
	SIF		pHF		eHF		AAF		Other	
	n	%	N	%	n	%	n	%	n	%
Czech R	25	47.2	19	35.8	2	3.8	5	9.4	2	3.8
Germany	13	50.0	9	34.6	2	7.7	2	7.7	0	0.0
Belgium	52	72.2	9	12.5	5	6.9	1	1.4	5	6.9
UK	13	38.2	2	5.9	11	32.4	4	11.8	4	11.8
All °	103	55.7	39	21.1	20	10.8	12	6.5	11	5.9

Legend: %: percent; n: number; SIF: standard infant formula; pHF; partially hydrolyzed formula; eHF: extensively hydrolyzed formula; AAF: amino acid formula; R: Republic; °: data for 185 infants (+83 partially breastfed infants) = 268 infants).

The time between the introduction of cow's milk formula and the onset of symptoms was recorded in only 37% (100/268) of subjects. According to the data, the time interval between ingestion of cow's milk and the onset of symptoms in the UK was only a few hours compared to a broad range of 0 up to 90 days in the other countries (Table 6: Time interval between ingestion of cow's milk and appearance of symptoms.

Table 6. Time between first intake of cow's milk and onset of symptoms per country.

	Time between Ingestion of Cow's Milk and Onset of Symptoms (Hours)					
	Mean	SD	Median	Min	Max	N
Czech Republic	219.8	431.4	60.0	0.1	2160.0	28
Germany	321.6	365.6	168.0	0.0	1008.0	10
Belgium	247.6	339.6	60.0	0.0	1080.0	38
UK	3.4	7.3	0.5	0.0	24.0	24
All	188.6	343.1	24.0	0.0	2160.0	100

Legend: SD: standard deviation; Min: minimum; Max: Maximum; n: number.

The CoMiSS was collected from the 268 subjects at the baseline visit. The score ranged from 0 to 28 (Figure 2).

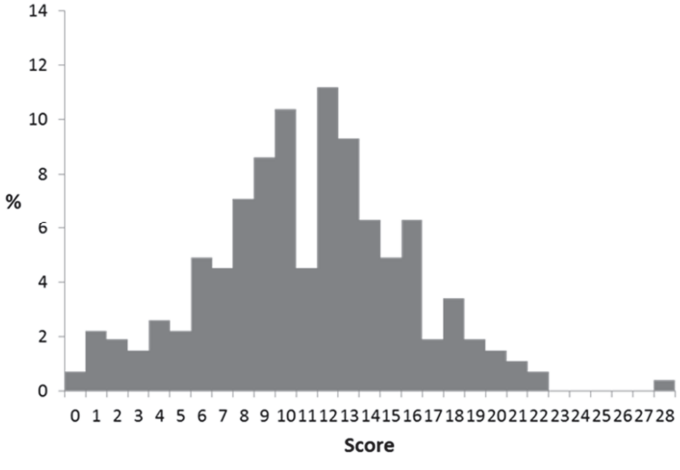

Figure 2. CoMiSS distribution at baseline.

Overall, the mean and median CoMiSS was 11.1 and 11.0, respectively, and 72.3% of subjects had a CoMiSS of >9 and 48.9% a CoMiSS ≥ 12. The CoMiSS score was lowest in the UK. Stratification of CoMiSS according to the cut-off value of 12 divided the infants in two roughly equal groups. However, country-specific CoMiSS stratification reflected that the majority of British and German subjects had scores below <12 (78% and 81%, respectively), while the majority of the Czech infants (82%) had CoMiSS of ≥12 (Table 7).

Table 7. CoMiSS < and >12 distribution per country at baseline and final visit.

	CoMiSS			
	At Baseline		Final Visit	
	<12	≥12	<12	≥12
	n (%)	n (%)	n (%)	n (%)
Czech R	15 (17.9)	69 (82.1)	82 (98.8)	1 (1.2)
Germany	29 (80.6)	7 (19.4)	32 (97.0)	1 (3.0)
Belgium	50 (53.8)	43 (46.2)	87 (97.8)	2 (2.2)
UK	43 (78.2)	12 (21.8)	38 (92.7)	3 (7.3)
All	137 (51.1)	131 (48.9)	239 (97.2)	7 (2.8)

Legend: n: number; %: percent; R: republic.

A cow's milk elimination diet was prescribed in 36% of the breastfeeding mothers (exclusive and mixed breastfeeding, n:164) and in 59% of the formula fed infants. An elimination diet in a breastfeeding mother was almost twice as frequently recommended if the CoMiSS was ≥12 than if CoMiSS was < 12 (24.8 vs. 47.3%, respectively). An eHF and an AAF were prescribed almost equally to 31% of formula fed infants (Table 8). An AAF was prescribed almost twice as frequently in subjects having a CoMiSS ≥ 12 than subjects having CoMiSS <12 (19.7% vs. 42.7%, respectively). Prescription of an eHF appeared almost equal for subjects that had CoMiSS < 12 and ≥12 (~30%). A pHF was recommended in ~6%, including 8% in the group with a CoMiSS ≥ 12.

Table 8. Actions undertaken at first visit, stratified by CoMiSS at baseline.

Action	CoMiSS Score at First Visit				All	
	<12		≥12			
	n	%	n	%	N	%
Elimination diet mother °	34	24.8	62	47.3	96	35.8
Elimination diet child *	68	49.6	91	69.5	159	59.3
pHF prescribed	5	3.6	11	8.4	16	6.0
eHF prescribed	42	30.7	40	30.5	82	30.6
AAF prescribed	27	19.7	56	42.7	83	31.0

Legend: pHF: partially hydrolysed formula; eHF: extensively hydrolysed formula; AAF: amino acid formula. °: exclusive and partial breastfeeding combined; *: partial breastfeeding and full formula feeding.

The prescription rate of pHF differed per country: 0% in the UK, 3% in Germany, 5% in Belgium and 11.9% in the Czech Republic. The prescription rate of eHF and AAF also differed from country to country (Table 9). In the Czech Republic, eHF was prescribed to only 8.3% of the infants, and AAF was recommended in 72.6%. In Germany, eHF and AAF prescription was comparable (27.8 vs. 22.2%, respectively). In Belgium and the UK, an eHF was much more frequently recommended than an AAF (54.8 and 25.5% vs. 8.6 and 10.9%, respectively.

Table 9. Actions undertaken at first visit, stratified by CoMiSS at baseline, per country.

	CoMiSS at First Visit				All	
	<12		≥12			
	n	%	n	%	N	%
Czech Republic						
Elimination diet mother	7	46.7	34	49.3	41	48.8
Elimination diet child	12	80.0	53	76.8	65	77.4
pH formula	1	6.7	9	13.0	10	11.9
eH formula	0	0.0	7	10.1	7	8.3
AA formula	12	80.0	49	71.0	61	72.6
Germany						
Elimination diet mother	5	17.2	2	28.6	7	19.4
Elimination diet child	12	41.4	6	85.7	18	50.0
pH formula	1	3.4	0	0.0	1	2.8
eH formula	6	20.7	4	57.1	10	27.8
AA formula	6	20.7	2	28.6	8	22.2
Belgium						
Elimination diet mother	11	22.0	18	41.9	29	31.2
Elimination diet child	27	54.0	29	67.4	56	60.2
pH formula	3	6.0	2	4.7	5	5.4
eH formula	25	50.0	26	60.5	51	54.8
AA formula	3	6.0	5	11.6	8	8.6
UK						
Elimination diet mother	11	25.6	8	66.7	19	34.5
Elimination diet child	17	39.5	3	25.0	20	36.4
pH formula	0	0.0	0	0.0	0	0.0
eH formula	11	25.6	3	25.0	14	25.5
AA formula	6	14.0	0	0.0	6	10.9

Legend: n: number; %: percent; pH: partial hydrolysate; eH: extensive hydrolysate; AA: amino acid.

The CoMiSS at the final visit was obtained in 246/268 (94%) subjects, and was significantly lower suggesting efficacy of the therapeutic actions taken (Figure 3: CoMiSS after intervention at final visit).

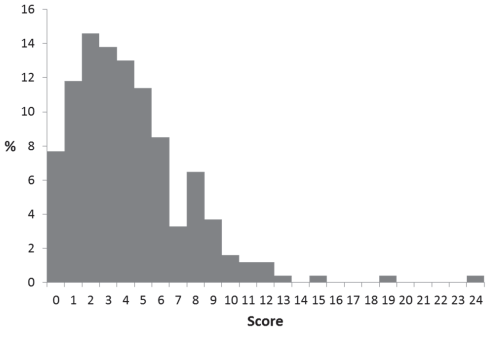

Figure 3. CoMiSS after intervention at final visit.

The overall mean of CoMiSS decreased from 11.1 at baseline to 4.2 at the final visit; the median was reduced from 11 to 4.0. At the final visit, only four infants had a CoMiSS ≥12, and 23 infants had a score of > 9. In formula fed infants, the change in CoMiSS was greater for AAF fed infant than for eHF fed infants (Table 10). The dietary intervention resulted in a significant decrease of the CoMiSS in the vast majority of infants. There was almost no difference between the change of the mean or median CoMiSS. In exclusively breastfed infants, the median CoMiSS decreased by 6.0 during the elimination diet, while the decrease was 11.0 in partially breastfed infants to whom also eHF or AAF was prescribed. Finally, the decreased score in formula fed infants observed with eHF was lower than with AAF (−6.0 vs. −10.0, respectively). A further analysis excluding infants who were on an eHF or AAF at inclusion did not result in a different outcome, as the number of infants on these formulas at inclusion was very low.

Table 10. Dietary intervention and change in CoMiSS.

Intervention	Change in CoMiSS Score at Final Visit					
	Mean	**SD**	**Median**	**Min**	**Max**	**n**
Elimination diet mother	−5.8	5.0	−6.0	−17.0	12.0	96
Partial breastfed and eHF or AAF	−10.0	6.2	−11.0	−17.0	0.0	6
eHF	−6.4	5.1	−6.0	−19.0	5.0	70
AAF	−9.5	4.5	−10.0	−27.0	−1.0	74

Legend: eHF: extensively hydrolysed formula; AAF: amino acid based formula; SD: standard deviation; n: number.

An open oral food challenge to confirm the diagnosis of CMA was performed in 17 infants, and was positive in 4 (24%). At the baseline visit, blood sampling, including IgE tests were much more frequently requested in infants with a CoMiSS ≥12. On the contrary, skin prick tests were less frequently requested in the group with CoMiSS ≥12. There is a large difference in performed diagnostic investigations according to country (Table 11). In Germany skin prick tests were not performed, whilst up to 17% of the infants in the Czech Republic had a skin prick test for cow's milk. Specific IgE for cow's milk was measured in almost half the children in the UK and Czech Republic (49.1 and 50.7%, respectively) whilst only 8.3% had this performed in Germany.

Table 11. Diagnostic actions requested stratified by country (%).

	SPT	**sIgE**
Czech Republic	17.0	50.7
Germany	0	8.3
Belgium	15.1	31.2
UK	3.6	49.1

Legend: SPT: skin prick test for cow's milk; sIgE: specific IgE for cow's milk;%: percent of infants.

At the end of the study, 77/84 (91.6%) health care providers completed the satisfaction questionnaire. Approximately 3 in 5 agreed that the CoMiSS was helpful to consider the diagnosis of CMA more rapidly. Seventy percent intended to continue using the CoMiSS tool and 77% would recommend CoMiSS to their colleagues. About 25% mentioned that the CoMiSS tool lengthens consultation time (Table 12).

Table 12. Satisfaction questionnaire for health care provider.

	Questions	Response Health Care Provider (%)				
		Fully Agree	Agree	=	Disagree	Strong Disagree
1	The time it took you on average to complete the CoMiSS tool did NOT significantly lengthen the total consultation time.	18	45	12	20	5
2	Based on the experience of your use of the CoMiSS tool, you think/believe that this tool is helpful in the diagnosis of CMPA.	25	61	9	5	0
3	You think/believe that the tool can help physicians to diagnose infants with CMPA faster.	17	64	13	6	0
4	You intend to continue using the CoMiSS tool in your practice.	21	49	18	9	3
5	You would recommend the tool to your colleagues.	19	57	17	4	3

Legend: =: neither agree or disagree.

4. Discussion

The CoMiSS was initially developed as the Symptom Based Score and was intended to facilitate comparability of the efficacy of two extensively hydrolyzed formulas in patients suspected of CMA, and included in a prospective, randomized, double-blind trial [13]. A group of key opinion leaders suggested this tool could be used as an awareness tool in order to increase the awareness of the most common symptoms of CMA to aid an earlier diagnosis [3]. This study confirms that the CoMiSS can be considered as a useful awareness tool for HCPs, what was already previously suggested in a review paper [14].

This real world collection of data highlights the differences in baseline characteristics in infants suspected to suffer from CMA per country, which can be related in part to the varied health care system practices (Tables 3–7). Therefore, the second visit was scheduled after 3 weeks, considering the recommendations for a diagnostic elimination diet during 2 to 4 weeks and local health care organization habits. This was an observational study, whose primary objective was to describe the diagnostic and therapeutic actions taken both overall and stratified by baseline CoMiSS in a general pediatric population consulting for symptoms possibly related to CMPA. The median age at inclusion was 18.4 weeks, but differed from 8.7 weeks in Belgium to 32.4 weeks in the UK, and, as a consequence, a large discrepancy in the mean duration of symptoms was observed, varying from 4.0 weeks in Belgium to 21.4 weeks in the UK. Moreover, a large difference in time between ingestion of cow's milk and appearance of symptoms was reported according to the country, and ranged from 0.5 h in the UK, over 60 h in Belgium and Czech Republic, to as long as 168 h in Germany. Systemic blood sampling was not part of this observational study (as it was the goal to highlight differences according to country), but the differences in baseline characteristics suggest that the children included in the UK presented mostly with IgE mediated allergy because of the short lapse of time between ingestion of cow's milk and appearance of symptoms, while the German infants mainly have non-IgE mediated allergy. These huge discrepancies in baseline characteristics contribute to a better understanding of the discrepancies in diagnosis, management and outcome of CMA according to country, and thus health care system. These baseline differences in population are likely to explain

the difference in CoMiSS scoring between countries. About 50% of the infants had a baseline CoMiSS $\geq$ 12, but this ranged from 19.4% in Germany to 82.1% in the Czech Republic (Table 8). Initially, an arbitrarily decided cut-off value of $\geq$12 had been proposed to predict the likelihood of CMA (3). A score of 12 requires the presence of a minimum of two severe symptoms and a score of >12 requires the presence of at least three symptoms and two organ systems [3]. Subsequent evidence from literature, both in a supposed healthy population and in symptomatic infants suggest that a cut-off of > 9 might be more appropriate [15–17]. Therefore, a further study exploring the efficiency of cut-off > 9 might be of interest.

A pHF was recommended as a therapeutic intervention in 6% of the infants, which is in disagreement with all existing guidelines [1]. The difference in prescription rate of pHF per country, ranging from 0% in the UK to 11.9% in the Czech Republic also illustrates the differences in education and training of the HCPs across Europe. Variation was also observed in prescription rates of eHF and AAF according to country. Differences in population selection, education and training as well as differences in reimbursement systems may contribute to these discrepancies. In the Czech Republic, there is full reimbursement of eHF and AAF, if prescribed by allergologists and pediatric gastroenterologists; general practitioners can only obtain reimbursement for 5 packages of AAF per patient. In Germany, there is full reimbursement in case of demonstrated IgE mediated allergy, while in non-IgE-mediated allergy, resolution of symptoms after 2 weeks of cow's milk exclusion needs to demonstrated and full reimbursement is obtained if the efficacy is well documented. In Belgium, only AAF is (almost) fully reimbursed, on condition allergy to an eHF is demonstrated or if symptoms are severe (failure to thrive, anaphylaxis). Inconsistencies in the diagnostic investigations performed per country again illustrate the differences in health care systems practices between countries. Since in the UK much more children are included with immediate type of reactions, suggesting IgE mediated allergy, it is logical that IgE levels are much more frequently determined than in countries such as Germany where the vast majority of children have delayed reactions, indicating non-IgE mediated allergy (Table 10). The discrepancy between UK and Germany in IgE and non IgE CMA can probably also be explained by difference in study sites, since in Germany mainly gastroenterologists did collaborate (and symptoms involving the GI tract are mainly non-IgE) while in the UK allergists were involved, who do see more frequently IgE mediated symptoms.

The elimination diet resulted in a decrease of 9 points or more in 51% of the infants (Table 11). In formula-fed infants, the decrease with AAF was larger than with eHF. According to this observational study, prescription of an AAF formula was the only factor with a relevant effect. As this is an open observational study, interpretation on efficacy may be biased. ESPGHAN and other guidelines recommend that the first line of prescription should be an eHF. However, this study suggested that primary HCPs frequently recommended AAF as the first formula. The reasons why this is observed has to be further investigated, as this could be due to differences in education and training, patient selection, availability of products, and reimbursement, which may all influence the choices made by the HCP.

5. Conclusions

This observational study suggests that CoMiSS is an efficient and reliable tool to facilitate awareness of the diagnosis of CMA in real life for HCPs. These results also show the discrepancies between countries, as baseline characteristics differ substantially according to country. It is very likely that the practice between different health care systems contributes to the variation observed in patient selection, diagnostic procedures and management.

Author Contributions: Y.V.: concept of study, analysis of data, writing original manuscript; S.B., A.E., P.F., N.M.: collection of data, analysis of data, approval manuscript; A.J.: concept of study, organization study, approval manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: No additional data.

Conflicts of Interest: Y.V. has participated as a clinical investigator, and/or advisory board member, and/or consultant, and/or speaker for Abbott Nutrition, Ausnutria, Biogaia, By Heart, CHR Hansen, Danone, ELSE Nutrition, Friesland Campina, Nestle Health Science, Nestle Nutrition Institute, Nutricia, Mead Johnson Nutrition, Phathom Pharmaceuticals, United Pharmaceuticals (Novalac), Yakult, Wyeth. A.E. is consultant and/or speaker for Nutricia, Nestlé Health Science, Nestlé Nutrition Institute, Infectopharm. M.N.: received funding to attend educational conferences and also honoraria for giving presentations from Nestlé Health Science. A.J. is an employee of Nestlé Health Science. The other authors reported no COI.

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Adherence to the Mediterranean Diet Improves Fatty Acids Profile in Pediatric Patients with Idiopathic Nephrotic Syndrome

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Abstract: The fatty acid profiles of patients with idiopathic nephrotic syndrome (INS) are different from that of healthy controls, even during remission, revealing an increase of the pro-inflammatory omega 6 series. It is still unknown whether the concomitance of nephrotic syndrome affects the potential positive effects of the Mediterranean diet on the levels of omega 3 and 6 fatty acids. We performed a cross-sectional study to evaluate the association between the adherence to the Mediterranean diet and fatty acid profile in 54 children with INS. The dietary habits were assessed through the validated Kidmed questionnaire. Patients with higher adherence had lower levels of linoleic acid and total omega-6. Moreover, a negative correlation between proteinuria and the anti-inflammatory omega-3 series was found. In conclusion, patients with INS with proteinuria and low adherence to the Mediterranean diet have an imbalance in the omega-6/omega-3 ratio that may benefit from following the Mediterranean diet.

Keywords: Mediterranean diet; nephrotic syndrome

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1. Introduction

Idiopathic nephrotic syndrome (INS) is the most common glomerular disease in children, characterized by the triad of oedema, proteinuria and hypoalbuminemia. The underlying biological mechanisms are not fully understood, but in most cases, they probably involve immunological processes [1].

Fatty acid profiles of subjects with INS, even during remission, is different from that of healthy controls [2], particularly with regard to omega-6 fatty acids levels, whose increase may indicate a state of persistent latent inflammation, ultimately dependent on the well-known imbalance of lymphocyte sub-populations [3]. A correct dietary balance between omega-6 and omega-3, in favor of the latter, is essential both in healthy subjects and in those with kidney disease [4].

The Mediterranean diet is one of the three more healthy diets in the world, as adherence to it positively correlates with the levels of omega 3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are known for their anti-inflammatory properties [5]. Moreover, in the general population, the Mediterranean diet is also able to counteract hypercholesterolemia, which is, however, a biochemical marker of nephrotic syndrome.

KidMed [6] is a questionnaire designed to assess adherence to the Mediterranean diet in pediatric subjects. It is a survey composed of 16 simple questions, and has been extensively validated by the scientific literature [7].

It is still largely unknown whether the concomitance of INS in each subject affects the potential positive effects of the Mediterranean diet on the levels of omega 3 and 6 fatty acids.

The aim of this study is to evaluate if adherence to the Mediterranean diet can influence the fatty acids profile in pediatric patients with INS, during proteinuria and remission, and if there is a correlation among patients' fatty acid pattern and the typical biochemical parameters of INS.

2. Patients and Methods

2.1. Patients

We performed a single-center, cross-sectional study, which included all pediatric (<18 years old) patients with INS who underwent a routine blood test at the Pediatric Nephrology, Dialysis and Renal Transplant Unit of Fondazione Cà Granda IRCCS Ospedale Maggiore Policlinico, Milan, Italy, between 27 October 2020 and 8 January 2021. The inclusion criteria were pediatric patients (<18 years old) with diagnosis of INS, as specified below. Patients whose parents/legal guardians did not consent to the study were excluded.

Patients were enrolled according to the Helsinki declaration statement.

2.2. Definition of INS

Idiopathic nephrotic syndrome was defined by the presence of oedema, proteinuria >40 mg/m^2/h in a 24 h urine collection, or a spot urine protein to creatinine ratio (uPr/uCr) of >2 mg/mg, and albuminemia <2.5 g/dl, in the absence of secondary causes of NS. Patients were further classified as steroid resistant or steroid dependent according to international guidelines [8].

2.3. Adherence to Mediterranean Diet

Adherence to the Mediterranean diet was evaluated by means of the Kid Med questionnaire. Patient's parents or legal guardians were asked to fill the questionnaire in during the visit. The questionnaire consists of 16 questions on eating habits, each to be answered Yes (score: 1 or −1) or No (Score: 0). A total score is eventually calculated, whose maximal and minimal values range from −4 to 12.

2.4. Fatty Acid Analysis

A blood sample of 200 µL was collected during routine checks. An aliquot of 50 µL was transferred into vials, methylated with 800 µL of HClMe 3N (Sigma Aldrich, Schnelldorf, Germany), incubated for 1 h at 90 °C and then refrigerated ad 4 °C for 10 min. Afterwards, 2 mL of KCl solution (Sigma Aldrich, Schnelldorf, Germany) and 330 µL hexane (Sigma-Aldrich, Schnelldorf, Germany) were added. Samples were first vortexed and then centrifuged at 3000 rpm for 10 min. Finally, the hexane layer (the upper layer) was collected from each vial and transferred into a gas chromatography vial for fatty acids profile evaluation with a Shimadzu Nexis GC-2030 (Shimadzu, Japan) gas chromatographer equipped with a 30 m fused silica capillary column FAMEWAX Restek (Restek, Centre Country, PA, USA). The gas chromatography results were analyzed using Labsolution software (Shimadzu, Japan).

Single fatty acids were expressed as relative percentage of total fatty acids.

Total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), total polyunsaturated fatty acids (PUFA), total omega-3 (N3) and total omega-6 (N6) were also calculated.

2.5. Biochemical Analyses

Urinary protein (UPr), urinary creatinine (UCr), serum triglycerides, total cholesterol and HDL cholesterol were measured and the uPr/Cr ratio was calculated as part of patients' routine check analyses.

2.6. Statistical Analysis

Data were correlated by two tailed Pearson bivariate analysis; PCA analysis was performed to confirm results. The paired T-test was used to compare fatty acid profiles between different patient groups. p-values < 0.05 were considered statistically significant. Statistical analysis was performed with software SPSS 21 (IBM).

2.7. Ethical Approval

The study protocol was approved by a local Ethical committee with document number 0035199-U.

3. Results

3.1. Patients

A total of 54 patients (mean age 11 years ± 4; 27 males, 27 females; mean body weight 23.1 ± 8.6 kg) were enrolled: 44 of them were of Italian origin, three of Central/South American origin, six of Northern African origin and one of Asian origin. 37 patients were steroid dependent, 29 were in remission while eight were in relapse and were receiving treatment with steroid-sparing drugs including mycophenolate mofetil and calcineurin inhibitors. 1Seventeen patients were initially steroid resistant: nine of them subsequently responded to calcineurin inhibitors and were in remission, while eight who did not respond to immunosuppressant medications were classified as multidrug resistant and were proteinuric.

Table 1 summarizes the biochemical data of the patients according to the presence or absence of proteinuria. As expected, patients with proteinuria had significantly higher levels of total cholesterol and lower levels of serum protein and albumin than those in remission.

Table 1. Biochemical data during proteinuria and remission.

	Proteinuria	**Remission**
	mean ± s.d.	mean ± s.d.
uPr/uCr * (mg/mg)	2.81 ± 2.13 *	0.24 ± 0.29
Triglycerides (mg/dl)	117.11 ± 61.72	90.09 ± 50.51
Total cholesterol (mg/dl) *	204.77 ± 52.75 *	158.16 ± 41.16
HDL cholesterol (mg/dl)	69.83 ± 23.26	62.10 ± 16.80
Total protein (g/dl) *	5.66 ± 0.71 *	6.72 ± 0.45
Total albumin (g/dl) *	3.33 ± 0.82 *	4.51 ± 0.31

uCr: urinary creatinine; uPr: urinary proteins. * p-value < 0.05 (T-test).

3.2. Adherence to Mediterranean Diet

48 out of 54 patients answered the questionnaire.

Patients' adherence to the Mediterranean diet was in the medium-high quartile, with an average score of 5.13 ± 2.2 (minimum value 1, maximum value 9, on a scale from −4 to 12). In detail, the number of patients who responded Yes or No to each Kidmed question is summarized in Table 2.

The population was then divided into two groups according to the KidMed score value (taking into account that the mean KidMed score was 5.1): those with high adherence (KidMed score ≥ 6), and those with low adherence (KidMed score < 4). All the cases with a score of 5) were excluded from the analysis.

No significant differences in Kidmed scores were found across different clinical groups: steroid dependent nephrotic syndrome (SDNS) vs. steroid resistant nephrotic syndrome

(SRNS); Proteinuria vs. Remission; SDNS in remission vs. SDNS with proteinuria; SRNS in remission vs. SRNS with proteinuria) (Table 3).

Table 2. Number of patients and responses (Yes/No) to the Kidmed questionnaire.

	Yes	No	Score
(1) Takes a fruit or fruit juice every day?	30	18	Yes + 1/no 0
(2) Has a second fruit every day?	20	28	Yes + 1/no 0
(3) Has fresh or cooked vegetables regularly once a day?	34	14	Yes + 1/no 0
(4) Has fresh or cooked vegetables more than once a day?	21	27	Yes + 1/no 0
(5) Consumes fish regularly (at least 2–3 times per week)?	21	27	Yes + 1/no 0
(6) Goes more than once a week to a fast-food (hamburger) restaurant?	5	43	Yes − 1/no 0
(7) Likes pulses and eats them more than once a week?	13	35	Yes + 1/no 0
(8) Consumes pasta or rice almost every day (5 or more times per week)?	38	10	Yes + 1/no 0
(9) Has cereals or grains (bread, etc.) for breakfast?	31	17	Yes + 1/no 0
(10) Consumes nuts regularly (at least 2–3 times per week)?	12	36	Yes + 1/no 0
(11) Uses olive oil at home?	44	4	Yes + 1/no 0
(12) Skips breakfast?	12	36	Yes − 1/no 0
(13) Has a dairy product for breakfast (yoghurt, milk, etc.)?	35	13	Yes + 1/no 0
(14) Has commercially baked goods or pastries for breakfast?	29	19	Yes − 1/no 0
(15) Takes two yoghurts and/or some cheese (40 g) daily?	6	42	Yes + 1/no 0
(16) Takes sweets and candy several times every day?	9	39	Yes − 1/no 0

Table 3. Kidmed score values in the clinical groups of Idiopathic nephrotic syndrome (INS).

	SD	SR	Remission	Proteinuria	SD in Remission	SD with Proteinuria	SR in Remission	SR with Proteinuria
KIDMED score	5.00 ± 2.48	5.37 ± 1.68	5.31 ± 2.36	4.56 ± 2.35	5.40 ± 2.40	3.60 ± 3.60	5.50 ± 1.80	5.25 ± 1.58

Additionally, we didn't detect any significant difference (p-value 0.53). in Kidmed scores between males (score 5.3 ± 2.1) and females (score 4.9 ± 2.3) of our cohort.

3.3. Fatty Acids Profile

When the population was divided into two groups according to the grade of adherence to the Mediterranean diet, the fatty acid profiles were significantly different with regard to linoleic acid and omega 6 levels, which were higher in subjects with low adherence, and saturated 22: 0 and 24: 0, which were higher in patients with high adherence. Total omega-3 and DHA levels were not statistically different in the two groups, but there was a tendency for both to be higher in patients with high adherence (Table 4).

Table 4. Fatty acid profile of patients with low or high adherence to the Mediterranean diet.

	Low Adherence (Kidmed Score 2.56 ± 1.21) *n* = 16	**High Adherence (Kidmed Score 7.67 ± 0.72) *n* = 15**	
	mean ± s.d.	mean ± s.d.	*p*-value
16.0 (Palmitic acid)	23.01 ± 1.57	22.99 ± 1.90	0.97
16:1 (Palmitoleic acid)	0.79 ± 0.34	0.85 ± 0.37	0.65
18:0 (Stearic acid)	11.34 ± 1.80	12.10 ± 2.58	0.35
18:1n9 (Oleic acid)	17.21 ± 2.73	17.53 ± 2.17	0.72
18:1n7 (Cis-vaccenic acid)	1.21 ± 0.22	1.29 ± 0.29	0.35
18:2n6 (Linoleic acid)	25.15 ± 5.34 *	21.54 ± 4.46	0.05
18:3n3 (Linolenic acid)	0.22 ± 0.15	0.20 ± 0.09	0.67
20:3n9 (Mead acid)	0.13 ± 0.13	0.18 ± 0.28	0.54
20:3n6 (DGLA)	1.65 ± 0.39	1.53 ± 0.28	0.32
20:4n6 (Arachidonic acid)	11.33 ± 2.01	11.63 ± 1.80	0.66
20:5n3 (EPA)	0.19 ± 0.17	0.21 ± 0.16	0.75
22:0 (Behenic acid)	0.80 ± 0.53 *	1.29 ± 0.51	0.01
22:5n3 (DPA)	0.66 ± 0.23	0.77 ± 0.17	0.12
24:0 (Lignoceric acid)	1.80 ± 0.67 *	2.39 ± 0.75	0.02

Table 4. *Cont.*

	Low Adherence (Kidmed Score 2.56 ± 1.21) *n* = 16	**High Adherence (Kidmed Score 7.67 ± 0.72) *n* = 15**	
22:6n3 (DHA)	2.39 ± 0.77	2.84 ± 0.65	0.08
24:1 (nervonic acid)	2.01 ± 0.50	2.47 ± 0.87	0.07
SFA	36.96 ± 2.84	38.77 ± 4.74	0.20
MUFA	21.22 ± 2.98	22.15 ± 1.95	0.31
PUFA	41.72 ± 3.80 *	38.90 ± 3.55	0.04
N3	3.45 ± 0.91	4.03 ± 0.74	0.06
N6 *	38.14 ± 4.18	34.70 ± 3.77	0.02

DGLA = Dihomo-gamma linoleic acid; EPA = Eicosapentaenoic acid; DPA = Docosapentenoic acid; DHA = Docosahexaenoic acid; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; N3 = total omega-3 fatty acids; N6 = total omega-6 fatty acids.* *p*-value < 0.05 at *T*-test.

The KidMed score values also showed a negative correlation with the levels of omega-6 (R^2 −0.32; *p*-value 0.02) and a positive correlation with the saturated fatty acids (SFA) 22:0 e 24:0 (R^2 and *p*-value 0.28/0.04 and 0.31/0.03 respectively).

3.4. Correlations of Biochemical Parameters with KidMed Score and Fatty Acids

No significant correlations were found between KidMed score values and all biochemical parameters of INS.

As regards fatty acids, proteinuria, expressed as uPr/uCr, negatively correlated with the omega 3 DHA (R^2 −0.32, *p*-value 0.03), with the total omega-3 (R^2 −0.35, *p*-value 0.02) and with the saturated fatty acid18:0 R^2 −0.3, *p*-value 0.04). This data was confirmed by PCA analysis (See Supplementary Materials).

There was a negative correlation between triglycerides and arachidonic acid (R^2 −0.39 *p*-value 0.012), the SFA 24:0 (R^2 −0.35, *p*-value 0.02) and total PUFAs (R^2 −0.32 *p*-value 0.04). Inversely, a positive correlation was found between triglycerides and the MUFA oleic acid (R^2 0.42, *p*-value 0.006) and total MUFAs (R^2 0.46, *p*-value 0.002).

Total cholesterol negatively correlated with arachidonic acid (R^2 −0.36 *p*-value 0.02) and 18:0 (R^2 −0.32 *p*-value 0.04), while it positively correlated with linoleic acid levels (R^2 0.36 *p*-value 0.019).

Total serum protein showed a positive correlation with 18:0 (R^2 0.44 *p*-value 0.03) and SFA (R^2 0.3 *p*-value 0.04) while serum albumin showed a positive correlation with 18:0 (R^2 0.42 *p*-value 0.04) and arachidonic acid (R^2 0.35 *p*-value 0.01)

4. Discussion

To the best of our knowledge, there is no data in the literature on the relation of the Mediterranean diet to nephrotic syndrome, despite the potential positive effects of the Mediterranean diet on various biochemical parameters (which are notoriously changed during INS), such as fatty acids, cholesterol and triglycerides.

Therefore, this study is the first which aimed to evaluate the dietary aspect of adherence to the Mediterranean diet in an important and widespread childhood renal disease like INS.

Our data show that this pediatric cohort, predominantly composed of Caucasian subjects of Italian origin, had a medium-high adherence to the Mediterranean diet, even if a small percentage of subjects (10%) significantly deviated, regardless of their ethnicity. The consumption of industrial snacks at breakfast (29 out of 48 subjects) and skipping breakfast (12 out of 48) impacted most negatively on the Kidmed score.

As expected, adherence to the Mediterranean diet, as evaluated by the Kidmed score, was independent of patients' clinical classification of INS and the presence of proteinuria. An analysis by sex and gender was conducted, even if previous studies did not find sex and gender differences in the adherence to the Mediterranean diet [7]. No significant difference was found in our cohort as regards the score on adherence to the Mediterranean diet for males compared to females.

In this study, adherence to the Mediterranean diet mainly influenced the blood levels of linoleic acid and total pro-inflammatory omega-6s, which were higher in patients with lower adherence than in those with higher adherence.

This is consistent with the correlation reported in the literature between maternal breast milk composition in omega 6 and adherence to the Mediterranean diet [9].

On the contrary, levels of omega 3s were higher, even if not significantly, in patients with higher Kidmed score than in those with a lower score. This data was confirmed in the group of patients without proteinuria, where the 11 patients who had a Kidmed score of more than 6 showed a higher level of omega-3 and DHA than the 8 who had a Kidmed score less than 5 (eleven vs. eight). We could not confirm this data in the population with proteinuria (nine and four patients, respectively),

Moreover, in our population, the levels of DHA and total omega-3, which are known for their anti-inflammatory properties, negatively correlated with proteinuria. As a consequence, we may hypothesize that a low level of these PUFAs contributes to INS progression and delay the remission of proteinuria.

Therefore, poor adherence to the Mediterranean diet and the state of proteinuria resulted in an increase of omega-6 fatty acids, in particular18:2n6 (linoleic acid), and a decrease of omega-3 and DHA, resulting in an imbalance of the N6/N3 ratio, in favor of a pro-inflammatory state.

As regards the increase of blood linoleic acid in patients with low adherence to the Mediterranean diet and the issue of possible dietary measures, it should be pointed out that linoleic acid is not considered at present a negative micronutrient per se. In effect, it has been established that its moderate intake is able to reduce total cholesterol and LDL concentrations [10] and to prevent the risk of development of cardiovascular disease [10,11]. However, the role of omega-6 metabolites is clearer, because they are involved in the inflammatory process in response to air pollution [12], and cause a shift in the microbiota, contributing to an increase of colonic inflammation [13]. Finally, a higher omega-6 dietary intake has been associated to an increase of pro inflammatory metabolites [14].

The importance of respecting a correct n-6/n-3 ratio in the diet, for the purpose of preventing chronic kidney disease, is essentially linked to the anti-inflammatory functions of linolenic acid (ALA, 18:3n-3) and longer-chain n-3 PUFAs [14]. According to this point

of view, it would be advisable to reduce the amount of omega-6 in the diet, particularly in patients with nephrotic syndrome, in order to improve the dietary n6/n3 ratio, As a matter of fact, it has been recently demonstrated that patients with INS showed an abnormal fatty acid profile and high also arachidonic acid blood levels during remission, which allows for the hypothesis of a persistent inflammatory state in this population, even in the absence of proteinuria.

High cholesterol and triglycerides levels were inversely correlated with arachidonic acid, and positively correlated with MUFAs. The inverse correlation with arachidonic acid could be due to the fact that hypercholesterolemia causes an increase in the production of LTB4 [15], a metabolite of arachidonic acid, with the consequent decrease of arachidonic acid levels. As regards MUFAs, several studies in animal models have shown that high MUFA levels are associated with an increase in total serum cholesterol [16,17].

Interestingly, the absence of correlation between adherence to the Mediterranean diet and total cholesterol suggests that the Mediterranean diet is not able to effectively counteract the hypercholesterolemia of proteinuric patients with INS, as is the case of healthy subjects [18]. This could be due to the fact that the hepatic compensation mechanism of hypoalbuminemia, which is responsible for hypercholesterolemia, is persistent over time and dissociated from diet.

5. Conclusions

In patients with INS, better adherence to the Mediterranean diet modifies the omega-6/omega-3 ratio in favor of the anti-inflammatory omega-3s and reduces blood linoleic acid levels. This data suggests that patients with INS, particularly in the phase of proteinuria, may benefit from following the Mediterranean diet.

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Dietary Determinants of Anemia in Children Aged 6–36 Months: A Cross-Sectional Study in Indonesia

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Abstract: Anemia has been acknowledged as worldwide problem, including in Indonesia. This cross-sectional study aims to explore dietary determinants as risk factors for anemia in children aged 6–36 months living in a poor urban area of Jakarta. The study was done in Kampung Melayu sub-district in Jakarta, Indonesia. Data was collected within two weeks in September–October 2020. A structured questionnaire for a 24-h recall and a semi-quantitative Food Frequency Questionnaire (FFQ) were used to collect the dietary intake data, and venous blood was withdrawn to determine the hemoglobin levels. Bivariate chi-square and multiple logistic regression tests were executed to explore the dietary determinant factors for anemia. We recruited 180 subjects. The average hemoglobin concentration was 11.4 ± 1.7 mg/dL; the anemia prevalence was 29.4%. The following variables were significantly associated with higher risk of anemia: no cow's milk formula consumption, inadequate intake of fats, protein, calcium, vitamin D, iron, zinc, vitamin A, vitamin C, vitamin B6, and vitamin B12. Only cow's milk formula consumption and zinc intake were revealed as the determinant factors of anemia. In conclusion, the prevalence of anemia was 29.4% among children aged 6–36 months old. Anemia was significantly associated with two dietary determinants as risk factors that are cow's milk formula consumption and zinc intake.

Keywords: anemia; cow's milk; cow's milk formula; zinc; toddler; Indonesia

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1. Introduction

Anemia has been acknowledged as a worldwide health problem that young children are specifically vulnerable. The data from the World Health Organization (WHO) shows that anemia prevalence in children aged 6–59 months in Indonesia are 43.9% in 2000 and 38.4% in 2019 [1]. A similar anemia prevalence (38.5%) is also reported from Indonesian national data in 2018 [2]. Another study in Indonesian rural area in 2009–2010 showed that the prevalence of anemia and iron deficiency anemia (IDA) in children aged 6–59 months were 56.9 and 29.4%, respectively [3]. The prevalence was higher than the WHO data in 2000 or the latest national data in 2018 that might indicate higher risk of anemia in the rural area. Childhood anemia contributes to poor motor and cognitive development resulting in poor school performance, and results in increased morbidity and mortality [4].

There are two types of anemia: nutritional and non-nutritional related. In nutritional anemia, there is insufficient intake of nutrients to meet the need for hemoglobin and erythrocyte synthesis. Special attention needs to be given to the consumption of iron-rich or iron fortified foods because iron deficiency is the common cause of anemia among under-five year old children [5]. It is estimated to contribute to 42% of anemia cases in under 5-year-old children worldwide [6]. Other nutrients that contribute to anemia are deficiencies of vitamin A, B2 (riboflavin), B6 (pyridoxine), B12 (cobalamin), C, D, E, folate, and copper [6]. Most anemia studies in under-five-year-old children highlighted

the relation with maternal factors, socio-economic factors, and failure to thrive related factors. A systematic review found that poor dietary diversity is one of the predictors for anemia in under-five-year-old children, along with failure to thrive, food insecurity and not being dewormed [7]. A study in Indonesia in 2017 found that the small quantity of lipid-based nutrient supplement was effective in improving the hemoglobin level and reduced the incidence of anemia in infants aged 6–12 months after the three-month intervention period [8]. The study showed that this supplement could fill the gap of iron intake as it contained 6 mg Fe and 30 mg vitamin C [8]. This shows the importance of dietary intake as a determinant factor in childhood anemia. Therefore, this new study aims to explore dietary determinant as risk factors of anemia among children aged 6–36 months living in a poor urban area of Jakarta.

2. Materials and Methods

Study design. This study is an observational analytical cross-sectional study.

Location and time. The poor urban Kampung Melayu sub-district in Jakarta, Indonesia, was purposively selected because it was the only area permitted by the local authority while other areas were closed due to COVID-19. Data collection was done within two weeks in September–October 2020, while strictly applying the COVID-19 health safety procedure.

Population and sample. Children aged 6–36 months were recruited from the selected Posyandu (i.e., community health post) after obtaining the signed informed consent from their parents. Those children who were seriously ill and/or needed special medication were excluded. It was calculated that at least 80 subjects were needed as a minimal sample size, considering an anemia prevalence of 29.4%, with a 95% degree of significance (Z_{alpha} = 1.96) and 90% degree of reliability.

Data collection. Socio-demographic characteristics of the subjects, i.e., age, sex, general health status, parents' education, and family income were collected using a structured questionnaire. Macronutrient intake was determined using a dietary intake assessment of a one-day 24-h recall, while for the micronutrient intake data was collected using semi-quantitative food frequency questionnaire (FFQ) over a period of the past two weeks [9]. Inadequate intake was defined as an intake that was below the Indonesian recommended daily allowance (RDA). Anemia was diagnosed using the cyanmethemoglobin method for venous blood to measure hemoglobin levels. The cut-off of hemoglobin level less than 11.0 g/dL is used, and we assumed that nutritional factors were likely to be the most important [10].

Data management and analysis. All data were recorded using a clinical record form before being entered into the spreadsheet using SPSS version 20.0. After data cleaning, data were analyzed using descriptive and inferential statistical tests to explore possible determinants of anemia using chi-square, and logistic regression analyses were performed in those with p-value < 0.020 according to the chi-square test [11]. A statistically significant level was determined using p-value less than 0.05.

Ethics: Data collection was done after receiving ethical approval released dated 27 April 2020 by the Ethical Committee Faculty of Medicine Universitas Indonesia (i.e., No. KET-438/UN2.F1/ETIK/PPM.00.02/2020) and obtaining informed consent from the parent.

3. Results

The subjects' recruitment and data collection were permitted by the local authority for two weeks only because of safety reasons. During the COVID-19 pandemic, under-five year old children were not allowed to go outside their house. Even the monthly Posyandu for child health and nutrition monitoring was closed. Thus, we collaborated with the Posyandus' volunteer health workers to screen the eligible subjects to participate in this study.

From the total of 185 participants (Table 1), we could not obtain a balanced inclusion according to age category, but sex distribution was similar. Regarding socio-demographic parental characteristics, the majority of fathers and mothers were mostly graduated from senior high school (64.9% and 58.9%, respectively) and had non-permanent jobs (61.6% and 96.8%, respectively), with a household income that was less than the recommended provincial minimal income (77.3%). These conditions matched the characteristics of a slum urban area in which the houses are very small and mostly rented with dense crowded neighborhoods.

Table 1. Socio-demographic characteristics of children aged 6–36 months.

Socio-Demographic Characteristics	Total Subject (185)
Age, month	22 (6–36)
Age group, *n* (%)	
6–11 month	27 (14.6)
12–23 month	75 (40.5)
24–36 month	83 (44.9)
Sex, *n* (%):	
Boy	90 (48.6)
Girl	95 (51.4)
Education of Father, *n* (%):	
Up to Junior high school	65 (35.1)
Senior high school and over	120 (64.9)
Education of Mother, *n* (%):	
Up to Junior high school	76 (41.1)
Senior high school and over	109 (58.9)
Occupation of Father, *n* (%)	
Not permanent	114 (61.6)
Permanent	71 (38.4)
Occupation of Mother, *n* (%)	
Not permanent	179 (96.8)
Permanent	6 (3.2)
Household income, *n* (%)	
Less than minimal income	143 (77.3)
Fulfill to minimal income	42 (22.7)

Legend: the population was homogeneous regarding their socio-economic characteristics.

Table 2 shows that the majority of subjects were reported to have exclusive breastfeeding experience for six months (78.4%), 63.2% subjects consumed cow's milk growing-up formula, and only 21.1% subjects took vitamin-mineral supplements.

Table 2. Feeding practice of children aged 6–36 months (*n* = 185).

Feeding Practice	n (%)
Exclusive BF practice for 6 months, *n* (%)	145 (78.4)
Intake of cow's formula milk, *n* (%)	117 (63.2)
Taking supplement, *n* (%)	39 (21.1)

Data in Table 3 reveals that more than 50% of the subjects had insufficient dietary intake of energy, carbohydrate, fats, calcium, vitamin D, and folate according to the Indonesian RDA. Insufficient dietary intake of iron and vitamin C were found in 48.1% and 30.3% of the subjects, respectively.

As shown in Table 4, the mean hemoglobin level was 11.4 ± 1.7 mg/dL, and the lowest hemoglobin value was found among subjects aged 6–11 months (10.9 ± 1.6 mg/dL). The prevalence of anemia (i.e., hemoglobin less than 11.0 mg/dL) was 29.4%, and the highest prevalence was found among those aged 6–11 months (42.3%). Significant difference of hemoglobin level was found related to the fathers' education and household

income. However, there is no significant difference in anemia prevalence based on socio-demographic characteristics.

Table 3. Nutrient intake of children aged 6–36 months (*n* = 185).

Nutrients	Mean ± SD or Median (Min–Max)	Inadequate Intake *n* (%)
Dietary energy intake, in Kcal/day	969.8 (90.5–2230.0)	130 (70.3)
Carbohydrate to total energy, in %	55.3 ± 9.7	147 (79.5)
Fats to total energy intake, in %	32.0 (8.0–51.0)	108 (58.4)
Protein to total energy intake, in %	12.0 (6.0–25.0)	35 (18.9)
Protein intake, in g/kg body weight	2.9 (0.6–8.3)	
Dietary calcium intake, in mg/day	481.5 (35–3381.8)	112 (60.5)
Dietary iron intake, in mg/day	7.4 (0.4–74,0)	89 (48.1)
Dietary zinc intake, in mg/day	4.5 (0.6–54.9)	58 (31.4)
Dietary vitamin A intake, in mcg/day	1021.8 (62.4–7041.4)	37 (20.0)
Dietary vitamin D intake, in mcg/day	2.9 (0–119.8)	172 (93.0)
Dietary B6 intake, in mg/day	0.8 (0.1–119.9)	37 (20.0)
Dietary B9 intake, in mcg/day	132.9 (15.7–597.8)	104 (56.2)
Dietary B12 intake, mcg/day	2.5 (0.2–2004.5)	50 (27.0)
Dietary vitamin C intake, mg/day	60.6 (4.1–445.4)	56 (30.3)

Table 4. Hemoglobin and anemia status of children aged 6–36 months (*n* = 180).

Subject's Sociodemographic Characteristics	*n*	Hemoglobin Level	Anemia Prevalence *n* (%)
Total	180	11.4 ± 1.7	53 (29.4)
Age group			
Age 6–11 month	26	10.9 ± 1.6	11 (42.3)
Age 12–23 month	74	11.5 ± 1.6	19 (25.7)
Age 24–36 month	80	11.3 ± 1.8	23 (28.7)
Sex			
Boy	89	11.3 ± 1.7	26 (29.2)
Girl	91	11.4 ± 1.7	27 (29.7)
Education of Father, *n* (%):			
Up to Junior high school	63	10.9 ± 1.7 *	23 (36.5)
Senior high school and over	117	11.6 ± 1.7	30 (25.6)
Education of Mother, *n* (%):			
Up to Junior high school	74	11.1 ± 1.7	25 (33.8)
Senior high school and over	106	11.5 ±1.7	28 (26.4)
Occupation of Father, *n* (%)			
Not permanent	112	11.3 ± 1.8	33 (29.5)
Permanent	68	11.4 ± 1.6	20 (29.4)
Occupation of Mother, *n* (%)			
Not permanent	174	11.4 ± 1.7	51 (29.3)
Permanent	6	10.7 ± 1.7	2 (33.3)
Household income, *n* (%)			
Less than minimal income	149	11.2 ± 1.7 *	48 (32.2)
Fulfill to minimal income	31	12.0 ± 1.4	5 (16.1)

* *p*-value < 0.05.

Using bivariate analysis (Table 5) to explore the dietary determinants of anemia among children aged 6–36 months, this study found significant associations between anemic status and inadequate dietary intake of fats (OR = 2.675), protein (OR = 3.3526), calcium (OR = 4.663), iron (OR = 3.681), zinc (OR = 3.960), vitamin A (OR = 4.525), vitamin C (OR = 2.797), vitamin B6 (OR = 2.860), vitamin B12 (OR = 3.290), and not consuming cow's milk formula (OR = 9.849).

Table 5. Determinants factors of anemia among the subjects (n = 180).

Determinant Factors	*n*	Anemia *n* (%)	*p*-Value	OR (CI95%)	OR (Logistic Regression) *
Energy intake					
Adequate	54	12 (22.2)	0.164		
Inadequate	126	41 (32.5)			
Carbohydrate intake					
Adequate	38	10 (26.3)	0.634		
Inadequate	142	43 (30.3)			
Fats intake					
Adequate	76	14 (18.4)	0.006	2.657	
Inadequate	104	39 (37.5)		(1.316–5.366)	
Protein intake					
Adequate	148	36 (24.3)	0.001	3.526	
Inadequate	32	17 (53.1)		(1.601–7.764)	
Calcium intake					
Adequate	71	9 (12.7)	<0.001	4.663	
Inadequate	109	44 (40.4)		(2.102–10.347)	
Vitamin D intake					
Adequate	13	1 (7.7)	0.074		
Inadequate	167	52 (31.1)			
Iron intake					
Adequate	94	16 (17.0)	<0.001	3.681	
Inadequate	86	37 (43.0)		(1.852–7.315)	
Zinc intake					
Adequate	124	25 (20.2)	<0.001	3.960	4.262
Inadequate	56	28 (50.0)		(2.000–7.842)	(p = 0.042)
Vitamin A intake					
Adequate	145	33 (22.8)	<0.001	4.525	
Inadequate	35	20 (57.1)		(2.087–9.811)	
Vitamin C intake					
Adequate	127	29 (22.8)	0.003	2.797	
Inadequate	53	24 (45.3)		(1.415–5.528)	
Vitamin B6 intake					
Adequate	145	36 (24.8)	0.006	2.860	
Inadequate	35	17 (48.6)		(1.334–6.130)	
Folate intake					
Adequate	80	19 (23.8)	0.134		
Inadequate	100	34 (34.0)			
Vitamin B12 intake					
Adequate	133	30 (22.6)	0.001	3.290	
Inadequate	47	23 (48.9)		(1.631–6.637)	
Type of milk consumed					
Cow's milk formula	113	14 (12.4)	<0.001	9.849	8.651
Non-cow's milk formula	67	39 (58.2)		(4.695–20.662)	(p < 0.001)

Legend: The first OR is the result from the bivariate analyses (chi-square test), while the second OR is the result from the logistic regression analysis, which was performed among those with p-value < 0.020 from the bivariate analyses (chi-square test). * Including p-value of <0.020 from the bivariate analysis after controlling for sex, father's education status and household income status.

Based on further analysis using the logistic regression (Table 5) after controlling for fathers' education and household income, this study found still two dietary determinant factors that significantly contributed to anemic status among children aged 6–36 months: inadequate dietary intake of zinc (OR = 4.262) and not consuming cow's milk formula (OR = 8.651).

4. Discussion

The socio-demographic characteristics of the area in this study matched the characteristics of a slum urban area. The prevalence of inadequate intake was found almost for all macro and micronutrient intake, namely carbohydrate, fats, calcium, vitamin D, folate, iron, and vitamin C. The prevalence of anemia among children aged 6–36 months was 29.4%, in which zinc intake and absence of cow's milk formula consumption were the key nutrient determinant factors that significantly associated with anemia.

The World Health Organization (WHO) has classified an anemia prevalence, estimated from serum levels of hemoglobin, between 20.0 to 39.9% as a significant moderate public health problem and a prevalence above 40% as of severe public health significance [10]. Thus, the anemia prevalence among children aged 6–36 months found in our study (29.4%) falls into the category of moderate public health significant. The prevalence of anemia in this study was lower than the reported prevalence in children aged 6–59 months in a rural area of Indonesia in 2014 (56.9%) [4] or the Indonesian national data in 2018 (38.5%) [2]. More concern should also be raised specifically for the age group 6–11 months because the prevalence of anemia in this age group reached 42.3%, what is classified by the WHO as of severe public health significance. The high anemia prevalence in this specific age group should also be carefully interpreted because the number of children in this age group was unfortunately much smaller than the other age groups. Furthermore, the study only provides a general description of anemia prevalence based on hemoglobin analysis with the assumption that nutritional factors were likely to be the most important factors. A shortcoming of our study design is the absence of other indicators of anemia, i.e., mean corpuscular volume (MCV), total count of erythrocytes, or reticulocyte count, thus limit the interpretation to a specific cause of anemia, i.e., iron deficiency anemia.

The high prevalence of anemia (41.1%) in a study done in Ethiopia was significantly associated with a number of variables, such as age group, living in an urban area, no formal education of mothers, primary education of mothers, low family monthly income, early introduction of complementary foods, and underweight [12]. Similarly, a cross-sectional study in the Lao People's Democratic Republic in 2017 reported a prevalence of anemia among children aged < 5 years of 43.0%, with three determinant factors identified: sex, underweight, and residence status [13]. Another cross-sectional study in Peru found that 53% children aged 6–35 months suffered from anemia that was associated with intestinal infection and poor access to safe drinking water [14]. In our study, the prevalence of anemia was not significantly associated with socio-demographic characteristics, but significantly associated with insufficient dietary intake of almost all relevant nutrients [15], i.e., more than 50% of inadequate dietary intake of fats, calcium, vitamin D, and folate intake, and more than 30% of inadequate dietary intake of iron, zinc, and vitamin C intake, also more than 15% inadequate intake of protein vitamin A, vitamin B6, and vitamin B12.

Due to the time restriction to conduct the study, it was not possible to conduct multiple 24-h recalls nor validate the semi-quantitative FFQ in this study. However, based on the FAO dietary assessment guideline, a one-day 24-h recall is still relevant to determine the mean intakes for a specific group or a population, i.e., children aged 6–36 months in this study. Given the time restriction, the semi-quantitative FFQ in this study was adapted from the existing one by adjusting the food list to target specific nutrients, which were known related to anemia. The existing semi-quantitative FFQ with Indonesian food list is commonly used in Indonesia, specifically for under-five-year-old children. The FFQ has the strength to capture a range of foods, specific nutrient(s), or a specific food group, including rarely consumed food items [16].

Parents had a sufficient level of education (i.e., graduated from senior high school), but more than 60% of the fathers and almost all the mothers had non-permanent jobs. Aligned with the parents' situation, more than 70% of the households had an income of less than the minimal recommendation for appropriate living in Jakarta. Furthermore, the limited household incomes might affect the availability and affordability of nutritious foods and increase child malnutrition resulting in anemia. In this study it was revealed

that children aged 6–36 months were at risk of anemia if they had inadequate dietary intake of both macro nutrients (i.e., fats, and protein), and micronutrients (i.e., calcium, iron, zinc, vitamin A, vitamin C, vitamin B6, and vitamin B12), and also if not consuming cow's milk formula. This showed that anemia among children aged 6–36 months old was not only associated with iron intake. Houghton et al. [17] concluded from a study among children aged 12–23 months in New Delhi that although iron deficiency was found to be the only nutrition factor significantly associated with anemia, most of the children who were classified as anemic were having multiple micronutrient deficiencies, including folate, vitamin D, vitamin B12, zinc, and vitamin A.

There was a negative association between dietary calcium intake and anemia because calcium inhibits iron absorption. However, the results from most multiple-meal studies suggested that calcium would have only a small effect on iron absorption unless habitual calcium consumption was very low. Thus, it was concluded that dietary calcium intake was unlikely to have a biologically significant impact on iron balance, and the mechanism by which calcium reduced iron absorption remained undetermined. It is predicted that the interaction of calcium with other meal components might have a role, for example, with the protein content in dairy milk or with phytate content in bread [18]. Inadequate dietary calcium intake in this study did not exist as one of the determinant factors of anemia.

For vitamin D, Chowdhury et al. [19] reported that vitamin D deficiency was associated with moderate anemia in young children in North India, while the effect was independent of iron deficiency. Up to now, the role of vitamin D in increasing the risk of anemia is still debated and needs to be evaluated in further studies.

Anemia is mostly associated with iron deficiency status. There is a significant positive correlation between dietary iron intake and hemoglobin values among children aged 24–36 months from the same area [20]. This shows the importance of an appropriate dietary intake in the prevention of anemia. However, a study among preschooler children in Brazil found that there was no significant difference between dietary intake of iron, energy, and protein between those children with or without iron depletion or iron deficiency anemia status [21]. This finding suggests that besides paying attention to adequate intake of dietary iron, other factors such as socio-economic of the household and childcare are also important as confounding variables [22].

As mentioned in the Brazilian study [21], inadequate intake of lipids and protein was also associated with anemia. Considering oil intake as an intake of lipid, a community-based cross-sectional study done in South-West Ethiopia found that iron deficiency anemia was significantly lower among subjects consuming oils [22]. Not-consuming protein-source foods, not-consuming dairy products, not-consuming discretionary calories, low family income and intestinal parasitic infections were predictors of IDA [22]. Dietary fat intake contributed as a source of energy and was important for fat-soluble vitamins, namely vitamin A that is known to reduce anemia [23]. With regard to dietary protein intake, it is well-known that protein plays a central role in iron metabolism through transferrin transports and ferritin stores of iron [24].

In this study, inadequate dietary zinc was associated with anemia among children aged 6–36 months old, and it remained as one of the two determinant factors for anemia. The odds of anemia were 3.8 times greater for children with inadequate dietary zinc intake. Dietary zinc intake was also found to be associated with anemia in children less than 24 months of age in a study done in Guatemala, in which the odds of anemia were more than 3 times greater for infants/toddlers with zinc deficiency [25]. Zinc is known as part of more than 100 enzymes and transcription factors involved in growth and cell division, as well as for erythropoiesis, stabilizing erythroid cell membranes, acting as catalyst of the enzyme that modulates erythroid transcriptional gene expression, supports the proliferation of immature erythroblasts, and influences the development of erythroid stem cells. Furthermore, by compromising the function of zinc-dependent antioxidant enzymes, the life span of red blood cells is reduced in the presence of zinc [26].

The odds of anemia in this study were 4.5 times greater for children with insufficient intake of vitamin A. Willows et al., found in their study among preschool children in Yunnan Province, China, that more anemic children had intakes below the estimated average requirement for vitamin A compared to non-anemic children [23]. It is known that besides reducing anemia, vitamin A may also reduce the prevalence of chronic infections, which can result in anemia.

The significant association between inadequate dietary vitamin C intake and anemia could be explained through the fact that vitamin C is known to facilitate ferric ion reduction to ferrous, which is more easily absorbed. Therefore, dietary vitamin C intake is beneficial in preventing anemia [23].

The insufficient dietary intake of vitamins B6 and B12 can be related to anemia because both vitamins are associated with homocysteine concentration. The decrease in those vitamins level correspond with an increase in homocysteine concentrations [26]. Furthermore, increased homocysteine levels are associated with iron deficiency anemia biomarkers, i.e., hemoglobin, hematocrit, and serum ferritin [27].

This study had odds of anemia 8.6 times greater for children not consuming cow's milk formula. The possible explanation was that cow's milk formula had been fortified with important micronutrients, including iron, to support child growth and development. Therefore, children that consumed cow's milk formula had a lower risk of anemia. Cow milk (unmodified) is known to have poor iron content and absorption, low vitamin C content, and in contrast, high casein and calcium content that could negatively influence iron absorption, and thus hemoglobin synthesis [28–30].

The present study has several limitations. First, anemia prevalence was only based on measurement of hemoglobin level without assessing other anemia indicator, i.e., MCV, total count of erythrocytes, or reticulocyte count. Second, the semi-quantitative FFQ was adapted from the existing one without validation due to restricted time given by the authority for data collection. Third, the absence of the possibility of checking for under- or over-reporting of nutrient intakes. However, this study had the strength in identifying significant dietary determinant factors of anemia in a homogenous socio-economic condition. This result could be used as a consideration to intervene anemia prevalence in the poor urban area.

5. Conclusions

In conclusion, this study shows that almost 30% of infants and toddlers in this selected population suffer from anemia. There are two determinant factors that contribute to the anemic status among children age 6–36 months, i.e., inadequate dietary intake of zinc and not consuming cow's milk formula. Therefore, the government should continue to assist communities to maintain a diversified dietary intake that will provide an adequate intake of both macro- and micronutrients. Further research must determine the appropriate local diet for children aged 6–36 months, especially for those living in a poor urban area.

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Estimated Prevalence and Care Pathway of Feeding and Eating Disorders in a French Pediatric Population

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Abstract: Feeding and Eating Disorders (FED) are mostly described in infants and adolescents but are less well-known in children. Information on the prevalence of FED in the general pediatric population is still limited. The aim of this study was to estimate the prevalence and the care pathway of FED in a population aged 0–18 years old, using the Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 classification. Two physicians interviewed 401 families using a questionnaire including demographics, BMI, dietary behavior data, and age-appropriate screening tools. Qualitative and quantitative variables were compared using the Chi^2 test and Student's t-test, respectively. After a headcount adjustment based on the French population by age group, the estimated prevalence rate was 3% [95%CI (1.7–5.1)] for Avoidant and Restrictive Food Intake Disorder (ARFID), and 9.7% [95%CI (7.2–13.0)] for Unspecified FED (UFED), which included other restrictive and compulsive FED. The median age for ARFID was 4.8 years (0.8–9 years), and 7.5 years (0.6–17 years) for UFED. The interviews did not identify cases of anorexia, bulimia, binge eating disorder, other specified FED, pica or rumination. Only 15.2% of children with an FED were receiving medical care. The development of validated pediatric screening tools, as well as the training of health professionals in children FED is necessary.

Keywords: feeding and eating disorders; pediatrics; prevalence

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1. Introduction

The knowledge of Feeding and Eating Disorders (FED) in children and adolescents has evolved significantly since the 2000s. Early descriptions of pediatric eating disorders (ED) were limited in the DSM-III (Diagnostic and Statistical Manual of Mental Disorders) to pica or rumination, while anorexia nervosa (AN) and bulimia nervosa (BN) were described in adolescents in the same way as in adults [1]. Later studies have contributed to a better understanding of FED in young children [2,3]. Accordingly, the DSM-IV included for the first time in 1998 a chapter dedicated to ED in children aged up to 6 years old [1], but many children were identified as having "ED not otherwise specified". Subsequently, the classification of GOSH (Great Ormond Street Hospital, London, UK) was proposed for children aged from 6 to 13 years old, which included early AN, restrictive eating, selectivity/neophobia, emotional food avoidance, functional dysphagia and food refusal [4,5]. The first cases of binge eating disorders (BED) in children were also described in the 2000s [6,7]. In order to include children over 6 years old [8], the new DSM-5 classification (2013) suppressed the age limit and proposed a classification based on types of FED [1]. This included new diagnostic categories, notably Avoidant and Restrictive Food Intake

Disorder (ARFID), BED, Other Specified FED (OSFED) and Unspecified FED (UFED). In contrast to AN and BN, ARFID is not associated with weight and shape concerns. ARFID includes restrictive eating, selective eating, phobia of swallowing and/or vomiting and food avoidance emotional disorder. The definition states that ARFID is an apparent lack of interest in eating or food and/or an avoidance based on the sensory characteristics of food and/or concern about aversive consequences of eating, associated with one (or more) of the following: significant weight loss, significant nutritional deficiency, dependance on enteral feeding or oral nutritional supplements, marked interference with psychosocial functioning. When ARFID is associated with a concurrent medical condition or mental disorder, its severity exceeds the eating disturbance routinely associated with these troubles, which makes classification difficult to follow in some practical cases [9]. OSFED include atypical AN, BN, BED, purging disorder, night eating syndrome and UFED associate clinically significant distress or impairment in social, occupational, or other important areas of functioning predominate but do not meet the full criteria for any of the disorders in the FED diagnostic class.

Although interest in FED has increased with DSM-5, prevalence studies in the general pediatric population are still limited, particularly in pre-pubertal children [10]. Pediatricians are actually aware of feeding disorders (FD) in infants, and of ED (AN and BN) in adolescents. In contrast, ED in toddlers (1–3 years old), and ED in the first and middle childhood (3–12 years old), are less well known. The aim of the present study was to estimate the prevalence of FED in a population of children and adolescents aged from 0 to 18 years old, using the DSM-5 classification. Secondary objectives were to correlate FED diagnosis with BMI, and to assess the care pathway. Our hypothesis was that patients with FED were poorly cared for, particularly in children aged from 1 to 12 years old.

2. Materials and Methods

2.1. Study Design

We carried out a non-interventional cross-sectional study in one French department (Seine-Maritime, Normandy), from 3 May 2019 to 5 March 2020. The protocol was approved by a Committee for the Protection of Persons on 18/03/2019 (N° RCB 2019-A00067-50). During general pediatric consultations, two pediatricians invited families to participate in the study with an information sheet. These consultations were conducted in a pediatric department. Patients were seen consecutively in order of arrival, without selection, until reaching a total of 400 patients, almost 100 patients in each of four age groups (0–1 year old, 1–6 years old, 6–12 years old and 12–18 years old). Physicians used an anonymized questionnaire to conduct the interview, which included demographic, medical, and age-appropriate dietary behavior data. Parents and adolescents also completed a part of the questionnaire with age-appropriate screening tools. The estimated participation time was 20 minutes. Physicians helped families if questions were not understood.

2.2. Inclusion Criteria

Children aged from 0 to 18 years were included, regardless of the reason for consulting and of their previous medical condition. Exclusion criteria were patients who were over 18 years of age on the day of the interview, parents or adolescents who were illiterate or unable to answer the questionnaire, patients who were not in a good physical or mental state to answer the questionnaire at that time or who did not agree to participate.

2.3. Data Collected in the Questionnaires

2.3.1. Demographic and Medical Data

Each questionnaire included, for all patients, demographic data (age, gender), medical data (physicians measured weight, height, body mass index (BMI), listed reason for consultation, personal history), and questions targeting parents' feelings about their child's current eating disorder (perception of the child's eating difficulties, which care pathway). Overweight and underweight were assessed according to the latest validated French growth curves [11]. After 2 years of age, children were considered underweight if BMI $\leq$ International Obesity Task Force (IOTF) 17 (grade II) and overweight if BMI $\geq$ IOTF 25.

2.3.2. Age-Appropriate Dietary Behavior Questions and Additional Screening Tools

Questionnaires also included age-appropriate dietary behavior questions and additional screening tools. These tools were extracted from the literature according to feasibility criteria, including completion time:

- For infants aged less than 1 year old, if the infant was fed only with milk, the type of feeding was specified (breast and/or bottle feeding), and parents completed the Baby Eating Behavior Questionnaire (BEBQ), which explores the enjoyment of food, food responsiveness, slowness in eating, satiety responsiveness and general appetite [12]. If the infant had started food, parents were asked about dietary diversification and then completed the Behavioral Pediatrics Feeding Assessment Scale (BPFAS), which evaluates the child's behavior and the parents' feelings about or strategies for dealing with eating problems, giving three scores (child, parent, total frequency score) [13,14]. BEBQ and BPFAS do not have a validated cut-off.
- For children aged from 1 to 12 years old, parents were asked about the child's eating behavior and then completed the BPFAS questionnaire.
- For adolescents, parents were asked about their eating behavior, and adolescents completed three screening questionnaires: the Sick Control One Fat Food, French (SCOFF-F), which is positive when the score is $\geq$2 [15,16], the Eating Attitudes Test EAT-26, which is positive when the score is $\geq$20, and explores dieting, bulimia and oral control [17,18], they are both predictive for eating disorders, and the Three-Factor Eating Questionnaire (TFEQ-R18), which does not have a cut-off, but evaluates cognitive restriction, uncontrolled eating and emotional eating [19,20].

2.4. Classification of Disorders According to DSM-5

After each interview, the physicians allocated one or more eating behavior characteristic to each patient ("FED items"), according to a predefined grid and in blind-fashion regarding screening tool scores, which were calculated later: no FED, breastfeeding difficulties, difficulty taking the bottle, refusal of the spoon, refusal of pieces, big eater, picky eater, neophobic/selective, restrictive with nutritional or psycho-social consequences, phobia of swallowing and/or vomiting, emotional food avoidance, hyperphagia, tachyphagia, nibbling between meals, compulsive eating, nocturnal nibbling, nocturnal hyperphagia, rumination, pica, AN, BN, BED, orthorexia. Then each patient with FED items was classified according to the DSM-5 classification (AN, BN, BED, ARFID, OSFED, pica, rumination, or UFED). Only significant and problematic isolated or associated FED items were used to classify patients' FED. For example, isolated items like tachyphagia or nibbling were not considered.

2.5. Statistical Analysis

A descriptive analysis was done for the entire cohort and for each age group. The analysis included sex ratio, age, BMI, FED prevalence rates, screening tool scores and the care pathway. Qualitative variables were described in percentages, and quantitative variables were described with medians (minimal-maximal) for age, or with means for score results ($\pm$standard deviation SD). When data on some items were missing in screening tool

questionnaires, raw scale scores were transformed to a 0–100 scale [(raw score − lowest possible raw score)/possible raw score range)/100]. A headcount adjustment based on the French population by age group [21] was carried out for the overall calculation of FED prevalence from 0 to 18 years old. Statistical analysis was done with the software XlstatBiomed 2020.3.1 (Addinsoft, Paris, France). The normality of the distribution was checked with the Shapiro-Wilk test ($p > 0.05$). Chi-square was used for comparisons of categorical data. Continuous variables were described with means, SD, and compared using the Student's t-test. Ninety-five percent confidence intervals (CI) were calculated for the prevalence of FED.

3. Results

3.1. Study Sample and Demographic Data

A total of 401 questionnaires were collected (Table 1). In the 0–1 year-old group, 58 infants were fed only with milk (only breastfeeding $n = 20$, breast and/or bottle-feeding $n = 11$, only bottle-feeding $n = 27$), and 43 older infants had started food. The reasons for consultations were very varied (medicine 77.3%, surgery 22.0%, psychological 2.7%). Only three consultations were nutrition-related (one follow-up for obesity and two infants for decreased appetite, without a known FED). The prevalence of overweight (including obesity) was 20.2% (markedly higher over the age of 6 years), and the prevalence of underweight was 4.5% (most prevalent in the 1–6 years age group) (Figure 1).

Table 1. Demographic data and BMI data of the pediatric population, by age group.

	Age Groups (Total $n = 401$ Patients)				
	0–1 Year Old $n = 101$	**1–6 Years Old $n = 99$**	**6–12 Years Old $n = 100$**	**12–18 Years Old $n = 101$**	p *
Sex ratio	1.2	0.86	1.2	1.1	0.58
Median age (min-max)	3.5 months (0.1–11.9)	3 years old (1–5.5)	8 years old (6–11.5)	14 years old (12–18)	
BMI overweight (including obesity)	8.9%	15.1%	28%	28.7%	<0.001
BMI grade II underweight	1.9%	12.1%	4%	0%	<0.001

* χ^2 test was used for comparisons of categorical data, Student's t-test was used for comparisons of continuous variables.

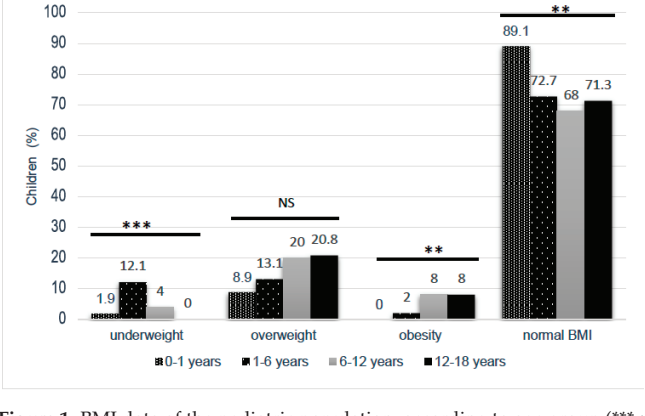

Figure 1. BMI data of the pediatric population, according to age group (*** $p < 0.001$, ** $p < 0.01$, NS no significant, χ^2 test).

3.2. FED Items

A total of 427 FED items were identified. The percentage of eating behavior characteristics was calculated according to the age groups concerned (Table 2). Since some items were age-specific, we could not compare the global prevalence of items across age groups; however, the highest number of items was observed in the 12–18 years age group. There were more restrictive FED items in the 1–6 years age group (picky eater, restrictive eater), and more compulsive FED items in adolescents (hyperphagia, nocturnal nibbling, tachyphagia, nibbling between meals). The item "selective eater" was found in all age groups with a frequency of 2–9%. Eighteen percent of selective eaters had severe selectivity (less than 10 different foods): they were aged between 3 and 4.5 years old, and one of them had an autism spectrum disorder.

Table 2. Prevalence of feeding and eating disorders (FED) items by age group.

***n* = FED Item/Patient (%)**	**Age Groups (Total *n* = 401 Patients, 427 FED Items)**				
	0–1 Year Old *n* = 101 Infants *n* = 23 Items	**1–6 Years Old *n* = 99 Children *n* = 112 Items**	**6–12 Years Old *n* = 100 Children *n* = 133 Items**	**12–18 Years Old *n* = 101 Adolescents *n* = 159 Items**	***p* (χ^2 Test)**
Breastfeeding withdrawal problems *n* = 2/31(6.4%)	2/31 breast-feeding (6.4%)	0/99 (0%)	NA	NA	NA
Difficulty taking the bottle *n* = 0 (0%)	0/101 (0%)	0/99 (0%)	NA	NA	NA
Refusal of the spoon *n* = 2/142 (1.4%)	2/43 infants who had started food (4.6%)	0/99 (0%)	NA	NA	NA
Refusal of pieces *n* = 10/343 (2.9%)	4/43 infants who had started food (9.3%)	6/99 (6%)	0/100 (0%)	0/101 (0%)	0.004
Picky eater *n* = 66/401 (16.4%)	6/101 (6%)	30/99 (30%)	17/100 (17%)	13/101 (13%)	<0.001
Neophobic/selective eater *n* = 22/343 (6.4%)	1/43 infants who had started food (2.3%)	7/99 (7%)	9/100 (9%)	5/101 (5%)	0.57
Restrictive eater *n* = 7/343 (2%)	0/101 (0%)	6/99 (6%)	1/100 (1%)	0/101 (0%)	0.01
Phobia of swallowing and/or vomiting *n* = 4/300 (1.3%)	NA	3/99 (3%)	1/100 (1%)	0/101 (0%)	0.16
Emotional food avoidance *n* = 1/300 (0.3%)	NA	0/99 (0%)	1/100 (1%)	0/101 (0%)	NA
Big eater *n* = 93/401 (23.2%)	8/101 (8%)	25/99 (25%)	29/100 (29%)	31/101 (31%)	0.001
Hyperphagia *n* = 20/343 (5.8%)	NA	0/99 (0%)	8/100 (8%)	12/101 (12%)	0.003
Compulsive eating *n* = 5/343 (1.4%)	NA	0/99 (0%)	1/100 (1%)	4/101 (4%)	0.07
Nocturnal nibbling *n* = 4/343 (1.1%)	NA	0/99 (0%)	0/100 (0%)	4/101 (4%)	0.02
Nocturnal hyperphagia *n* = 1/343 (0.3%)	NA	0/99 (0%)	0/100 (0%)	1/101 (1%)	0.37
Tachyphagia *n* = 101/343 (29.4%)	NA	21/99 (21%)	30/100 (30%)	50/101 (50%)	<0.001
Nibbling between meals *n* = 89/343 (25.9%)	NA	14/99 (14%)	36/100 (36%)	39/101 (39%)	<0.001
Anorexia or bulimia nervosa, binge eating disorder, rumination, pica, orthorexia *n* = 0/300	NA	0/99 (0%)	0/100 (0%)	0/101 (0%)	NA

Abbreviations: FED feeding and eating disorders, NA not applicable.

3.3. Prevalence of FED According to DSM-5

In a second step, we classified patients having FED according to the DSM-5 classification and the estimated prevalence was calculated for each age group (Table 3). We identified 2.7% of patients with ARFID, median age 4.8 years (0.8–9 years) and 8.7% of patients with UFED, median age 7.5 years (0.6–17 years), $p = 0.09$. Children who had a marked selective eating behavior, but without other ARFID diagnostic criteria, were classified as UFED. The interviews did not identify any other FED characterized by DSM-5, such as AN, BN, BED, OSFED, pica or rumination. After a headcount adjustment based on the French population by age group, the total prevalence of FED estimated from this cohort was 12.7% [95%CI (9.8–16.3)]. The prevalence of ARFID was 3.0% [95%CI (1.7–5.1)] and the prevalence of UFED was 9.7% [95%CI (7.2–13.0)].

Table 3. Prevalence of patients with feeding and eating disorders (FED) according to the DSM-5 classification.

	Age Groups (Total *n* = 401 Patients)				
DSM-5 FED *n* = 46/401 (11.4%)	**0–1 Year Old *n* = 101**	**1–6 Years Old *n* = 99**	**6–12 Years Old *n* = 100**	**12–18 Years Old *n* = 101**	***p* (χ^2 Test)**
ARFID *n* = 11/401 (2.7%)	*n* = 1 (1%)	*n* = 6 (6%)	*n* = 4 (4%)	n = 0	0.09
Selective *n* = 3 (including 1 refusal of pieces)	1	0	2	0	
Emotional food avoidance *n* = 1	0	0	1	0	
Restrictive eater *n* = 7 (including 1phobia SV, 3 selective eaters)	0	6	1	0	
Unspecified FED *n* = 35/401 (8.7%)	*n* = 6 (6%)	*n* = 8 (8%)	*n* = 9 (9%)	*n* = 12 (12%)	
Compulsive eating *n* = 4	0	0	1	3	
Nocturnal snacking/hyperphagia *n* = 5 (including 1 selective)	0	0	0	5	
Refusal of pieces + phobia SV *n* = 1	0	1	0	0	
Refusal of pieces + selective *n* = 2	0	2	0	0	
Selective eater *n* = 12	0	1	7	4	
Phobia SV + selective eater *n* = 1	0	1	0	0	
Phobia SV *n* = 1	0	0	1	0	
Refusal of pieces *n* = 6	3	3	0	0	
Refusal of the spoon *n* = 1	1	0	0	0	
Breastfeeding withdrawal problem *n* = 1	1	0	-	-	
Refusal of the spoon + breastfeeding withdrawal problem *n* = 1	1	0	-	-	
Anorexia nervosa, bulimia nervosa, binge eating disorder, rumination, pica, other specified FED *n* = 0	0	0	0	0	

Abbreviations: ARFID avoidant and restrictive food intake disorder, DSM-5 Diagnostic and Statistical Manual of Mental Disorders-5th edition, FED feeding and eating disorders, *n* number, Phobia SV Phobia of swallowing and/or vomiting.

3.4. FED and BMI

Patients with ARFID were more often underweight (91%) than patients with UFED (0%) and patients without FED (2.2%) ($p < 0.001$). Children aged between 1 and 18 years who had tachyphagia were more often overweight (40%) than children without tachyphagia (16%) ($p < 0.001$). Patients with nibbling were more often overweight (34.8%) than those without (19.4%) ($p = 0.004$). Compulsive eaters were more often overweight (80%) than non-compulsive eaters (23%) ($p = 0.001$). Among the 29 overweight adolescents, 13.8% were compulsive eaters.

3.5. FED and Analysis of Screening Tools

The means of BEBQ, BPFAS and TFEQ scores according to the DSM-5 classification of patients are presented in Figure 2. Parents answered all questions except for the BPFAS in infants, where a few responses were missing. For infants who had not started food, BEBQ scores were consistent with the parents' interview (no FED in this group). For all other children, patients with ARFID had the highest BPFAS scores. For adolescents, 23 SCOFF-F and 2 EAT-26 scores were positive. Among positive SCOFF-F, 3 patients had an UFED, and 20 patients had no FED diagnosis, but only one of them also had a positive EAT-26 score.

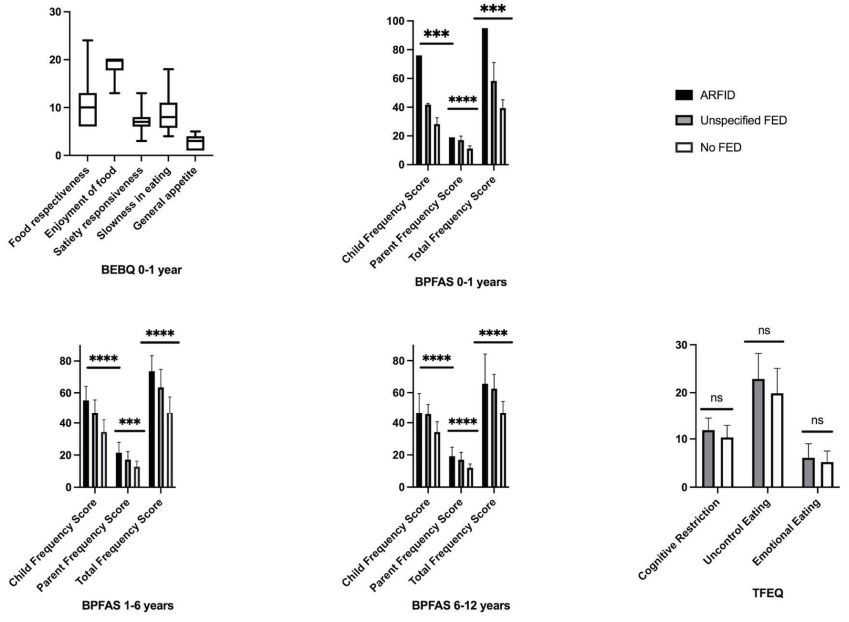

Figure 2. Means of BEBQ (baby eating behavior questionnaire), BPFAS (Behavioral Pediatrics Feeding Assessment Scale) and TFEQ-R18 (Three-Factor Eating Questionnaire) scores (±standard deviation) according to the age and DSM-5 classification of the patients (*** $p < 0.001$, **** $p < 0.0001$, Student's t-test).

3.6. FED Care Pathway

Among the cohort, 20% of parents felt their child had feeding or eating difficulties. More difficulties were found with adolescents than with other age groups. Overall, 79.7% of families felt this eating difficulty was problematic or alarming, but only 22.3% of children were receiving professional care, mostly infants, with the 1–6 and 6–12 years-old groups receiving the least care (Table 4). When considering only the FED classified according to the DSM-5, 87.8% of parents felt it was problematic or alarming (ARFID 88.9%, UFED 87.5%), but only 15.2% of children were receiving professional care (ARFID 18.0%, UFED 14.2%), with the least care in the 1–6 and 6–12 years-old groups (20% and 11% respectively), in contrast to the infants group (50%) and the adolescents group (25%).

Table 4. Parents' feelings about their child's current eating difficulties, and the care pathway.

	Age Groups (Total *n* = 401 Patients)				
Parents' Feelings	**0–1 Year Old *n* = 101**	**1–6 Years Old *n* = 99**	**6–12 Years Old *n* = 100**	**12–18 Years Old *n* = 101**	***p***
Having a child with current eating difficulties (20%)	11.0%	20.0%	19.0%	30.0%	0.021
Perception of the child's eating difficulties					
It is not a problem (20.3%)	18.2%	17.6%	23.5%	20.7%	<0.001
It is a problem (52.7%)	45.4%	64.7%	52.9%	48.3%	
It is alarming (27%)	36.4%	17.6%	23.5%	31.0%	
Child receiving professional care (22.3%)	54.5%	15.0%	15.8%	26.9%	0.007
General practitioner (29.4%)	66.7%	33.3%	66.7%	-	
Pediatrician (35.2%)	33.3%	66.7%	-	28.6%	
Psychiatrist (5.9%)	-	-	-	28.6%	
Dietician (11.7%)	-	-	33.3%	42.8%	

4. Discussion

In this large cohort, FED were diagnosed in at least 12.7% of the children and adolescents, including 3% with ARFID. Few studies have assessed the prevalence of FED in the general pediatric population [22]. Most epidemiological studies included adults, using the DSM-IV classification, or the DSM-5 posteriori on previous DSM-IV data, including sometimes adolescents [23–25]. The main limiting factor in assessing pediatric FED prevalence is probably the lack of tools that can screen all FED, regardless of age.

4.1. Prevalence of ARFID and Related Subtypes

ARFID is described from the age of 1 year until adolescence, and patients with ARFID are generally younger than those with AN or BN [26–31]. For infants, ARFID should not be confused with a normal and transient neophobia at the age of 6–9 months, or with a marked selective eating behavior that would not include the associate clinical features described in the ARFID DSM-5 definition and could be then classified as UFED.

Two studies screened ARFID in school children. ARFID was identified in 3.2% of 1444 Swiss children aged from 8 to 13 years [32], using the Eating Disturbances in Youth-Questionnaire (EDY-Q). In 4816 Taiwanese children aged from 7 to 14 years old, ARFID prevalence was estimated at 0.5%, using a mental-disorder questionnaire with psychiatric interviews [33]. For the same age group, ARFID prevalence was 2.8% in our study. Percentages of sub-types in our study were 63% (restrictive), 54.5% (selective), and 10% (phobia eaters), thus rather similar to 39%, 60.9% and 15.2 %, respectively, in the Swiss study [32].

Three studies have investigated the 8–18 years age group. A retrospective study in a gastroenterological pediatric cohort [28] identified 1.5% ARFID in 2250 children, (57.6% restrictive, 21% selective, 9% phobias eaters). The EDY-Q, without clinical interview, detected 3.7% ARFID in 190 girls followed in a pediatric gynecology clinic [34] and 0.9% ARFID in 111 children from a pediatric hospital, as compared to 2.4% in a general population sample [35]. Thus, our estimated prevalence of 3% of ARFID fits well in the range of other European studies.

For toddlers, data on the prevalence of ARFID are lacking. Before the diffusion of DMS-5, a study in 400 children aged 1–4 years old identified 15.4% selective, 11.2% restrictive, and 0.25% phobia eaters [36], versus 5.9%, 4.7% and 3.5% respectively, in our study for the same age group. The "selective" and "restrictive" profiles are often called "picky eater" in the literature and are most frequent between 2 and 6 years old [36–38]. Recently, a retrospective study on insurance databases of children up to 5 years in the USA reported FD in 21–34 children for 1000 child-years [39], which underlines the need for active screening of FD [40].

4.2. FED Other Than ARFID

In our study, we did not identify AN, BN, BED or OSFED. As a matter of fact, their prevalence is generally low, but we cannot exclude that some adolescents had debutant FED not revealed during the interview. AN prevalence is estimated at approximately 0.3–0.9% in adolescents and 0.2% in children [10,24,33]. The prevalence of BN, BED and pica/rumination is not well-known and is estimated at 0.3% [41,42], 1–3% [6,43], and 1.5–10% [10,44,45], respectively. BN and BED are more frequent in adolescents and adults [46]. In overweight children/adolescents, one metanalysis reported 22% of compulsive disorders [47]. Night Eating Disorders are not well documented [48,49]. In pediatric FED series, AN remains the most common FED (40–70%), followed by ARFID (5–22.5%), and BN (4–12%) [27,29–31,50–52]. It is probably because AN results generally in a lower BMI than ARFID and is thus more likely to be identified and treated than ARFID [27,30,32,33,53].

A majority of children with FED in our cohort were classified as UFED. Indeed, the DSM-5 classification remains criticized because definitions of specified FED are not always usable in children [9,43]. Pediatric FEDs have multifactorial origins (aversive environmental factors, association with organic and/or mental diseases), resulting in complex

clinical features with varied levels of severity [27,54]. It is estimated that 25–45% of young children in the general population experience FD at some point, 5–10% requiring intensive management [8,55,56]. Recently, some authors have therefore proposed a functional or a severity approach of FD in four areas (medical, nutritional, oral developmental and psychosocial) [57,58]. It is important to better identify children with UFED, because although it is less severe than other disorders, they may require parental guidance in order to prevent the disorder from worsening to full ARFID or even ED [55,59,60].

4.3. Insufficient Care of ARFID and UFED

In our study, only 15.2% of children with identified ARFID and UFED were already receiving care. These FED are probably not well-known by physicians and are difficult to diagnose [61]. Delays in ARFID diagnosis of up to 33 months, much longer than for AN, have been reported [27,30]. In our experience, BPFAS is helpful in detecting restrictive, selective eating in children between 10 months and 10 years old, but not specifically ARFID. In children after 10 years, SCOFF or EAT26 are helpful tools to screen for AN and BN, while TFEQ is helpful to screen for compulsive ED. Although SCOFF has also been validated in adolescents, we observed in our study that some questions of the SCOFF-F were not always easy to understand for some adolescents [15]. Several tools have been proposed to screen for ARFID in children, but none is validated for all age groups thus far [32,62,63]. Therefore, only structured interviews allow for the diagnosis of FED.

4.4. Strength and Limitations of the Study

Our study is one of the rare pediatric studies based on clinical interviews to estimate prevalence rates and the care pathway of FED in a general population. Our population was quite representative of the French population in terms of sex ratio and BMI [64]. Our observed high prevalence of overweight in children aged between 6 and 18 years old is consistent with that observed in the Normandy region of France and European countries [65,66]. Our population size was sufficient to detect most FED represented by UFED and ARFID, but too small to give an estimate of the less prevalent AN, BN or BED in pediatrics. Interviews alone may also have limitations, because some differential diagnoses of FED may remain unidentified. In addition, some adolescents could under-declare their symptoms, and parents may also misperceive their child's eating behavior [36,55,67].

5. Conclusions

Although ARFID and UFED are present in 12.7% of pediatric patients, we can conclude that they remain largely underdiagnosed because the care pathway is not correlated with the parents' request for care. We suppose that the clinical features of ARFID and UFED are not well known and are difficult to identify by untrained physicians, leading to delayed care. Moreover, this study shows that the DSM-5 classification remains poorly adapted to children, because the most frequent disorders (UFED) are the least well defined. UFED requires further clinical studies to better characterize less severe atypical subtypes of FED, which are common in children. The development of validated screening tools, as well as the training of health professionals in all clinical forms of pediatric FED are necessary. Early screening for pediatric FED should be performed to prevent both nutritional and psychopathological consequences.

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Data Availability Statement: Data can be made available upon request. However, sharing of the data may require approval and some access restrictions may apply. Requests may be sent to the corresponding author.

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Clinical Impact of Nutritional Status and Sarcopenia in Pediatric Patients with Bone and Soft Tissue Sarcomas: A Pilot Retrospective Study (SarcoPed)

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Abstract: Background: We evaluated nutritional and sarcopenia status and their clinical impact in pediatric patients affected by bone and soft tissue sarcomas. Methods: Body mass index (BMI), prognostic nutritional index (PNI), and total psoas muscle area (tPMA) at diagnosis and after 12 months were analyzed. tPMA was measured from single cross-sectional computed tomography (CT) images at L4–L5. Age-specific and sex-specific tPMA Z-scores were retrieved from an online calculator. Results: A total of 21 patients were identified between February 2013 and December 2018. Twelve patients (57.1%) experienced sarcopenia at diagnosis, although not statistically associated with overall survival (OS) ($p = 0.09$). BMI Z-score, PNI, and tPMA Z-score significantly decreased between diagnosis and after 12 months of treatment ($p < 0.05$). Univariate analysis showed significant associations between poor OS and the presence of metastasis ($p = 0.008$), the absence of surgery ($p = 0.005$), PNI decrease ($p = 0.027$), and the reduction in tPMA > 25% ($p = 0.042$) over the 12 months. Conclusions: Sarcopenia affects more than half of the patients at diagnosis. Decreased PNI during 12 months of treatment has significant predictive value for OS. The role of tPMA derived from CT scan among pediatric patients with sarcoma should be investigated in further prospective and larger studies.

Keywords: soft tissue sarcoma; bone sarcoma; pediatric patients; chemotherapy; psoas muscle area (PMA); sarcopenia; personalized medicine

1. Introduction

Nutritional status plays a key role in the growth, response to treatment, related complications, quality of life, cost of care, and hospital stay of pediatric patients admitted for various types of illness [1]. In 2013, the Academy of Nutrition and Dietetics of the American Society of Parenteral and Enteral Nutrition (ASPEN) defined pediatric malnutrition status as "an imbalance between nutritional needs and nutrient intake, resulting in a cumulative deficit of energy, protein, or micronutrients that may adversely affect growth, development, and other clinical outcomes" [2].

Pediatric hospital malnutrition is still an underestimated problem, although it results in significant morbidity and mortality among hospitalized children. Several studies have reported a prevalence of 6–51% of this condition among hospitalized pediatric patients [3,4]. The European Society for Clinical Nutrition and Metabolism at Risk (ESPEN) and the European Society for Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommend screening for nutritional risk in hospitalized children on admission to the ward to facilitate the identification of nutritionally at-risk children and to allow the physician to prepare an appropriate nutritional support plan [5–7].

In children hospitalized for cancer, malnutrition constitutes a very common complication, and it is influenced by the type and extent of disease, the intensity of treatment, and the patient's living conditions [8]. In these patients, the inflammatory response of the underlying disease induces high-energy expenditure and protein catabolism with a net lean body mass loss. In addition, gastrointestinal symptoms such as nausea, vomiting, and lack of appetite (caused by the therapy and/or the tumor) further aggravate the state of protein-energy malnutrition, sometimes exacerbating the toxic effects of therapy and susceptibility to complications [9].

Early identification of the risk and presence of malnutrition, coupled with rapid and personalized nutritional intervention, could, in turn, enhance treatment adherence, reduce treatment complications, and improve patients' quality of life [10–12].

The prognostic nutritional index (PNI) is calculated using the serum albumin concentration and peripheral blood lymphocyte count. PNI has been recognized as a valid indicator of the nutritional and immune status among cancer patients and as an independent prognostic indicator of various malignant tumors [13–16].

Numerous studies have tried to quantify the effect of malnutrition on the survival of pediatric cancer patients, showing a close relationship between the body mass index (BMI) and mortality [17]. However, the BMI is a nutritional indicator based on the weight and height of the patient, without taking into account the real body composition and the proportions of fat and lean tissue. On the other hand, the loss of muscle tissue and the alteration of body composition may occur as an effect of malnutrition, regardless of weight changes [18]. In pediatric patients with neoplasms, sarcopenia may coexist in the context of malnutrition, amplifying its negative impact on the patients' prognosis. Sarcopenia is a pathological condition, characterized by a progressive and generalized reduction in the quantity, quality, and strength of muscle mass, more or less associated with reduced physical performance. It is a major cause of physical disability, poor quality of life, loss of self-sufficiency, and death [19,20]. Despite that sarcopenia is generally associated with aging (primary sarcopenia) [21], there are many inflammatory conditions (such as chronic inflammatory diseases and cancer) yielding its occurrence even at a young age as an effect of the catabolic state (secondary sarcopenia) [22]. The development of sarcopenia in adult cancer patients is closely linked to an increase in mortality and morbidity, independently from the BMI variation [23,24]. In the pediatric age, the presence of sarcopenia has been linked to the mortality and morbidity of numerous chronic diseases and in patients with aggressive and disabling malignant pathologies, even if the evidence is still scarce [25,26].

There are several ways to assess muscle mass and possible sarcopenia status in clinical practice [27]. In adults, the skeletal muscle mass index (SMI) is calculated from the skeletal muscle area (SMA) (cm^2) divided by the square of the patient's height (m^2) [28–30]. SMI is indicative of sarcopenia if less than 55 cm^2/m^2 in males and 39 cm^2/m^2 in females [31,32].

This method has been widely adopted for the analysis of muscle mass in adult patients with neoplasia, as well as in patients with liver disease, patients in intensive care units, and patients undergoing surgery [33–36]. Recently, Lurz et al. generated age- and sex-specific curves related to total psoas muscle area (tPMA) derived at L3–L4 or L4–L5, which can be used for pediatric patients aged 1 to 16 years. From them, the Z-scores of the PMA can be derived through a calculator available online (https://ahrc-apps.shinyapps.io/sarcopenia/ (accessed on 15 December 2021)) and easily identify the presence of sarcopenia [37]. Although Lurz et al. analyzed tPMA at both L3–L4 and L4–L5 levels, tPMA at L4–L5 seems to be more relevant in the pediatric age group, because at this level the psoas muscle shape is also rounder than at L3–L4, allowing for more accurate contour drawing. In addition, L4–L5 is the reference level for the assessment of visceral adipose tissue, so an analysis at this level is a reliable measure of both skeletal muscle and adipose tissue [38,39]. Bone and soft tissue sarcomas of childhood are highly aggressive pathologies, which require intensive chemotherapy treatments and can be burdened by a high rate of immobilization resulting from surgical procedures or pain. Prolonged immobilization further causes a reduction in muscle mass and strength, with a consequent possible worsening of the state of sarcopenia [40].

In the present study, we evaluate body composition changes, sarcopenia assessed by the evaluation of PMA obtained from computed tomography (CT) scans, and their impact on the survival and prognosis of pediatric patients with bone and soft tissue sarcomas.

2. Materials and Methods

2.1. Study Endpoints

The primary endpoint was:

- To describe the clinical characteristics, nutritional status (defined by the BMI and the PNI), and the presence of sarcopenia (defined by the measurement of tPMA detected by axial CT images of the L4–L5 vertebrae) at diagnosis and after 12 months of chemotherapy in pediatric patients with bone and soft tissue sarcomas.

The secondary endpoints were:

- To evaluate the association between clinical characteristics, nutritional status, and the presence of sarcopenia (at diagnosis) with overall survival (OS);
- To evaluate the association between the presence of sarcopenia (at diagnosis) with any infectious complications due to treatment (defined as the number of hospitalizations for febrile neutropenia in the 12 months of observation).

2.2. Study Design, Patients' Characteristics, Ethical Approval, Inclusion, and Exclusion Criteria

In this observational pilot study, data from twenty-two pediatric patients with a new diagnosis of bone and soft tissue sarcoma, admitted at the Pediatric Oncology Unit of the Fondazione Policlinico Agostino Gemelli IRCCS from February 2013 to December 2018, were retrospectively analyzed.

The ethical approval was obtained from the Ethics Committee of our institution (Protocol ID 4211, Approval Letter Number 36155/21). Informed consent was obtained from the parents or the legal guardians of the enrolled patients. The study was carried out following the Helsinki Declaration of Human Rights.

Inclusion criteria were: diagnosis of bone or soft tissue sarcomas (Ewing sarcoma, rhabdomyosarcoma, desmoplastic tumor), age between 1 and 16 years, and staging of the disease by CT scan of the abdomen at diagnosis and after 12 months of treatment.

The exclusion criterion was the absence of CT images available at the time of data analysis.

2.3. Clinical Data and Nutritional Assessment

For each patient, the following data were collected:

- Demographic characteristics (age, sex, ethnicity);

- Data related to the neoplastic disease (histology, primarily affected site, date of diagnosis, and presence of metastasis at diagnosis);
- Type and duration of the treatment (chemotherapy protocol, radiotherapy, and high-dose chemotherapy with autologous transplantation);
- Surgery during the 12 months of observation;
- Five-year OS defined as the time from the day of diagnosis to death from any cause in five years after diagnosis;
- Data on treatment-related infectious complications (febrile neutropenia that required hospitalization and intravenous antibiotic therapy) in the 12 months of observation.

For each patient, the assessment of nutritional status was performed according to the following variables: BMI, BMI Z-score, PNI, and tPMA at diagnosis and after 12 months.

The BMI was calculated with the formula: weight (kg)/height (m^2). The BMI Z-score was calculated with "PediTools-Clinical tools for pediatric providers", available on the internet (https://peditools.org/, last (accessed on 20 September 2021)). The change in BMI (ΔBMI) over the 12 months was calculated with the formula: ((BMI after 12 months—BMI at diagnosis)/(BMI at diagnosis)) × 100.

The PNI, as an indicator of the nutritional and inflammatory status, was calculated according to the formula: (10 × serum albumin (g/dL)) + (0.005 × number of lymphocytes/μL). The change in the PNI (ΔPNI) over the 12 months was calculated with the formula: ((PNI after 12 months − PNI at diagnosis)/(PNI at diagnosis)) × 100.

To evaluate the presence of sarcopenia, tPMA was measured from single cross-sectional abdominal CT images at the level of L4–L5 at diagnosis and after 12 months of treatment. The tPMA expressed in mm^2 was obtained using the SliceOmatic software v5.0 (Tomovision, Montreal, QC, Canada) and was measured at the level of L4–L5 because most clinically relevant and useful according to Lurz et al. [37]. A calculator available online (https://ahrc-apps.shinyapps.io/sarcopenia/) (accessed on 15 December 2021) was used to obtain the tPMA Z-scores in relation to sex and age of the patient. The degree of sarcopenia was defined by the following parameters: mild for <−1 Z-score, moderate for <−2 Z-score, severe for <−3 Z-score. The change in tPMA (ΔtPMA) over the 12 months was calculated with the formula: ((tPMA after 12 months—tPMA at diagnosis)/(tPMA at diagnosis)) × 100.

2.4. Sample Size and Statistical Analysis

Given the lack of data on the presence and degree of sarcopenia in the population of interest, we set the sample size for this pilot study at $n = 22$ patients. This number intercepts various expected proportions, with a confidence level of 95% and a margin of error ranging from a minimum of 4.16% (for an expected proportion of 1%) to a maximum of 20.89 (for an expected proportion of 50%).

Continuous data are given as median and interquartile range (IQR: 25–75° percentile) and were compared using Mann–Whitney U tests. Categorical data are presented as absolute and percentage frequencies and were compared using the Chi-squared test or Fisher Exact Test, where appropriate. The study of the correlations was carried out using Pearson's test.

OS was estimated by the Kaplan–Meier method and was tested with the log-rank test. OS was defined as the time between diagnosis and death, expressed in months. A Cox regression proportional hazards model was constructed to assess the prognostic significance of covariates. The hazard ratio (HR) with 95% CI was reported as an estimate of the risk of death. All statistical analyses were performed using XLSTAT version 2021.3.1 by Addinsoft. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Clinical Characteristics, Nutritional Status, Sarcopenia at Diagnosis and 12 Months

A total of 22 patients diagnosed with a bone or soft tissue sarcoma and treated at our center were included between February 2013 and December 2018. Only one patient was excluded, due to the impossibility of obtaining the CT images at12 months after diagnosis.

Demographic and clinical characteristics of the enrolled population are reported in Table 1.

Table 1. Clinical characteristics, nutritional status, and sarcopenia at diagnosis (n = 21).

Characteristics	Number of Patients (%) or Median IQR
Age (months)	125.6 (78.7; 181.5)
Sex (male)	11 (52.4)
Histology	
Ewing sarcoma	14 (66.6)
Rhabdomyosarcoma	6 (28.6)
Desmoplastic tumor	1 (4.8)
Primary localization	
Bone	13 (61.9)
Soft tissue	8 (38.1)
Presence of metastases at diagnosis	9 (42.9)
BMI (kg/m^2)	17.4 (15; 19.6)
BMI (percentile)	41.7 (0.9–86.2)
BMI Z-Score	−0.23 (−0.89; 0.85)
PNI	48.1 (45.2; 53.2)
tPMA L4–L5 (mm^2)	13.21 (8.79; 19.38)
tPMA L4–L5 Z-Score	−1.01 (−1.71; −0.35)
Nonsarcopenic patients	9 (42.8)
Sarcopenic patients	12 (57.1)
Mild	8 (38.1)
Moderate	4 (19.1)
Severe	0 (0)
Progressive disease	13 (61.9)
Deaths	11 (51.4)

Abbreviations: tPMA, total area of the psoas muscle; BMI, body mass index; IQR, interquartile range; PNI, prognostic nutritional index.

Eleven (52.4%) patients were male, and ten (47.6%) were female, with a median age of 125.6 (78.7; 181.5) months. There were 14 (66.6%) diagnoses of Ewing sarcoma, 6 (28.6%) of rhabdomyosarcoma, and 1 (4.8%) of desmoplastic tumor.

At diagnosis, no patient could undergo complete resection of the primitive mass. As per EpSSG RMS 05 protocol, all patients with rhabdomyosarcoma or desmoplastic tumor who did not have metastases at diagnosis and who could not undergo complete resection of the mass were classified as stage III, while those with metastases were classified as stage IV. Similarly, all patients with Ewing's sarcoma without metastases at diagnosis and who could not undergo complete resection of the mass were classified as stage III, and those with metastases were classified as stage IV.

At diagnosis, the median of the BMI Z-score was −0.23 (−0.89; 0.85), the median of PNI was 48.1 (45.2; 53.2), and the median of the tPMA Z-score was −1.01 (−1.71; −0.35). At diagnosis, 12 (57.2%) were sarcopenic, 8 (38.1%) mild, and 4 (19.1%) moderate sarcopenic. None were diagnosed with severe sarcopenia.

Patients with Ewing sarcoma were treated according to the EURO-EWING99 protocol: 4 (28.6%) of them received high-dose chemotherapy with busulfan and melphalan, followed by autologous stem cell transplantation, 3 (21.4%) underwent radiotherapy, and 13 (92.9%) underwent surgical excision of the primary residual mass. Patients with rhabdomyosarcoma and desmoplastic tumor were treated according to the EpSSG RMS 05 protocol. None received high-dose chemotherapy or radiotherapy. One patient with rhabdomyosarcoma and desmoplastic tumor required primary tumor surgery, followed by chemotherapy according to the EpSSG NRSTS 05 protocol.

At the time of the analysis, 11 patients (52.4%) were dead, and 13 patients (61.9%) had disease progression.

Table 2 shows the comparison of nutritional variables at diagnosis and 12 months after the beginning of treatment. The differences in terms of presence of sarcopenia at diagnosis

and after 12 months of treatment were not statistically significant, whereas significant differences were observed for the BMI Z-score, the PNI, and the tPMA Z-score.

Table 2. Comparison of nutritional status variables and sarcopenia status rates at diagnosis and after 12 months of treatment.

Variables	Diagnosis (Median (25°; 75°) or Number of Patients (%))	12 Months (Median (25°; 75°) or Number of Patients (%))	*p* Value
BMI (kg/m^2)	17.4 (15; 19.6)	17.0 (13.6; 19.2)	0.25
BMI (percentile)	41.7 (0.9–86.2)	12.2 (3.9–72.9)	0.28
BMI Z-score	−0.23 (−0.89; 0.85)	−1.17 (−2.11; 0.48)	0.01
PNI	48.1 (45.2; 53.2)	39.6 (35.7; 45.6)	0.001
tPMA L4–L5 (mm^2)	13.2 (8.8; 19.4)	11.5 (9.95; 18.48)	0.31
tPMA L4–L5 Z-score	−1.01 (−1.71; −0.35)	−1.46 (−2.57; −1.11)	0.005
Nonsarcopenic patients	9 (42.8)	5 (23.8)	0.32
Sarcopenic patients			
Mild	8 (38.1)	8 (38.1)	0.999
Moderate	4 (19.1)	8 (38.1)	0.29
Severe	0 (0)	0 (0)	-

Abbreviations: tPMA: total area of the psoas muscle; BMI: body mass index; PNI prognostic nutritional index.

Figure 1 represents the change of tPMA, measured from single cross-sectional abdominal CT images at the level of L4–L5 in the same patient at diagnosis and after 12 months of treatment.

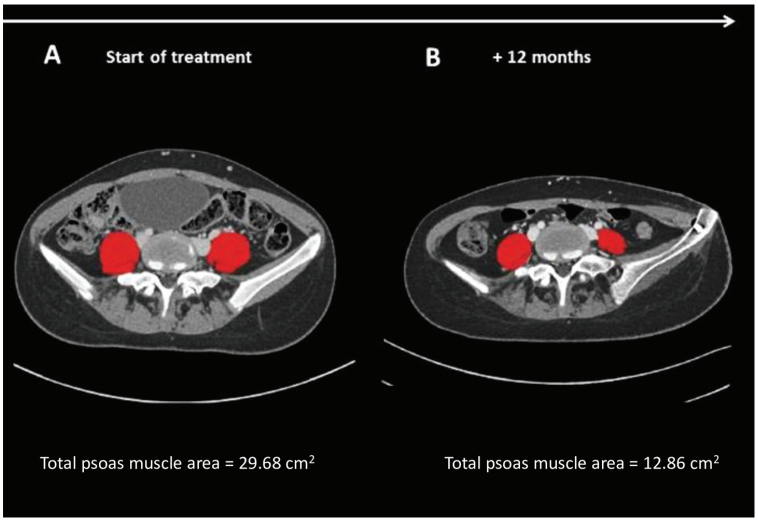


Figure 1. tPMA (in red color) measured from single cross-sectional abdominal CT images at the level of L4–L5 in the same patient, at diagnosis, and after 12 months of treatment.

3.2. Association between Clinical Characteristics, Nutritional Status, Sarcopenia, and Overall Survival

Among the sarcopenic patients at diagnosis, seven (58.3%) were affected by Ewing sarcoma and five (41.7%) by rhabdomyosarcoma ($p = 0.16$).

Table 3 describes the potential associations between clinical and nutritional variables and OS, as a result of Cox proportional hazards analysis. The presence of mild or moderate sarcopenia at diagnosis was not statistically associated with OS (HR = 3.19 (0–82–12.36); $p = 0.09$). In the univariate analysis, the presence of metastasis, the absence of surgery

during the 12 months, the ΔPNI, and a reduction of PMA greater than 25% over 12 months were significantly associated with poor OS.

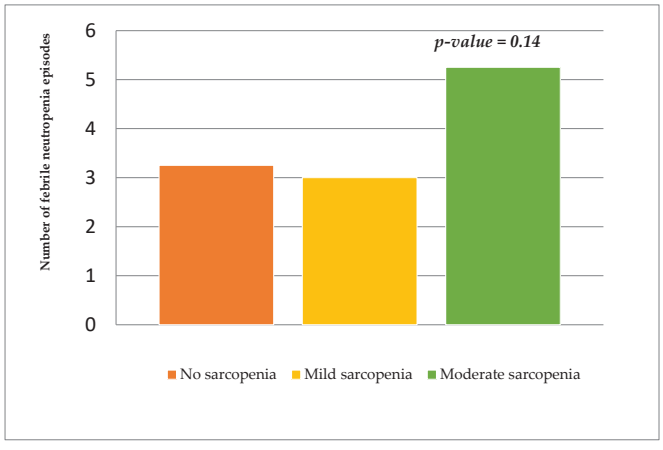
Table 3. Univariate analysis of clinical characteristics, nutritional status, and sarcopenia for overall survival.

	HR (95%CI)	*p* Value
Absence of sarcopenia at diagnosis	0.63 (0.18–2.15)	0.461
Mild sarcopenia at diagnosis	0.8 (0.24–2.75)	0.726
Moderate sarcopenia at diagnosis	3.19 (0.82–12.36)	0.09
Ewing sarcoma	0.35 (0.1–1.15)	0.08
Rhabdomyosarcoma	2.86 (0.86–9.4)	0.08
Presence of metastases at diagnosis	6.07 (1.59–23)	0.008
High-dose chemotherapy	0.39 (0.05–3.13)	0.38
Radiotherapy	0.49 (0.06–3.83)	0.49
Absence of surgery	0.17 (0–0.6)	0.005
BMI at diagnosis	1.02 (0.96–1.07)	0.52
PNI at diagnosis	1 (0.95–1.07)	0.76
tPMA L4–L5 Z-score at diagnosis	1 (0.52–2)	0.95
ΔBMI	1.01 (0.97–1.01)	0.59
ΔPNI	0.966 (0–0.99)	0.027
ΔtPMA L4–L5	0.97 (0.94–1)	0.122
ΔtPMA L4–L5 > −25%	4.14 (1.05–16.3)	0.042

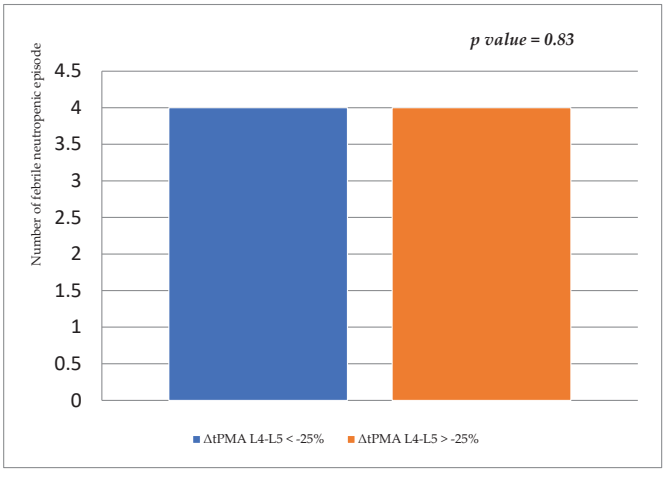
Abbreviations: tPMA: total area of the psoas muscle; BMI: body mass index; PNI prognostic nutritional index; ΔBMI (the change of BMI over the 12 months of treatment); ΔPNI (the change of PNI over the 12 months of treatment); ΔtPMA (the change of PMA over the 12 months); ΔtPMA L4–L5 > −25% (reduction of PMA greater than 25% over 12 months).

3.3. Association between Sarcopenia and Infectious Complications

The median number of febrile neutropenic episodes that require hospitalization and intravenous antibiotic therapy was 3 (IQR = 3–7) in nonsarcopenic patients, 2.5 (IQR = 0.5–4.5) in mild sarcopenic patients, and 5 (IRQ 1–5.5) in moderate sarcopenic patients. The comparison between the median number of febrile neutropenic episodes that require hospitalization and intravenous antibiotic therapy was not statistically different among the nonsarcopenic, mild sarcopenic, and moderate sarcopenic groups at diagnosis, as shown in Figure 2a. Additionally, the median number of febrile neutropenic episodes that require hospitalization and intravenous antibiotic therapy was 2 (IQR = 2–5.75) in patients with ΔtPMA L4–L5 < −25% and 2 (IQR = 3.5–5) in patients with ΔtPMA L4–L5 > −25%. The comparison between the median number of febrile neutropenic episodes that require hospitalization and intravenous antibiotic therapy was not statistically different among the patients with ΔtPMA L4–L5 < −25% and patients with ΔtPMA L4–L5 > −25%, as shown in Figure 2b. The study of correlations using Pearson's test showed that there was no association between Δ tPMA L4–L5 and the number of febrile neutropenic episodes ($r = -0.25$; $p = 0.06$) and between ΔBMI and the number of febrile neutropenic episodes ($r = -0.3$; $p = 0.1$). A weak association was observed between ΔPNI and the number of febrile neutropenic episodes ($r = 0.1$; $p = 0.01$).



(**a**)



(**b**)

Figure 2. (**a**) Number of febrile neutropenic episodes in nonsarcopenic, mildly sarcopenic, and moderately sarcopenic patients at diagnosis. (**b**) Number of febrile neutropenic episodes in the patients with ΔtPMA L4–L5 < −25% and patients with ΔtPMA L4–L5 > −25%.

4. Discussion

This is a pilot retrospective study investigating sarcopenia and nutritional status at diagnosis among inpatient children affected by cancer and their potential clinical impact. The recently published study of Lurz et al. defining pediatric reference curves for tPMA allowed us to diagnose sarcopenia in children using CT scan [37]. Indeed, by using the reference curves we observed that sarcopenia in children with cancer at diagnosis is a greatly more widespread condition than we might have expected. We can speculate that the cause of this phenomenon could be found in the pathogenetic mechanisms of sarcopenia. Secondary sarcopenia is caused by the coexistence of multiple pathogenic mechanisms involving the increase in apoptotic activity in muscle cells, the production of inflammatory cytokines such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-6, the presence of oxidative stress, and accumulation of oxygen radicals, low energy and protein intake [41]. Even before the tumor diagnosis, it is possible that these mechanisms, especially the

neoplastic, inflammatory, microenvironmental (TNF, IL-1, proteolysis-inducing factor), and physical inactivity [42], play a role in the pathogenesis of sarcopenia. In our opinion, such a high rate of sarcopenia at diagnosis in children with soft tissue and bone sarcomas could also be related to the diagnostic delay of these tumors [43].

By comparing the mean value of tPMA at diagnosis and after 12 months, we observed a statistically significant reduction in the tPMA Z-score. This change during treatment may probably be caused by an inadequate intake of energy or proteins (due to anorexia, nausea, vomiting, malabsorption related to the oncologic treatments), already associated with the inflammatory state and hypomobility [42]. A reduced intake of nutrients during cancer treatment can be associated with sarcopenia diagnosis or can worsen its severity if already present before initiating treatment. As observed by Chindapasirt et al., sarcopenia may produce a vicious circle, causing the worsening of the patient's inflammatory status [44]. In our population, this resulted in the fact that after 12 months of treatment only 23% of the patients did not develop sarcopenia. A previous report also showed an increased rate of pediatric sarcopenia status during treatment of acute lymphoblastic leukemia, but the authors assessed the skeletal muscle mass using dual-energy X-ray absorptiometry scans without exactly defining sarcopenia status at diagnosis [45]. In our study, the incidence of sarcopenia in children with cancer and its variations during treatment have been precisely defined from the analysis of CT scan routinely used at diagnosis for the staging of sarcomas. This means that the diagnosis of sarcopenia was easily made without the need for supplementary procedures and additional costs.

Furthermore, BMI Z-score and PNI significantly decreased from diagnosis to 12 months after treatment. The mechanisms underlying the reduction of BMI Z-score and PNI could be similar to those of the reduction in muscle mass in children with cancer. PNI is a nutritional and inflammatory marker associated with survival in numerous studies of cancer patients. It is calculated on the basis of albumin values, which are compromised both by the inflammatory status and the reduced protein intake and by lymphocytes, whose values may also be affected by myelotoxic chemotherapy [14].

The univariate analysis showed that metastatic disease, the absence of surgery during the 12 months, the ΔPNI and ΔtPMA L4–L5 > −25% adversely affected the prognosis of the population studied. This has not been shown for the change in BMI. Indeed, BMI is not a marker of muscle mass, especially in inflammatory conditions. The decline in visceral and muscle proteins generally occurs with an increase in extracellular volume, so body weight and BMI may vary little until cachexia becomes evident [46]. It has also been observed that pediatric patients with poor prognosis frequently have a high BMI due to an excess of fat or edema. In recent decades, sarcopenic obesity has emerged as a pathological condition with a loss of muscle tissue associated with an increase in adipose tissue and body weight [47]. The presence of sarcopenic obesity may adversely affect the prognosis of adult patients with cancer [47,48]. The lack of significance of the variation of the BMI Z-score as a negative prognostic factor in our population is probably the result of this mechanism.

As we have observed in children with soft tissue and bone sarcomas, a relationship between tPMA and survival has also been demonstrated in children with hepatoblastoma and neuroblastoma [49,50]. Ritz et al. observed that tPMA in children with hepatoblastoma was significantly lower than in healthy controls, even in the presence of normal body weight and height. Moreover, children with tPMA under the fifth percentile showed an increased risk of relapse and liver transplant [49]. Finally, Kawakubo et al. analyzed the rate of change in the tPMA of L3 level on CT images before and after treatment in fifteen children with high-risk neuroblastoma. They found shorter progression-free survival (PFS) and OS in the group of patients who presented a reduced skeletal muscle mass during the treatment [50].

Unlike the study conducted by Rayar et al. in children with acute lymphoblastic leukemia, the presence of sarcopenia at diagnosis and the reduction of tPMA during treatment seem not to influence the number of infectious episodes recorded during the observation [45]. However, Rayar et al. conducted their studies among children with

leukemia during the induction treatment when infections are very frequent due to the intensity of the treatment to leukemia itself, which compromises the immune system [45]. Moreover, during treatment, children with leukemia undergo a modification of the gut microbiome, which can affect the nutritional status of the patients, causing an increase in infectious episodes [51]. In soft tissue and bone sarcomas, the treatment has a decisively lower myeloablative effect compared to the induction phase of leukemia with a consequent lower incidence of infectious episodes.

Our results failed to show a significant reduction in skeletal muscle mass during treatment as a prognostic value. It could be due to the small sample size of the population studied, representing the main limitations of this pilot study. However, we showed a significant reduction in tPMA between diagnosis and 12 months after treatment at an age in which muscle mass and weight should physiologically increase. Moreover, this is the first study that used tPMA measurement in the clinical setting as a marker of sarcopenia in childhood cancer. We acknowledge that tPMA is easy to use and can be determined through a routinely abdominal CT. Since all patients with soft tissue and bone sarcoma undergo numerous disease stages with abdominal CT, tPMA can be simply performed also during the treatment and the follow-up visits. Further studies with a more robust design and higher sample size are warranted to confirm or rebut our results.

5. Conclusions

Sarcopenia may occur in pediatric patients with soft tissue and bone sarcoma already at diagnosis. The diagnosis of sarcopenia can be easily performed by measuring the tPMA from single cross-sectional abdominal CT images at the level of L4–L5, according to tPMA Z-scores specific for the sex and age of the patients. Our study showed a reduction of tPMA Z-scores between diagnosis and at 12 months of treatment. This could be crucial for OS, tolerance, and the response to the treatment; therefore, careful surveillance should be performed, to allow the implementation of nutritional and physical rehabilitation measures. Furthermore, significant differences in BMI Z-scores and PNI may be observed after 12 months of treatment, and consequently, nutritional and immune status should be closely monitored throughout the treatment.

Prospective and larger studies may help to better investigate the role of tPMA derived from CT scan in this particular setting of patients, to implement their use in the clinical practice, allowing a therapeutic approach as personalized as possible.

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Informed Consent Statement: Informed consent was obtained from all subjects' parents involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author for any academic use upon citation of this article. The data are not publicly available due to privacy and permission restricted to the publication of this article only.

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nutrients

MDPI

Systematic Review

Effect of Protein-Rich Breakfast on Subsequent Energy Intake and Subjective Appetite in Children and Adolescents: Systematic Review and Meta–Analysis of Randomized Controlled Trials

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Abstract: Breakfast has been labeled "the most important meal of the day", especially for children and adolescents. Dietary protein intake may benefit and regulate appetite and energy balance. However, few meta–analyses have been conducted to examine the effect of protein–rich (PR) breakfast on both children and adolescents. This meta–analytic study was conducted to examine the effect of consuming a PR breakfast on short–term energy intake and appetite in children and adolescents. PubMed, Embase, Cochrane Central Register of Controlled Trials, China Biology Medicine disc (CBM), and China National Knowledge Infrastructure (CNKI) were searched for randomized controlled trials (RCTs) published in January 1990–January 2021. The inclusion criteria applied were RCTs in children and adolescents (7–19 year) comparing PR breakfast consumption with normal protein (NP)/traditional breakfast consumption. Finally, ten studies were included in the analysis, eight studies examined the effect of consuming PR breakfast on SEI (n = 824), and nine studies examined the effect on appetite (fullness = 736, hunger = 710). Our meta-analysis using the random–effects model shows that participants assigned to consume PR breakfast had lower SEI (MD, −111.2 kcal; 95% CI: −145.4, −76.9), higher fullness (MD, 7.4 mm; 95% CI: 6.0, 8.8), and lower hunger (MD, −8.5 mm; 95% CI: −9. 7, −7.3) than those assigned to consume NP/traditional breakfast. However, there was considerable inconsistency across the trial results. Our review suggests that the consumption of PR breakfast could be an excellent strategy for weight management by declining SEI and suppressing appetite, and provides new evidence of the relationship between energy balance and obesity. However, since most eligible studies were of low quality, the results ought to be interpreted cautiously.

Keywords: protein; breakfast; subsequent energy intake; appetite; fullness; hunger; meta–analysis; children; adolescents

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1. Introduction

The prevalence of obesity has risen continuously over the past decades in low– and middle–income countries as well as in many high-income countries [1–3]. Obesity is a worldwide health concern in children and adolescents resulting from long-term imbalance of energy (energy intake > expenditure intake) [4]. Obesity and obesity–related disorders such as cardiovascular diseases and type–2 diabetes is increasing steadily worldwide [5–7]. Moreover, the main risk factors for attributable DALYs globally, in 2019, was child and maternal malnutrition, which accounted for 11.6 % of all global DALYs that year [8].

Strategies for obesity prevention and management are multiple, including bariatric surgery, drug therapies, physical activity, and so on [9]. Among them, dietary recommendation is an effective strategy for the prevention and treatment of obesity among children and adolescents [10]. Particularly, breakfast, the most important meal of the day, has played a pivotal role in weight management and energy balance [11]. Furthermore, dietary protein is essential to the health of individuals of all ages, and is especially critical for the growth and development of children and adolescents. Thus, protein–rich (PR) breakfast consumption might be a useful strategy for weight management [12]. However, there is no consensus on the definition for PR breakfast. Given this lack of consensus, and to maximize identified articles for this review, we defined a PR breakfast as any breakfast containing more protein than the normal protein (NP)/traditional breakfast, and there were no restrictions on protein sources, protein doses, protein type, and macronutrient composition of breakfast.

PR breakfast promotes weight loss in children and adolescents possibly through regulating appetite and subsequent food intake (SFI) [13–17], whereas the effect of PR breakfast on appetite and subsequent energy intake (SEI) is inconsistent. Recent studies among children and adolescents have challenged the conclusion of PR breakfast and by checking the findings of six randomized controlled trials (RCTs) that did not show the effect on reductions of SEI [13,14] and subjective appetite [14–17]. To our knowledge, RCTs' meta-analysis has not been conducted to evaluate the effects of PR breakfast on appetite and SEI in both children and adolescents.

Thus, this study aimed to search for the evidence of children and adolescents from RCTs to identify the effect of PR breakfast on subjective appetite and SEI for a better understanding of the relationship between energy balance and obesity, focused on the studies published in the last thirty years.

2. Materials and Methods

Our systematic review followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta–Analyses) statement [18].

2.1. Search Strategy and Study Selection

We gathered literature from January 1990 through January 2021 by conducting a systematic search in PubMed, Embase, Cochrane Central Register of Controlled Trials, China Biology Medicine disc (CBM), and China National Knowledge Infrastructure (CNKI). We also searched ClinicalTrials.gov in order to identify any unpublished or ongoing RCTs. We adjusted a 31–year search limit because dietary patterns from three decades ago may have changed dramatically over the past several decades [19]. Additionally, relevant reviews and studies of all references were also screened for other relevant citations. We restricted the search to RCTs of children and adolescent studies. The search strategy is described in detail in Tables 1 and 2. Two reviewers examined inclusion and exclusion criteria independently by screening the titles, abstracts, and then the full–text of the articles. Search terms included "Breakfast", "Child, Preschool", "Minors", "Students", "randomized controlled trial", "ready to eat cereals/RTEC", etc.

Table 1. Description of the PICOS (Participants, Interventions, Control, Outcomes) statement.

PICOS	Descriptions
Participants	Children and adolescents older than 7 and younger than 19 years; Both sexes; All nationalities
Interventions	The intervention group consumed a protein-rich breakfast; No restrictions regarding the dose or intervention duration were applied.
Control/Comparator group	The control group consumed a normal protein or traditional breakfast;
Outcomes	Subsequent energy intake or subjective appetite components (fullness and hunger)
Setting	Randomized controlled or crossover trials

Table 2. Search strategy for Pubmed.

1	((((“Breakfast”[Mesh]) OR ((((((((((((Breakfasts[Title/Abstract]) OR (Breakfast Time[Title/Abstract])) OR (Breakfast Times[Title/Abstract])) OR (Time, Breakfast[Title/Abstract])) OR (Times, Breakfast[Title/Abstract])) OR (Morning Meal[Title/Abstract])) OR (Meals, Morning[Title/Abstract])) OR (Morning Meals[Title/Abstract])) OR (meal timing[Title/Abstract])) OR (Cereal[Title/Abstract])) OR (RTEC[Title/Abstract])) OR (Ready To Eat Cereals[Title/Abstract])) OR (breakfast cereal[Title/Abstract]))) AND ((“Breakfast”[Mesh]) OR ((((((((((((Breakfasts[Title/Abstract]) OR (Breakfast Time[Title/Abstract])) OR (Breakfast Times[Title/Abstract])) OR (Time, Breakfast[Title/Abstract])) OR (Times, Breakfast[Title/Abstract])) OR (Morning Meal[Title/Abstract])) OR (Meals, Morning[Title/Abstract])) OR (Morning Meals[Title/Abstract])) OR (meal timing[Title/Abstract])) OR (Cereal[Title/Abstract])) OR (RTEC[Title/Abstract])) OR (Ready To Eat Cereals[Title/Abstract])) OR (breakfast cereal[Title/Abstract]))))
2	(((((“Child, Preschool”[Mesh]) OR (“Adolescent”[Mesh])) OR (“Minors”[Mesh])) OR (“Students”[Mesh])) OR ((((((((((((((((((((((((((Preschool Child[Title/Abstract]) OR (Children, Preschool[Title/Abstract])) OR (Preschool Children[Title/Abstract])) OR (Children[Title/Abstract])) OR (Adolescence[Title/Abstract])) OR (Teens[Title/Abstract])) OR (Teens[Title/Abstract])) OR (Teenagers[Title/Abstract])) OR (Teenager[Title/Abstract])) OR (Youth[Title/Abstract])) OR (Youths[Title/Abstract])) OR (Adolescents, Female[Title/Abstract])) OR (Adolescent, Female[Title/Abstract])) OR (Female Adolescent[Title/Abstract])) OR (Adolescents, Male[Title/Abstract])) OR (Female Adolescents[Title/Abstract])) OR (Adolescent, Male[Title/Abstract])) OR (Male Adolescent[Title/Abstract])) OR (Male Adolescents[Title/Abstract])) OR (juvenile adult[Title/Abstract])) OR (Minor[Title/Abstract])) OR (Minors[Title/Abstract])) OR (Student[Title/Abstract])) OR (School Enrollment[Title/Abstract])) OR (Enrollment, School[Title/Abstract])) OR (Enrollments, School[Title/Abstract])) OR (School Enrollments[Title/Abstract]))))
3	((randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized [tiab] OR placebo [tiab] OR clinical trials as topic [mesh: noexp] OR randomly [tiab] OR trial [ti]))
4	1 AND 2 AND 3
5	Filters: from 1 January 1990–1 January 2021

2.2. Selection Criteria

The studies included in this review meet the following criteria: (1) subjects include children and adolescents aged 7–19 years old with no restrictions regarding sex, races, or health status; (2) having the intervention that after overnight fasting the subjects consumed a single breakfast meal; (3) studies with explicit breakfast composition, specifically protein content; (4) investigating the effect of PR breakfast on SEI or subjective appetite components (fullness or hunger); (5) use visual analogue scale questionnaire (VAS) to evaluate different aspects of subjective appetite; (6) reporting means and standard error (SE) or standard deviation (SD) or 95% confidence intervals (CI) for SEI and/or fullness and hunger; (7) randomized controlled or crossover trials study design; and (8) studies published in English or Chinese. The articles were excluded if they meet any of the exclusion criteria: (1) articles without sufficient data like reviews, guidelines, case reports, non–human studies, etc.; (2) participants with diabetes, cancer, or other specific conditions that impacted subjective appetite or postprandial metabolism; (3) trials among groups that used other interventions such as health education and promotion, exercise, drug treatments, and dietary supplements; (4) articles without sufficient relevant outcome data; and (5) full–text articles or originals were not available.

2.3. Data Extraction

Two reviewers (Qiu and Zhang) independently extracted data of the included studies and any disagreements were resolved by discussion until resolved, including: (1) First authors' names, publication year, country, study design, duration; (2) Sex and age of participants, body mass index (BMI) percentage of female participants, subject health status; (3) Intervention and control group (Composition of the whole breakfast); (4) Subsequent lunch intake details; and (5) Study results for SEI and subjective appetite (including fullness or hunger). A third reviewer (Long) checked the extracted data. All reported SE were converted to SD. If data were not available in digital form, we used WebPlotDigitizer (WebPlotDigitizer. 2020. https://automeris.io/WebPlotDigitizer/; accessed date: 30 January 2021) to approximatively estimate it from corresponding graphs. WebPlotDigitizer is an open-source, semi–automatic digitization, web–based, free online tool. All the available images files from the original publications were imported to WebPlotDigitizer. The study results for SEI and subjective appetite (including fullness or hunger) were then extracted.

2.4. Appraisal of the Quality of Studies

Two reviewers (Qiu and Zhang) independently evaluated the quality of eligible studies using the Cochrane Collaboration's tool (ROB 1). This tool assesses the risk of bias according to the following domains: randomization, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, missing outcome data, selective reporting, and other sources of bias. The risk of bias for each item was classified as high, low, or unclear. A trial with low risk of all items was rated the overall quality at low risk of bias, at least one item was at high risk was judged as having a high risk of bias overall, otherwise the overall quality was at unclear risk.

2.5. Data Synthesis

Mean differences ± SDs of SEI and subjective appetite, comparing consuming PR breakfast with NP/traditional breakfast were used to calculate the overall effects of eligible studies. Differences in SEI and appetite were analyzed using weighted mean difference (WMD). Due to clinical and methodological between–study heterogeneity, all effect size calculations used a random–effects model. Between–study heterogeneity was evaluated using I^2. Subgroup analysis was based on sex (girl, boy, and both), study design (cross–over and parallel), subject health status (non–overweight and overweight), economic status of country (High–income country and Medium– and Low–income country). Publication bias of SEI was assessed by funnel plots. Sensitivity analysis was performed by the leave–one–out method on studies that may cause bias in the results. All statistical analyses were conducted in R 4.0.3 (packages meta and robvis). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Literature Search and Screening

The search of the five electronic databases identified 5076 records of which 1403 articles were remained after duplicate removal. After screening titles and abstracts, 3605 studies were excluded because they did not meet inclusion and exclusion criteria. Then, 71 studies underwent full–text screening, and 61 studies were excluded after full-text evaluation. Finally, ten studies were included in the analysis (Figure 1) [13–17,20–24]. We searched two ongoing trials from ClinicalTrials.gov that potentially meet our inclusion criteria and are included in future updates of this review (NCT01192100 and NCT03146442).

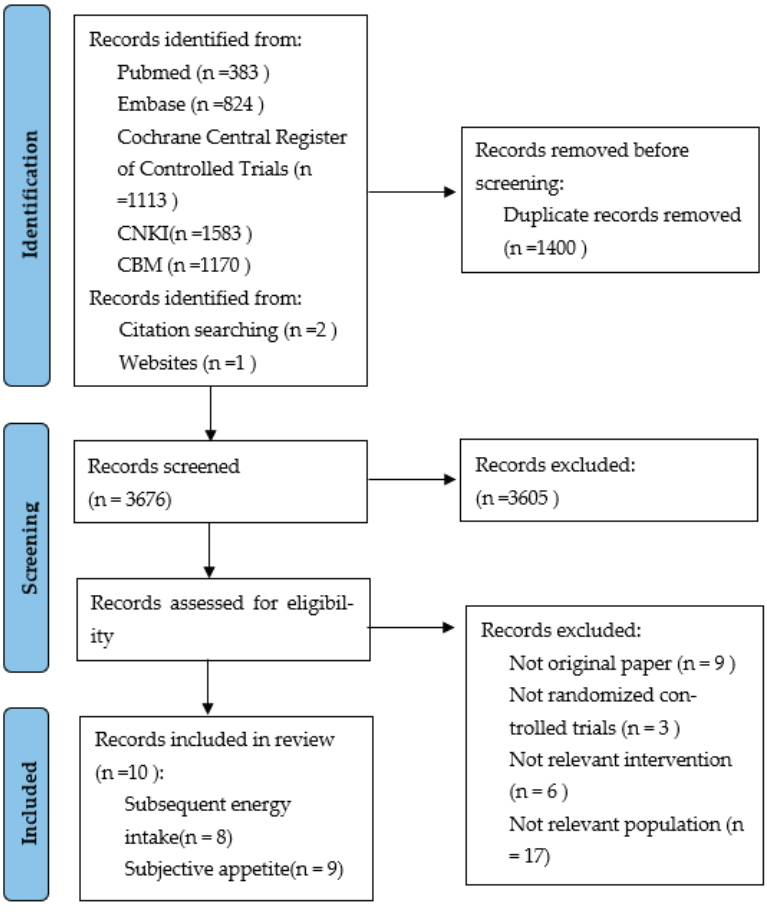

Figure 1. Flow diagram of the literature search process.

3.2. Study Design Characteristics

The characteristics of the included studies are summarized in Table 3. The interventions of all included studies are a PR breakfast. The included studies were published between 2010 and 2020. The sample size ranged from 13 to 156 subjects, with a mean age ranged from 9 to 19 years. Most studies were carried out in the high–income countries [13–16,20–22] and the middle–income countries [17,23,24]. All studies were conducted on healthy children and adolescents. Six trials included specifically with obesity/overweight subjects [13,17,21–24]; the remaining trials included a population with any weight range, including normal weight, overweight, and obese subjects [13–16,20]. Of the 10 studies, eight studies examined the effect on SEI [13–17,20,23,24] and nine studies examined it on subjective appetite measured by VAS [13–17,21–24].

Table 3. Characteristics of included studies in the systematic review.

Author (Country, Year)	Study Design (Duration)	Participants			Intervention Group (Composition of the Whole Breakfast)	Control Group (Composition of the Whole Breakfast)	Composition of the Intervention Breakfast	Subsequent Lunch Intake	Appetite (Mean ± SD/SE *)	
		Population [1]	Age (Mean ± SD)	BMI (Mean ± SD)					Intervention (mm)	Control (mm)
Baum (US, 2015) [13]	Crossover (9 days)	[*n* = 16; 44%], Nonoverweight	9.9 ± 1.2	16.7 ± 1.6	PRO (344 kcal, 18 g protein, 45 g CHO, 16 g sugars, 1 g fiber, 10.5 g fat)	CHO [2] (327 kcal, 3 g protein, 55 g CHO, 39 g sugars, 0.5 g fiber, 11 g fat)	Egg whites, butter, orange juice, white bread	Buffet-style meal served at 240 min	Hunger Post–breakfast: 32.8 ± 8.2 * Pre–lunch: 77.6 ± 3.5 * Fullness Post–breakfast: 68.9 ± 7.5 * Pre–lunch: 23.3 ± 4.2 *	Hunger Post–breakfast: 50.8 ± 6.1 * Pre–lunch: 82.6 ± 4.3 * Fullness Post–breakfast: 42.7 ± 6.1 * Pre–lunch: 21.1 ± 5.3 *
		[*n* = 13; 46%], Overweight/obese	9.5 ± 1.4	22.7 ± 4.0	PRO (344 kcal, 18 g protein, 45 g CHO, 16 g sugars, 1 g fiber, 10.5 g fat)	CHO * (327 kcal, 3 g protein, 55 g CHO, 39 g sugars, 0.5 g fiber, 11 g fat)	Egg whites, butter, orange juice, white bread	Buffet-style meal served at 240 min	Hunger Post–breakfast: 37.0 ± 9.4 * Pre–lunch: 85.2 ± 5.0 * Fullness Post–breakfast: 70.0 ± 9.0 * Pre–lunch: 13.0 ± 4.7 *	Hunger Post–breakfast: 41.5 ± 10.1 * Pre–lunch: 81.6 ± 7.0 * Fullness Post–breakfast: 38.2 ± 9.2 * Pre–lunch: 15.4 ± 6.3 *
Bellissimo (Canada, 2020) [20]	Crossover (25 days)	[*n* = 17; 47%], Nonoverweight	12.0 ± 1.65	20.8 ± 3.7	HP (450 kcal, 45 g protein, 30 g CHO, 2 g fiber, 17 g fat)MP (450 kcal, 30 g protein, 45 g CHO, 3 g fiber, 17 g fat)LP (450 kcal, 15 g protein, 61 g CHO, 5 g fiber, 17 g fat)	C (450 kcal, 7 g protein, 69 g CHO, 3 g fiber, 17 g fat)	Egg yolk, egg whites, butter, cheese, home fries, ketchup	Pizza lunch according to one's preference served at 210 min	NA	NA

Table 3. *Cont.*

Author (Country, Year)	Study Design (Duration)	Participants			Intervention Group (Composition of the Whole Breakfast)	Control Group (Composition of the Whole Breakfast)	Composition of the Intervention Breakfast	Subsequent Lunch Intake	Appetite (Mean ± SD/SE *)	
		Population [1]	Age (Mean ± SD)	BMI (Mean ± SD)					Intervention (mm)	Control (mm)
Douglas (US, 2019) [21]	Crossover (15 days)	[*n* = 19, 100%], Overweight	19 ± 1	29.0 ± 3.8	SKIP-HP (350 kcal, 35 g protein)	SKIP-NP (350 kcal, 13 g protein)	Yogurt parfaits, bagels, breakfast burritos, cereals, etc.	NA	Hunger Post–breakfast: 9.9 ± 9.7 Pre–lunch: 50.8 ± 19.7 Fullness Post–breakfast: 72.0 ± 22.2 Pre–lunch: 28.3 ± 16.8	Hunger Post–breakfast: 12.7 ± 15.9 Pre–lunch: 61.1 ± 20.5 Fullness Post–breakfast: 83. 5 ± 11.3 Pre–lunch: 30.2 ± 22.5
		[*n* = 18, 100%], Overweight	19 ± 1	28.9 ± 2.9	CONSUME-HP (350 kcal, 35 g protein)	CONSUME-NP (350 kcal, 13 g protein)	Yogurt parfaits, bagels, breakfast burritos, cereals, etc.	NA	Hunger Post–breakfast: 10.3 ± 17.9 Pre–lunch: 70.8 ± 14.6 Fullness Post–breakfast: 80.9 ± 14.9 Pre-lunch: 21.0 ± 14.5	Hunger Post–breakfast: 7.3 ± 9.2 Pre–lunch: 46.4 ± 22.5 Fullness Post–breakfast: 75.8 ± 19.3 Pre–lunch: 34.8 ± 17.8
Kral (US, 2016) [15]	Crossover (3 weeks)	[*n* = 40, 47.5%], Overweight/Nonoverweight	9.4 ± 0.8	NA Overweight or obese (45%)	Egg (350 kcal, protein % energy: 21)	Oatmeal (350 kcal, protein % energy: 14%)Cereal (350 kcal, protein % energy: 8%)	Scrambled eggs (prepared with 1/8 tsp. table salt), toasted whole wheat bread, diced peaches, and milk (1% fat)	Lunch (chicken nuggets, macaroni and cheese, green beans (prepared with 3 g of salted butter), ketchup, applesauce, chocolate chip cookies, and milk) served at 180 min	Hunger Post–breakfast: 19.0 ± 4.4 * Pre–lunch: 83.1 ± 4.4 * Fullness Post–breakfast: 60.4 ± 4.9 * Pre–lunch: 8.4 ± 4.9 *	Hunger (Oarmal) Post–breakfast: 14.1 ± 3.5 * Pre–lunch: 69.4 ± 4.9 * Fullness (Oarmal) Post–breakfast: 59.0 ± 5.5 * Pre–lunch: 16.0 ± 3.8 * Hunger (Cereal) Post–breakfast: 22.5 ± 5.5 * Pre–lunch: 77.0 ± 4.6 * Fullness (Cereal) Post–breakfast: 57. 7 ± 6.0 * Pre–lunch: 14.4 ± 7.1 *

Table 3. *Cont.*

Author (Country, Year)	Study Design (Duration)	Participants			Intervention Group (Composition of the Whole Breakfast)	Control Group (Composition of the Whole Breakfast)	Composition of the Intervention Breakfast	Subsequent Lunch Intake	Appetite (Mean ± SD/SE *)	
		Population [1]	Age (Mean ± SD)	BMI (Mean ± SD)					Intervention (mm)	Control (mm)
Leidy (UK, 2010) [16]	Crossover (17 days)	[*n* = 13, 46%], Overweight/Nonoverweight	14.3 ± 1.1	23.5 ± 3.6	PR * (512 ± 26 kcal, 49.1 ± 2.5 g protein, 62.8 ± 3.2 g CHO, 30.7 ± 1.6 g sugar, 2.1 ± 0.1 g fiber, 7.5 ± 0.4 g fat)	PN * (513 ± 26 kcal, 18.1 ± 0.9 g protein, 95.3 ± 4.9 g CHO, 31.1 ± 1.6 g sugar, 2.0 ± 0.1 g fiber, 7.5 ± 0.4 g fat)	Whey Pancakes (whey protein powder, skim milk, margarine, egg–whites, butter, etc.)	Buffet lunch served at 240 min	Fullness Post–breakfast: 54.7 ± 8.1 * Pre–lunch: 32.1 ± 5.8 *	Fullness Post–breakfast: 48.7 ± 6.0 * Pre–lunch: 18.5 ± 4.0 *
Leidy (US, 2013) [22]	Crossover (5 weeks)	[*n* = 20, 100%], Overweight	19 ± 4.5	28.6 ± 3.1	HP (350 kcal, 35.1 g protein, 35.1 g CHO, 18 g sugar, 6.1 g fiber, 7.8 g fat)	NP (350 kcal, 13 g protein, 57 g CHO, 18 g sugar, 6.1 g fiber, 7.8 g fat)	Egg, Beef, Dairy, Plant–based, etc.	NA	Hunger Post–breakfast: 7.1 ± 5. 7 * Pre–lunch: 45.3 ± 3.6 * Fullness Post–breakfast: 76.3 ± 2.2 * Pre–lunch: 35.0 ± 2.9 *	Hunger Post–breakfast: 10.5 ± 2.3 * Pre–lunch: 49.7 ± 5.3 * Fullness Post–breakfast: 71.0 ± 5.1 * Pre–lunch: 28.0 ± 3.6 *
Liu (US, 2015) [14]	Parallel (9 days)	[*n* = 15, 60%], Overweight/Nonoverweight	15.6 ± 4.26	NA Overweight or obese (40%)	Egg (342 kcal, 16.8 g protein, 32.2 g CHO, 16.6 g fat)	Bagel (336 kcal, 11 g protein, 48.6 g CHO, 10 g fat)	Scrambled, toast, jelly	Lunch (baked chicken, macaroni and cheese, green beans, mandarin oranges, rolls, and milk) served at 180 min	Hunger Post–breakfast: 23.0 ± 6.0 * Pre–lunch: 42.2 ± 6.2 * Fullness Post–breakfast: 66.4 ± 6. 9 * Pre–lunch: 49.6 ± 7.3 *	Hunger Post–breakfast: 25.2 ± 6.2 * Pre–lunch: 49.0 ± 6.0 * Fullness Post–breakfast: 68.44 ± 6. 7 * Pre–lunch: 49.55 ± 4. 9 *

Table 3. *Cont.*

Author (Country, Year)	Study Design (Duration)	Participants			Intervention Group (Composition of the Whole Breakfast)	Control Group (Composition of the Whole Breakfast)	Composition of the Intervention Breakfast	Subsequent Lunch Intake	Appetite (Mean ± SD/SE *)	
		Population [1]	Age (Mean ± SD)	BMI (Mean ± SD)					Intervention (mm)	Control (mm)
Mehrabani (Iran, 2015) [23]	Crossover (16 days)	[*n* = 34, 0%], Overweight	11.14 ± 0.8	27.62 ± 2.7	LFM (401.24 kcal, 19.08 g protein, 49.055 g CHO, 0.458 g fiber, 15.407 g fat)	W (297.74 kcal, 10.931 g protein, 37.185 g CHO, 0.458 g fiber, 12.779 g fat) AJ (411.44 kcal, 11.276 g protein, 65.195 g CHO, 1.016 g fiber, 13.022 g fat)	Low–fat milk, Iranian whole wheat bread, Walnut, Low–fat cheese	Buffet-style meal served at 300 min	Hunger Post–breakfast: 12.6 ± 1.0 * Pre–lunch: 72.6 ± 1.0 * Fullness Post–breakfast: 86.4 ± 0.9 * Pre–lunch: 21.1 ± 1.4 *	Hunger (W) Post–breakfast: 22.8 ± 1.6 * Pre–lunch: 79.4 ± 1.0 * Fullness (W) Post–breakfast: 74.0 ± 1.5 * Pre–lunch: 16.4 ± 0.9 * Hunger (AJ) Post–breakfast: 14.7 ± 1.2 * Pre–lunch: 75.9 ± 0.8 * Fullness (AJ) Post–breakfast: 83.5 ± 1.0 * Pre–lunch: 19.5 ± 1.3 *
Wang (China, 2014) [17]	Parallel (9 days)	[*n* = 56, 46%], Overweight	14.1 ± 2.1	32.2 ± 1.7	Egg (386 kcal, 12.2 g protein, 29.3 g CHO, 15.9 g fat)	Steamed bread (386 kcal, 8.2 g protein, 44.7 g CHO, 11.5 g fat)	Boiled eggs, White rice, Milk	Lunch (pork with Chinese cabbage, apple, and rice, etc.) served at 240 min	Hunger Post–breakfast: 23.2 ± 0.2 Pre–lunch: 41.1 ± 0.4 Fullness Post–breakfast: 64, 9 ± 0.7 Pre–lunch: 45.2 ± 0.6	Hunger Post–breakfast: 23.1 ± 0.2 Pre–lunch: 52.3 ± 0.5 Fullness Post–breakfast: 65.0 ± 0.8 Pre–lunch: 35.1 ± 0.8
Wang (China, 2015) [24]	Parallel (3 months)	[*n* = 156, 49%], Overweight	14.3 ± 2.2	32.0 ± 1.7	Egg (386 kcal, 12.2 g protein, 29.3 g CHO, 15.9 g fat)	Steamed bread (386 kcal, 8.2 g protein, 44.7 g CHO, 11.5 g fat)	Boiled eggs, White rice, Milk	Lunch (pork with Chinese cabbage, apple, and rice, etc.) served at 240 min	Hunger Post–breakfast: 22.1 ± 0.1 Pre–lunch: 40.6 ± 0.6 Fullness: Post–breakfast: 65.1 ± 0.8 Pre–lunch: 45.0 ± 0.6	Hunger Post–breakfast: 22.30 ± 0.3 Pre–lunch: 51.20 ± 0.3 Fullness: Post–breakfast: 64.9 ± 0.9 Pre–lunch: 34.9 ± 0.9

[1] [total number completed: % girl], subject health status; PRO: protein-based breakfast; CHO [2]: carbohydrate-based breakfast; HP: high protein; MP: medium protein; LP: low protein; C: control; SKIP-HP: habitually skipped higher-protein breakfast; SKIP-NP: habitually skipped normal-protein breakfast; CONSUME-HP: habitually consumed higher-protein breakfast; CONSUME-NP: habitually consumed normal-protein breakfast; PR: protein-rich breakfast; PN: normal-protein breakfast; HP: high-protein breakfast; NP: normal-protein breakfast; LFM: a fixed breakfast with low-fat milk; W: a fixed breakfast with water; AJ: a fixed breakfast with apple juice; NA: Not Applicable; BMI: body mass index; CHO: Carbohydrate; US = United States; UK = United Kingdom; 1 kcal = 4.18 kJ; SD = standard deviation; SE * = standard error.

3.3. Risk of Bias across Studies

The risk of bias assessments for all included studies was presented in Table 4, Figures 2 and 3. Due to lack of allocation concealment, blinding of participants and personnel, and blinding of subjective and objective outcome assessment, the primary issues were at a high risk of bias among the ten RCTs. Most RCTs reported information regarding randomization sequence generation was at unclear risk. Of ten included studies, nine were categorized as high risk, and one as unclear risk.

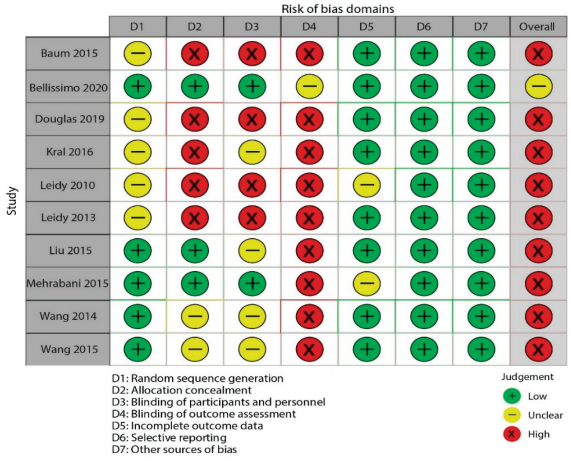

Figure 2. Study quality and risk of bias assessment of included studies in the meta−analysis (traffic light).

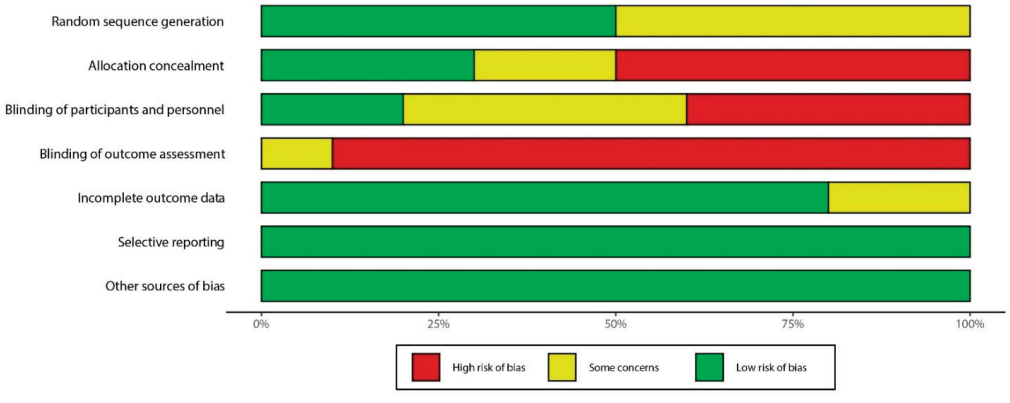

Figure 3. Study quality and risk of bias assessment of included studies in the meta−analysis (summary).

Table 4. Study quality and risk of bias assessment of included studies in the meta-analysis.

Study ID	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Blinding of Outcome Assessors	Incomplete Outcome Data	Selective Reporting	Other Bias	Overall Quality
Baum 2015	Unclear risk	High risk	High risk	High risk	Low risk	Low risk	Low risk	High risk
Bellissimo2020	Low risk	Low risk	Low risk	Unclear risk	Low risk	Low risk	Low risk	Unclear risk
Douglas2019	Unclear risk	High risk	High risk	High risk	Low risk	Low risk	Low risk	High risk
Kral2016	Unclear risk	High risk	Unclear risk	High risk	Low risk	Low risk	Low risk	High risk
Leidy2010	Unclear risk	High risk	High risk	High risk	Unclear risk	Low risk	Low risk	High risk
Leidy2013	Unclear risk	High risk	High risk	High risk	Low risk	Low risk	Low risk	High risk
Liu2015	Low risk	Low risk	Unclear risk	High risk	Low risk	Low risk	Low risk	High risk
Mehrabani2015	Low risk	Low risk	Low risk	High risk	Unclear risk	Low risk	Low risk	High risk
Wang2014	Low risk	Unclear risk	Unclear risk	High risk	Low risk	Low risk	Low risk	High risk
Wang2015	Low risk	Unclear risk	Unclear risk	High risk	Low risk	Low risk	Low risk	High risk

3.4. Findings from Meta–Analysis

3.4.1. Protein–Rich Breakfast and Subsequent Energy Intake

The effect of PR breakfast on SEI was examined in eight studies [13–17,20,23,24]. At the end of the trials (range 9 days to 3 months), we observed that participants who were assigned to consume PR breakfast had a lower SEI than those assigned to consume NP/traditional breakfast (MD, −111.2 kcal; 95% CI: −145.4 to −76.9; $p < 0.01$) (Figure 4), namely, consuming PR breakfast elicits the decrease of SEI. However, we did detect considerable inconsistency across trial results ($Tau^2 = 1294.9$, $I^2 = 67.0\%$, $Q = 36.3$). The funnel plot showed some asymmetry (Figure A1). After the elimination of one trial, the results were largely robust to the traditional sensitivity analysis. The heterogeneity was significantly reduced (MD, −100.0 kcal; 95% CI: −120.5 to −79.5; $Tau^2 = 213.6$, $I^2 = 24.0\%$, $Q = 14.5$). In addition, we performed a subgroup analysis based on study design, sex, economic status of country, and baseline body mass index (Table 5). Thus, we presumed that the trial of Mehrabani et al. [23] was the source of heterogeneity.

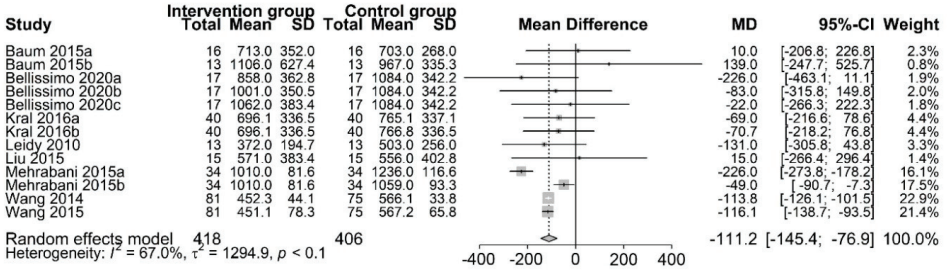

Figure 4. Random–effects meta–analysis of relationships between protein–rich breakfast and subsequent energy intake (kcal). Data for Baum 2015a [13] are based on non–overweight participants, whereas data for Baum 2015b [13] are based on overweight participants. Bellissimo 2020a [20], Bellissimo 2020b [20], and Bellissimo 2020c [20] are based on different subsets of subjects who identified as different dose protein breakfast consumers. Kral 2016a (Oatmeal vs. Scrambled eggs) [15], Kral 2016b (Cereal vs. Scrambled eggs) [15], Mehrabani 2015a (LFM vs. W) [23], and Mehrabani 2015b (LFM vs. AJ) [23] are based on different subsets of subjects who identified as different control groups, respectively. Other studies were defined as Leidy 2010 [16], Liu2015 [14], Wang2014 [17], and Wang 2015 [24], respectively.

Table 5. Results of subgroup–analysis for subsequent energy intake (kcal) and protein–rich breakfast.

	Number of Comparisons	WMD (95% CI)	Heterogeneity I^2 (%)	*p* between
Study–design				
Crossover	10	−116.9 (−145.6, −88.3)	75%	$p < 0.0001$
Parallel	3	−114.1 (−124.9, −103.4)	0%	0.66
Sex				
Girl	0			
Boy	2	−125.3 (−156.8, −93.9)	97%	$p < 0.0001$
Both	11	−113.2 (−123.9, −102.6)	0%	0.82
Economic status of country				
High–income country	9	−70.09 (−137.8, −2.4)	0%	0.83
Medium–and–low–income country	4	−115.49 (−125.7, −105.3)	90%	$p < 0.0001$
Baseline body mass index (BMI)				
Non–overweight/Overweight	8	−76.70 (−145.5, −8.0)	0%	0.87
Overweight/Obese	5	−115.31 (−125.5, −105.1)	88%	$p < 0.0001$

3.4.2. Breakfast and Subjective Appetite

Protein–Rich Breakfast and Fullness

Fullness was reported according to the effect of PR breakfast in nine studies, including two time points of post–breakfast and pre–lunch [13–17,21–24].We found that participants who were assigned to consume PR breakfast had a higher fullness than those assigned to consume NP/traditional breakfast, in random–effects meta–analysis of the post–breakfast (MD, 2.3 mm; 95% CI:0.8, 3.8; $p < 0.01$) and pre–lunch group (MD, 7.4 mm; 95% CI: 6.0, 8.8; $p < 0.01$) (Figure 5), although there was substantial inconsistency across trial results ($Tau^2 = 1.8$, $I^2 = 84.0\%$, $Q = 74.7$ and $Tau^2 = 1.5$, $I^2 = 83.2\%$, $Q = 71.4$, respectively). The meta–analysis results for the pooled effects of the post–breakfast and pre–lunch groups were robust in the sensitivity analysis. Similarly, we also conducted a subgroup analysis (Table 6). To assess the impact of study design, we exclude the crossover design of the trial and found a large change in the mean difference (post–breakfast: 5.8 mm, 95% CI: 3.9, 7.7; pre–lunch: 2.39 mm, 95% CI: 0.3, 4.5). However, we found that it did not have a significant impact on the heterogeneity of the post-breakfast and pre–lunch group.

Protein–Rich Breakfast and Hunger

The effect of PR breakfast on hunger was examined in eight studies [13–15,17,21–24]. A random–effects meta–analysis revealed that the hunger did not differ between trials in the post–breakfast group (MD, −0.2 mm; 95% CI: −0.7, 0.2; $p < 0.01$) (Figure 6). However, we found that participants who were assigned to consume PR breakfast had a lower hunger than those assigned to consume NP/traditional breakfast in the pre–lunch group (MD, −8.48 mm; 95% CI: −9.7, −7.3; $p < 0.01$) (Figure 6), although there was significant inconsistency across trial results ($Tau^2 = 1.1$, $I^2 = 90.5\%$, $Q = 116.0$). The meta–analysis result of the pre–lunch group was steady in the sensitivity analysis. Likewise, the results of all other subgroup analyses were not significant (Table 7).

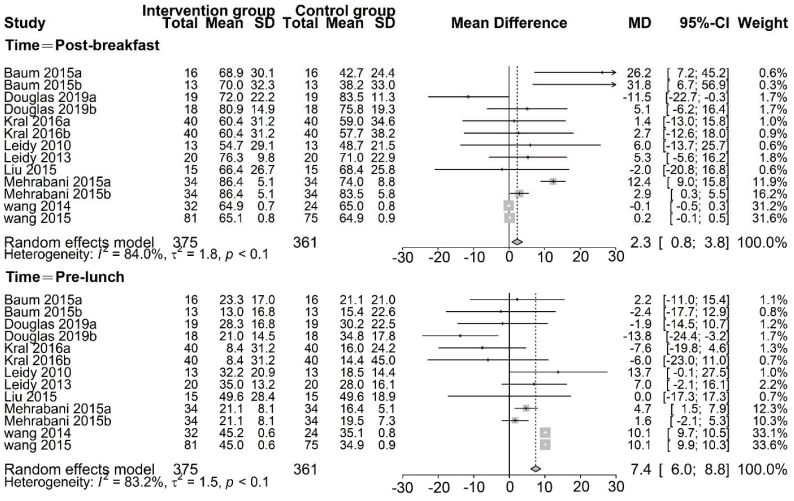

Figure 5. Random–effects meta–analysis of relationships between protein–rich breakfast and fullness (mm). Data for Baum 2015a are based on non–overweight participants, whereas data for Baum 2015b are based on overweight participants. Bellissimo 2020a [20], Bellissimo 2020b [20], and Bellissimo 2020c [20] are based on different subsets of subjects who identified as different dose protein breakfast consumers. Data for Douglas 2019a [21] are based on different subsets of subjects who habitually skipped breakfast, whereas data for Douglas 2019b [21] are based on different subsets of subjects who habitually consumed breakfast. Kral2016a (Oatmeal vs. Scrambled eggs) [15], Kral2016b (Cereal vs. Scrambled eggs) [15], Mehrabani 2015a (LFM vs. W) [23], and Mehrabani 2015b (LFM vs. AJ) [23] are based on different subsets of subjects who identified as different control groups, respectively. Other studies were defined as Leidy 2010 [16], Liu2015 [14], Wang2014 [17], and Wang 2015 [24], respectively.

Table 6. Results of subgroup–analysis for fullness (mm) and protein–rich breakfast.

	Number of Comparisons	WMD (95% CI)	Heterogeneity I^2 (%)	*p* between
Subgroup analyses for fullness and protein–rich breakfast (post–breakfast)				
Study–design				
Crossover	10	6.0 (4.1, 7.9)	76%	$p < 0.0001$
Parallel	3	0.1 (−0.1, 0.3)	0%	0.46
Sex				
Girl	3	−0.3 (−6.7, 6.2)	65%	0.06
Boy	2	6.4 (4.3, 8.5)	95%	$p < 0.0001$
Both	7	−0.1 (−0.5, 0.3)	58%	0.03
Economic status of country				
High–income country	9	2.5 (−2.2, 7.1)	57%	0.02
Medium–and low– income country	4	0.2 (−0.0, 0.4)	95%	$p < 0.0001$
Baseline body mass index (BMI)				
Non–overweight/Overweight	5	5.9 (−1.8, 13.5)	29%	0.23
Overweight/Obese	8	0.2 (−0.0, 0.4)	90%	$p < 0.0001$
Subgroup analyses for fullness and protein–rich breakfast (pre–lunch)				
Study–design				
Crossover	10	2.4 (0.3, 4.5)	53%	0.02
Parallel	3	10.1 (9. 9, 10.3)	0%	0.52
Sex				
Girl	3	−1.8 (−7.9, 4.2)	76%	0.01
Boy	2	3.4 (1.0, 5.8)	37%	0.21
Both	8	10.1 (9.9, 10.3)	59%	0.02
Economic status of country				
High–income country	9	−0.8 (−5.1, 3.4)	46%	0.06
Medium–and low– income country	4	10.1 (9.9, 10.3)	90%	$P < 0.0001$
Baseline body mass index (BMI)				
Non–overweight/Overweight	5	0.6 (−5.9, 6.9)	33%	0.2
Overweight/Obese	8	10.0 (9.8, 10.2)	88%	$P < 0.0001$

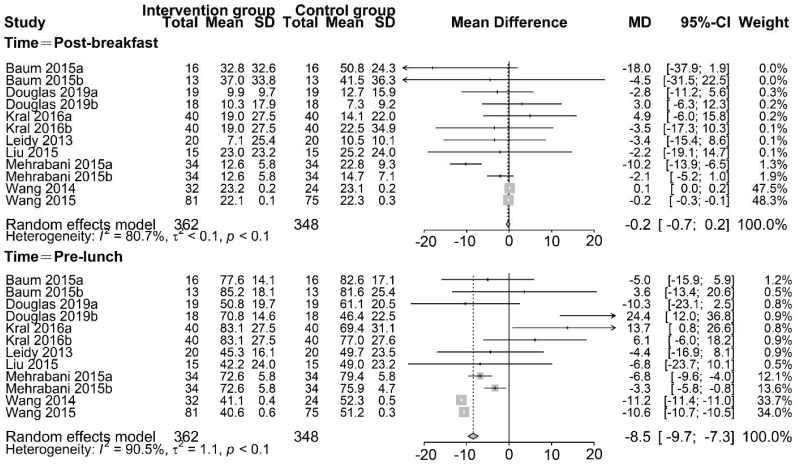

Figure 6. Random–effects meta–analysis of relationships between protein–rich breakfast and hunger (mm). Data for Baum 2015a [13] are based on non–overweight participants, whereas data for Baum 2015b [13] are based on overweight participants. Bellissimo 2020a [20], Bellissimo 2020b [20], and Bellissimo 2020c [20] are based on different subsets of subjects who identified as different dose protein breakfast consumers. Data for Douglas 2019a [21] are based on different subsets of subjects who habitually skipped breakfast, whereas data for Douglas 2019b [21] are based on different subsets of subjects who habitually consumed breakfast. Kral 2016a (Oatmeal vs. Scrambled eggs) [15], Kral 2016b (Cereal vs. Scrambled eggs) [15], Mehrabani 2015a (LFM vs. W) [23], and Mehrabani 2015b (LFM vs. AJ) [23] are based on different subsets of subjects who identified as different control groups, respectively. Other studies were defined as Liu2015 [14], Wang2014 [17], and Wang 2015 [24], respectively.

Table 7. Results of subgroup–analysis for hunger (mm) an d protein–rich breakfast (pre-lunch).

	Number of Comparisons	WMD (95% CI)	Heterogeneity I^2 (%)	p between
Study–design				
Crossover	9	−3.8 (−5.5, −2.0)	78%	$p < 0.0001$
Parallel	3	−10.8 (−10.9, −10.6)	88%	0.0002
Sex				
Girl	3	3.5 (−3.7, 10.8)	88%	0.0002
Boy	2	−4.9 (−6.8, −3.0)	70%	0.07
Both	7	−10.8 (−10.9, −10.6)	86%	$p < 0.0001$
Economic status of country				
High–income country	8	2.9 (−1.7, 7.5)	70%	0.0002
Medium–and low– income country	4	−10.7 (−10.9, −10.6)	95%	$p < 0.0001$
Baseline body mass index (BMI)				
Non–overweight/Overweight	4	2.3 (−4.0, 8.7)	52%	0.1
Overweight/Obese	8	−10.7 (−10.9, −10.6)	92%	$p < 0.0001$

4. Discussion

This systematic review and meta–analysis of 10 studies examined the effect on SEI and appetite in children and adolescents consuming a PR breakfast. We found new evidences to support the opinion that the PR breakfast consumption decreased SEI compared with consuming NP/traditional breakfast. Furthermore, there was an evidence indicated that consumption of a PR breakfast can increase fullness and decrease hunger. When we conducted a subgroup analysis based on study design, sex, economic status of country,

and baseline body mass index, the results of the pre–lunch group were similar. In addition, this review and meta–analysis provided the first evidence demonstrating the effect of PR breakfast on SEI and subjective appetite components (hunger and fullness), and provided new evidences of the relationship between energy balance and obesity.

4.1. Principal Findings

Energy imbalance seems to be an independent risk factor in the etiology of obesity [25]. Meta–analysis of RCTs showed decreased SEI in participants who consumed a PR breakfast compared with those who consumed NP/traditional breakfast among children and adolescents. The sensitivity analysis indicated that the trial of Mehrabani et al. [23] was responsible for the most of the heterogeneity. We compared Mehrabani 2015a with Mehrabani 2015b and found that the breakfast composition and energy contribution were greatly different between the trials. In addition, the included studies [15–17,19,22,23] showed that consuming a PR breakfast reduced SEI, but not total energy intake (TEI) [15,16,20]. However, the previous study supported a negative association between dietary protein and TEI [26]. Thus, the significant reduction in SEI in participants suggests that serving a PR breakfast may be a strategy to regulate energy balance in children and adolescents.

We also found that fullness was higher and hunger was lower in groups consuming PR breakfast than those consuming an NP/traditional breakfast among children and adolescents. And we observed that the effect of a PR vs. an NP/traditional breakfast had a higher fullness at post–breakfast. Some included studies [13,14,17,24] had reported appetite at 30 min post–breakfast. Of these, only one trial [13] reported that the normal weight children consuming PRO breakfast had significantly lower glucose values at 30 min than those children consuming CHO breakfast, this suggested that diets higher in protein and lower in carbohydrate had been shown to improve glycemic control. Thus, appetite regulation is likely one of the mechanisms that are responsible for better glycemic control. The subgroup analysis was performed in our research to investigate the possible explanations for the heterogeneity of satiety in the post–breakfast group and the pre–lunch group. The results of fullness may be affected by some aspects of the study design. However, study design does not influence the results of hunger. This difference may be due to the difference in time intervals. One of the included studies, conducted in Iran, found the greatest differences in appetite scores at 4 h after breakfast intake and these differences remained significant at 5 h [23]. The finding may be explained via food between the preloads and their subsequent meals, while the related indicators were not measured in other studies [13–17,20–23]. In addition, a recent systematic review indicated that consuming a high–protein diet may influence subjective appetite by enhancing fullness, while hunger-reduction observed in the high protein diet did not convert to appetite [27].

We saw methodological differences across the trials of the length of measurement period, energy content, and macronutrient composition of breakfast. And these differences may in part account for the heterogeneities. The effects of PR breakfast on SEI and appetite are greatly influenced by various protein doses and forms [28]. There is no consensus on breakfast protein recommendations for children and adolescents. Most included studies were conducted with adolescents, and the recommended dietary allowance for dietary protein is 0.85 g protein kg^{-1} day^{-1} for adolescents aged 14–18 years [29]. However, in our included studies protein dose of interventions ranged from 12.2 g to 58 g. In addition, protein type may also be a critical factor impacting the heterogeneities. In the most trials, the protein was administered in a semi–solid/solid form. Four of the included studies examined the effect of an egg breakfast [14,15,17,24]. Previous studies showed complete proteins can drive thermogenesis, thus affecting the synthesized effect [30]. Another explanation might be due to the differences in breakfast size, meal frequency and habitual breakfast patterns [31,32]. Taken together, the data did not support enough the potential mechanisms of the effect of dietary protein breakfast. Further high–quality studies are needed to fill the important gap.

According to the protein leverage hypothesis, the body preferentially consumes protein in three main nutrients (carbohydrate, fat, and protein). If a breakfast lacks adequate protein, then we had to attempt to acquire a higher amount of protein from more food, leading to an increased risk of obesity [33]. Furthermore, protein increases diet–induced thermogenesis (DIT) more than carbohydrates and fats in adults due to the high energy costs related to protein synthesis and changes in substrate utilization conducing to fat oxidation [34,35]. Two of the included studies examined carbohydrate oxidation and fat oxidation [13,19]. One of the two studies, conducted in non–overweight individuals, found the protein–rich treatment had lower carbohydrate oxidation and greater fat oxidation compared to the control breakfast. However, the other study conducted in non–overweight and overweight subjects, respectively, researchers found greater fat oxidation but no difference in carbohydrate oxidation between different meals. Furthermore, the differences in results between the trials are also likely to be attributed to confounders from other compositions in breakfast, such as fiber and fat [36]. However, previous studies showed that protein has better appetite suppressive effects than other nutrients [37]. Another study indicated that dietary protein content was negatively related to TEI irrespective of whether fat or carbohydrate was the diluents of protein [25]. Thus, it is presumed that the consumption of higher protein at breakfast could assist in weight management because of declined SEI and suppressed appetite.

4.2. Quality of Evidence

For some reasons, we consider the quality of evidence to be low. All included studies were at unclear risk, or high risk of bias in at least one risk of bias item. More strictly conducted trials could draw more decisive conclusions. We also observed considerable heterogeneity among the results of subjective components (hunger and fullness). Firstly, although the VAS can serve as a useful supplementary method to measure food intake, it is lack of uniform scale and appetite rating is subjective [38]. Furthermore, the heterogeneity may be partly due to the age difference of intervention objects. The degree of refinement of brain structure and function varies in children and adolescents of different ages, although we narrowed the inclusion of age [39].

Most of the studies included in this review were conducted in the US, UK, and Canada [13–16,19–21]. Protein sources and nationally habitual breakfast patterns in these countries may differ greatly from those in other countries that do not follow the western dietary patterns, such as China or Japan. Veldhorst et al. [40] found that different protein sources can affect SEI and subjective appetite. Thus, the findings concerning SEI and appetite should be interpreted with caution.

4.3. Limitations

This meta–analysis had some limitations. At first, a further obvious limitation is that there is not adequate numbers of literatures and subjects included in the review, which will be resolved in the future. Second, the search strategy should have reported short–term energy intake or appetite as search terms. This omission might influence the number of included articles. Additionally, we set a 31–year limit according to the previous studies [19,41]. However, we should ideally have performed search from inception until January 2021 and then do meta–regression for year of reporting to ascertain such change, which will provide a time series like interpretation. Third, the included trials lasted from 9 days up to 3 months. Although the divergence in SEI between PR breakfast eaters and NP/traditional breakfast eaters was about 111 kcal. In some studies [15,16,21], total energy intake (TEI) was not different between the groups, whereas in others [13,14,20–24] TEI was not measured. And short– and long–term protein consumption could also produce different effects on appetite [42]. Thus, it is hard to draw conclusions about SEI and subjective appetite based on existing results. More long–term trials are needed to identify whether these changes cause long–term alterations in routine energy regulation and appetite control when the PR breakfast is consumed in daily. Fourth, the included studies examined a

series of hormones associated with energy balance and appetite regulation, including ghrelin and PYY (serum peptide YY). Six of the included studies examined changes in hormones [14,16,17,21,22,24]. The levels of ghrelin and PYY did not differ significantly between the intervention and control groups. We have no explanation for this phenomenon, and this needs to be further researched.

5. Conclusions

As the quality of the eligible studies was mostly low, the results ought to be interpreted cautiously. Currently, the meta–analysis reveals consuming a protein–rich breakfast has an impact on decreased subsequent energy intake, decreased hunger and increased fullness among children and adolescents. And our review provides a better understanding of the relationship between energy balance and obesity by regulation of short–term energy intake or appetite. More high–quality RCTs are needed to prove whether those children and adolescents against obesity should consume protein–rich breakfast and identify the suitable dosage of protein.

Author Contributions: M.Q. and Y.Z. designed the whole study and completed the literature search and data collection; and M.Q. and Z.L. conducted the meta-analysis; M.Q. wrote the original draft and review & editing; Y.H. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data (reviewed articles) used, were from already published empirical articles, retrieved from databases. All data generated or analysed during this study were included in this published article.

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Conflicts of Interest: No potential conflict of interest was reported by the authors.

Appendix A

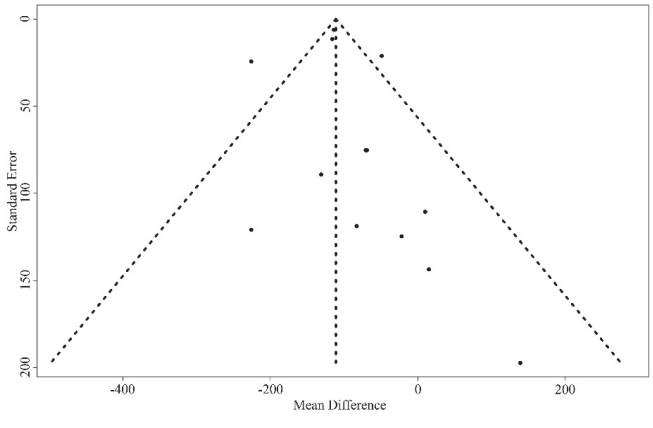

Figure A1. Funnel plot for random effects meta-analysis of mean difference in subsequent energy intake (kcal).

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Review

Probiotics in Pediatrics. A Review and Practical Guide

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Abstract: The potential benefit of the administration of probiotics in children has been studied in many settings globally. Probiotics products contain viable micro-organisms that confer a health benefit on the host. Beneficial effects of selected probiotic strains for the management or prevention of selected pediatric conditions have been demonstrated. The purpose of this paper is to provide an overview of current available evidence on the efficacy of specific probiotics in selected conditions to guide pediatricians in decision-making on the therapeutic or prophylactic use of probiotic strains in children. Evidence to support the use of certain probiotics in selected pediatric conditions is often available. In addition, the administration of probiotics is associated with a low risk of adverse events and is generally well tolerated. The best documented efficacy of certain probiotics is for treatment of infectious gastroenteritis, and prevention of antibiotic-associated, *Clostridioides* difficile-associated and nosocomial diarrhea. Unfortunately, due to study heterogeneity and in some cases high risk of bias in published studies, a broad consensus is lacking for specific probiotic strains, doses and treatment regimens for some pediatric indications. The current available evidence thus limits the systematic administration of probiotics. The most recent meta-analyses and reviews highlight the need for more well-designed, properly powered, strain-specific and dedicated-dose response studies.

Keywords: probiotics; pediatrics; children

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1. Introduction

The human microbiome is a topic of great research interest and its role in host protection, physiology and the development of a normal and balanced immune system has been extensively proven. Probiotics are suggested as a therapeutic or preventive option for a variety of childhood diseases [1–3].

The term probiotic was defined in 2001 by an Expert Panel of the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO). In 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) convened an Expert Panel reviewing the field of probiotics and the literature. The result was a consensus statement reiterating the following definition: "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". This is the widely accepted scientific definition of probiotics [4].

Increased knowledge and awareness of the possible benefits of probiotics has resulted in an exponential growth of available commercial products in a wide range of forms. Because most of them are classified as food supplements, they have to fulfill less stringent criteria and quality control procedures than medicinal products. This may raise concern about their safety [1,5,6]. Case reports were reported of probiotic administration causing systemic infections and immune stimulation in susceptible populations [7,8]. The possibility of transfer of genes coding for antibiotic resistance has been suggested. Less severe adverse events such as transient gastrointestinal symptoms, including excessive gasiness, bloating or diarrhea, do occur [7,8]. However, adverse events remain exceptionally seldom and are mostly limited to high risk populations. There is overwhelming evidence that probiotics are safe for use in the general population [7,8].

In humans the most frequently used probiotic bacteria belong to the lactic acid-producing genera *Lactobacillus* (including several new genera formerly under the *Lactobacillus* umbrella) or *Bifidobacterium*. Other genera, including *Streptococcus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Bacillus* and *Escherichia* and some non-bacterial yeast strains of *Saccharomyces* are also used [9,10].

Due to variation between available studies, including heterogeneity of the strains, the dosage, matrix, administration route, indication, as well as the research protocol, no broad consensus on the use of probiotics could be reached. The American Gastroenterology Association (AGA) and the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recently published different evidence-based recommendations [7,11]. Regarding necrotizing enterocolitis, the AGA, ESPGHAN and the American Academy of Pediatrics (AAP) came to different conclusions [7,12,13]. These caveats result in the necessity for further research. More well designed trials are needed before evidence-based recommendations can be proposed on the indications for pediatric administration of probiotics.

There is substantial evidence suggesting that the effectiveness of probiotics is specific regarding strain and disease [14]. Moreover, also the matrix in which the probiotic is administered is a factor influencing efficacy [15]. Health-care providers have to consider both factors when prescribing the appropriate probiotic in a given patient. This paper gives an overview on the available literature on the prophylactic and therapeutic use of probiotics in well-defined clinically relevant pediatric conditions to guide pediatricians in decision-making. The focus will be on strain-specific effects of probiotics.

2. Methods

We searched the Cochrane Library and MEDLINE for randomized controlled trials (RCT) or their meta-analyses and evidence-based clinical practice guidelines published between 1 January 2000 and 30 April 2021. The search terms used were "infant" and/or "child" and/or "pediatric" and "probiotics" and appropriate search terms for the selected conditions. Only articles in English were selected (Table 1).

Table 1. PICO search strategy.

Population	**Infant Child**	
Intervention	Probiotic therapy	
Comparison	Placebo Probiotic therapy Standard treatment	
Outcome	AGE AAD and CDAD Nosocomial diarrhea Infantile colic Regurgitation IBS Constipation	IBD *H. pylori* NEC LOS Atopic dermatitis Asthma Allergic rhinitis

Legend: PICO: population, intervention, control, and outcomes; AGE: acute gastro-enteritis; IBD: inflammatory bowel disease; AAD: antibiotic associated diarrhea; CDAD: *Clostridioides* difficile associated diarrhea; NEC: necrotizing enterocolitis; LOS: late onset sepsis; IBS: irritable bowel syndrome.

3. Results: Evidence of Efficacy of Probiotics

3.1. Diarrhea

3.1.1. Acute Gastroenteritis

Acute infectious gastroenteritis (AGE) was defined by ESPGHAN as a decrease in the consistency of stools and/or increase in the frequency of evacuations (typically ≥ 3 in 24 h), with or without fever or vomiting [16]. Episodes of acute infectious diarrhea remain a major disease burden in children throughout the world. Most episodes are self-

limiting. Treatment includes rehydration with oral rehydration solutions (ORS) and rapid refeeding [16,17]. Meta-analyses and systematic reviews on probiotics in AGE are listed in Table 2.

A Cochrane review including 63 RCTs in a total of 8014 participants, primarily infants and children, evaluated the effect of administering probiotics for the treatment of AGE in all age groups [17]. The most common organisms evaluated were *Lacticaseibacillus (L.) rhamnosus* GG ATCC53103 (13 studies), *Saccharomyces (S.) boulardii* CNCM I-745 (10 studies) and *Enterococcus lactic acid bacteria* (LAB) SF68 (5 studies). This Cochrane review concluded that "probiotics reduce the duration of diarrhea by approximately 25 h, as well as the risk of diarrhea lasting ≥four days", but failed to recommend specific strains [17]. The use of probiotics was not associated with any adverse event [17]. However, this review included both pediatric and adult studies and included five studies using dead microbes (which are not defined probiotics, but now called "postbiotics") [17].

L. rhamnosus GG ATCC53103 and *S. boulardii* CNCM I-745 are the two most studied strains. They should be initiated early in the course of acute infectious diarrhea. *L. rhamnosus* GG ATCC53103 should be administered at a minimal dose of 10^{10} colony-forming units (CFU) per day, typically for 5–7 days. The use of *L. rhamnosus* GG ATCC53103 had no effect on stool volume, but is associated with a significantly reduced duration of diarrhea, mean stool frequency on day two and risk of diarrhea lasting ≥four days [2,18]. *S. boulardii* CNCM I-745 should be given at a dose of 250–750 mg a day, for 5–7 days [18]. A recent meta-analysis, including 29 RCTs, concluded that adding *S. boulardii* CNCM I-745 to standard rehydration therapy reduced the duration of diarrhea with one day and efficiency was only seen when administered within 72 h after the onset of symptoms [19]. Another meta-analysis by Feizizadeh et al. showed on the other hand no clear beneficial effect in children with acute diarrhea. Additional studies are required [20].

Evidence is also available for *Limosalactobacillus (L). reuteri* DSM 17938, daily dose of 1×10^8 to 4×10^8 CFU for 5–7 days, *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246, 10^{10} CFU of each strain twice daily for five days [11]. A recent meta-analysis confirmed the reduction of duration of diarrhea and hospitalization when *L. reuteri* DSM 17938 was administered [21]. A RCT in 2002 evaluated the effect of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246, 10^{10} CFU of each strain twice daily for five days on acute diarrhea in children in a cohort of 30 children. In the treatment group the duration of diarrhea was significantly reduced [22].

Table 2. Meta-analyses and systematic reviews on probiotics in acute gastroenteritis.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Allen et al., 2010 Cochrane Review [17]	8014 (6489/352/1173)	*L. rhamnosus* GG ATCC53103 *S. boulardii* CNCM I-745 *Enterococcus* LAB	NA	↓ duration of diarrhea +/− 25 h
Szajewska et al., 2014 ESPGHAN guidelines [18]	NA	*L. rhamnosus* GG ATCC53103 *S. boulardii* CNCM I-745	≥10^{10} CFU/d, 5–7 d 250–750 mg/d, 5–7 d	↓ duration of diarrhea
Szajewska et al., 2020 ESPGHAN guidelines [11]	NA	*L. reuteri* DSM 17938 *L. rhamnosus* 19070-2 and *L. reuteri* DSM 2246	1×10^8–4×10^8 CFU, 5–7 d 10^{10} CFU 2×/d, 5 d	↓ duration of diarrhea
Collinson et al., 2020 Cochrane Review [23]	12,127 (11,526/412/189)	Several	NA	Uncertain effect
Su et al., 2020 AGA guidelines [7]	NA	Several	NA	Recommendation against use of probiotics
Vassilipoulou et al., 2021 [24]	3469 (3469/0/0)	Several	Several	No sufficient clinical impact
In summary: Recent reviews conclude there is insufficient evidence to recommend the systematic administration of probiotics to prevent AGE, although—as listed in the Table above—many meta-analyses and systematic reviews recommend some specific strains.				

Legend: NA: Not available; CFU: Colony-Forming Units; L: *Lactobacillus*; S: *Saccharomyces*; ESPGHAN: European Society of Pediatric Gastroenterology, Hepatology and Nutrition.

The ESPGHAN Working Group recommended the use of certain probiotics for treating pediatric AGE [11]: *S. boulardii* CNCM I-745 (low to very low certainty of evidence); *L. rhamnosus* GG (very low certainty of evidence); *L. reuteri* DSM 17938 (low to very low certainty of evidence); and *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 (very low certainty of evidence). The Working Group recommended strongly against *L. helveticus* R0052 and *L. rhamnosus* R0011 (moderate certainty of evidence) and weakly against *Bacillus clausii* strains O/C, SIN, N/R, and T (very low certainty of evidence) [11], highlighting not only the importance of strain specificity but also of disease specificity.

The AGA on the other hand reported the lack of evidence for the benefit of probiotics in pediatric AGE in a review including 89 studies. Their conclusion is based on the fact that studies performed in other geographical regions cannot be extrapolated to the general population because of differences in genetics, diet, sanitation and endemic enteropathogens [7].

A recent Cochrane review included RCT's comparing a specified probiotic agent with a placebo or no probiotic in patients (adults and children) with acute diarrhea, proven or presumed to be caused by an infectious agent [23]. Eighty-two studies (12,127 participants of whom 11,526 children and 412 adults) were included. This Cochrane review included large trials with low risk of bias and came to a similar conclusion as the AGA [7], that probiotics probably make little or no difference to the number of people who have diarrhea lasting 48 h or longer, and whether probiotics reduce the duration of diarrhea remains uncertain [23]. Last but not least, one more meta-analysis concluded that probiotics (and synbiotics) did not reduce the duration of diarrhea in children in developed countries [24]. Thus, the most recent high-quality reviews all concluded that there is a lack of demonstrated efficacy for probiotics to reduce the duration of diarrhea in children in developed countries.

Regarding prevention of diarrhea, according to a review, *L. reuteri* is reported to be effective in reducing the episodes of AGE in children attending day care centers [25]. The administration of *L. rhamnosus* GG was reported to have the potential to reduce the overall incidence of healthcare-associated diarrhea, such as caused by rotavirus [26]. A RCT assessed the effect of administering *L. reuteri* DSM 17938, 10^8 CFU daily for three months, to 336 healthy Mexican children. A significant reduction in the number of episodes of diarrhea and days with diarrhea was reported. These effects were seen during the study period and at 3-month follow-up. The number of medical visits, antibiotic use, absenteeism from day school and parental absenteeism were also significantly lower, with important cost savings [27].

In summary: Although older meta-analyses recommend some specific strains to shorten the duration of AGE, the most recent reviews all conclude that there is no evidence of benefits. There is insufficient evidence to recommend the systematic administration of probiotics to prevent AGE.

3.1.2. Antibiotic-Associated Diarrhea

Antibiotic treatment is known to disturb the gastrointestinal microbiome, resulting in a range of symptoms, including diarrhea and crampy abdominal pain. Antibiotic-associated diarrhea (AAD) is defined as three or more liquid stools in 24 h in subjects during or within six to eight weeks after antibiotic treatment and is attributed to the administration of these drugs after exclusion of other possible etiologies. However, not all studies on probiotics in AAD did apply this definition [28]. The risk of AAD is higher in young children and with certain antibiotics, such as aminopenicillins with or without clavulanate, cephalosporines, clindamycin and other antibiotics against anaerobes [28,29]. Meta-analyses and systematic reviews on probiotics in antibiotic-associated diarrhea are listed in Table 3.

A Cochrane systematic review evaluated 33 studies with 6352 children. The participants received probiotics (*Lactobacilli* species, *Bifidobacterium* species, *Streptococcus* species or *S. boulardii* CNCM I-745 or combinations), placebo or other treatments thought to prevent AAD [30]. Global analysis concluded that probiotics may be effective for preventing AAD: the incidence of AAD was 8% in the probiotic group, compared to 19% in the control

patients [30]. *L. rhamnosus* GG ATCC53103 and *S. boulardii* CNCM I-745 at 5 to 40 billion CFU per day appear the most appropriate probiotic strains to prevent AAD, but this analysis misses strain-specificity [30]. McFarland and colleagues reported a strain-specific effect in the prevention of adult AAD for *Lactobacillus* species, such as a mixture of *L. acidophilus* CL1285, *L casei* LBC80R, and *L. rhamnosus* CLR2, or *L. casei* DN114001 and *L. reuteri* 55730, while other *Lactobacillus* strains did not show efficacy [14]. Ma et al. evaluated the effect of probiotics on *Clostridium difficile*-associated diarrhea, and concluded based on 11 RCTs, including 4692 patients, that *L. casei* ranked the best in reducing AAD (odds ratio (OR) 0.32, 95% CrI 0.14–0.74) [31]. The impact of probiotic and disease specificity was highlighted in a recent paper in coronavirus disease 2019 (COVID-19) patients [32].

Table 3. Meta-analyses and systematic reviews on probiotics in antibiotic-associated diarrhea.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Szajewska et al., 2016 ESPGHAN guidelines [33]	NA	*L. rhamnosus* GG ATCC 53103 *S. boulardii* CNCM I-745	Uncertain >250 mg and <500 mg	↓ incidence of AAD
Guo Q et al., 2019 Cochrane review [30]	6352 (6352/0/0)	*L. rhamnosus* GG ATCC 53103 and *S. boulardii* CNCM I-745	5–40 × 10^9 CFU/d for the duration of antibiotic treatment	↓ incidence of AAD
Ma et al., 2020 [31]	4692 (NA)	*L. casei*	50–100 × 10^{10} CFU/d	↓ incidence of AAD

Legend: AAD: Antibiotic-associated diarrhea; NA: Not available; CFU: Colony-Forming Units; L: *Lactobacillus*; S: *Saccharomyces*.

If the use of probiotics for preventing AAD is considered in the presence of risk factors such as the choice of antibiotic agent, duration of treatment, patient's age, comorbidities, need for hospitalization or previous episodes of AAD, ESPGHAN recommends using *L. rhamnosus* GG ATCC53103 or *S. boulardii* CNCM I-745 [33].

In summary: the routine administration of specific strains can be considered in the presence of risk factors such as age of the patient, antibiotic administered, and other comorbidities. However, different guidelines recommend different strains.

3.1.3. *Clostridioides* Difficile-Associated Diarrhea (CDAD)

The majority of AADs are mild to moderate. A fulminant form of pseudomembranous colitis usually develops in children with underlying chronic conditions, such as cystic fibrosis, inflammatory bowel disease or cancer and is due to a causative agent often identified as *Clostridioides* difficile (*C. difficile*) [28,29]. Meta-analyses and systematic reviews on probiotics in CDAD are listed in Table 4.

Moderate-quality evidence suggests that probiotics are associated with a lower risk of *C. difficile* infection [34]. A Cochrane review including 39 RCTs and 9955 participants with 1141 children concluded that administration of probiotics together with antibiotics reduces the risk of *Clostridioides* difficile-associated diarrhea (CDAD) by approximately 60%. In patients at high risk, the beneficial effect of probiotics is even more pronounced [35]. However, this Cochrane review failed to recommend specific strains and does therefore not help to clinical decision making because some strains resulted in a favorable effect while others did not [35].

The AGA does recommend the use of certain strains or combinations in the prevention of *C. difficile* infection, based on the 39 trials evaluated by this Cochrane review, their more recent review in 2020 could not identify any new RCTs. Subgroup analysis of individual probiotics or combinations showed a reduced risk of *C. difficile* infection with *S. boulardii* CNCM I-745; a 2-strain combined product with *L. acidophilus* CL1285 and *L. casei* LBC80R; a 3-strain product with *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Bifidobacterium (B.) bifidum* and a 4-strain combination of *L acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. bifidum* and *Streptococcus salivarius* subsp. *thermophilus* [7]. The ESPGHAN Working Group recommends choosing *S. boulardii* CNCM I-745 if probiotics are considered for preventing CDAD [33].

Table 4. Meta-analyses and systematic reviews on probiotics in *Clostridioides difficile*-associated diarrhea.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Szajewska et al., 2016 ESPGHAN guidelines [33]	NA	*S. boulardii* CNCM I-745	>250 mg and <500 mg in children	↓ risk of CDAD
Goldenberg et al., 2017 Cochrane Review [35]	9955 (1114/7036/1805)	Several	NA	↓ risk of CDAD
Su et al., 2020 AGA guidelines [7]	NA	Several, but also S. *boulardii* CNCM I-745	NA	↓ risk of CDAD
In summary: There is soms evidence for *S. boulardii* CNCM I-745 to reduce the risk of CDAD.				

Legend: CDAD. *Clostridioides difficile*-associated diarrhea; NA: Not available; CFU: Colony-Forming Units; S: *Saccharomyces.*

The recent technical review by the AGA, identified five RCTs regarding probiotics as additional treatment to metronidazole or vancomycin in CDAD, showing a benefit for *S. boulardii* CNCM I-745, *L. plantarum* 299v or a 4-strain combined product with *L. acidophilus* ATCC 700396, *L. paracasei* subsp. *paracasei* ATCC 335, *B. animalis* subsp. *lactis* ATCC SD5219 and ATCC SD5219. The fact that *L. rhamnosus* ATCC on the other hand increased the recurrence of *C. difficile* infection highlights again the importance of disease specificity, showing that further studies are needed to identify these probiotic strains as well as patient groups that may benefit [7].

In summary: The routine administration of specific strains can be considered in the prevention and management of CDAD. Although different guidelines recommend different strains, *S. boulardii* CNCM I-745 is recommended in all.

3.1.4. Nosocomial Diarrhea

Nosocomial or hospital-acquired infections are defined as infections that develop during a hospital stay, meaning that they are not present or incubating on hospital admission. Gastrointestinal infections account for the majority of them [36]. Meta-analyses and systematic reviews on probiotics in nosocomial diarrhea are listed in Table 5.

Table 5. Meta-analyses and systematic reviews on probiotics in nosocomial diarrhea.

	N° Patients (Children/Adults/NS)	Probiotic	Dose and Duration	Outcome
Szajewska et al., 2011 [26]	1092 (1092/0/0)	*L. rhamnosus* GG ATCC 53103	at least 10^9 CFU/d	↓ risk of nosocomial diarrhea
Hojsak et al., 2018 ESPGHAN guidelines [37]	NA	*L. rhamnosus* GG ATCC 53103	at least 10^9 CFU/d for the duration of hospital stay	↓ risk of nosocomial diarrhea
In summary: There is evidence for *L. rhamnosus* GG ATCC53103to reduce the risk of nosocomial diarrhea				

Legend: CFU: Colony-Forming Units; L; *Lactobacillus.*

The ESPGHAN Working Group on Probiotics and Prebiotics provided recommendations on the role of probiotics in the prevention of nosocomial diarrhea in children based on a systematic review of available systematic reviews and RCTs [37]. *L. rhamnosus* GG ATCC53103 administration for the duration of the hospital stay and at a minimal daily dose of 10^9 CFU reduced the risk of nosocomial diarrhea from 13.9% to 5.2%, including rotavirus gastroenteritis [26]. A RCT, including 90 children, showed that administration of *L. rhamnosus* GG ATCC53103 at 6×10^9 CFU/day in combination with zinc, vitamin B and C during 2 weeks, beginning at the first day of the hospitalization, significantly decreased nosocomial infections (48.9% in the control and 24.4% in the treatment group) [38].

The effect of *L. reuteri* was evaluated in 2 RCTs and these showed no effect on overall incidence of nosocomial diarrhea and symptomatic rotavirus infection. A trial in 2012, including 106 children, found that *L. reuteri* DSM 17938 did not significantly reduce the risk of developing diarrhea or rotavirus infection >72 h after hospitalization [39]. Another research group confirmed in 2015 that administration of *L. reuteri* at a daily dose of 10^8 CFU for the duration of the hospitalization, was not effective in reducing the incidence of

nosocomial diarrhea. Nosocomial diarrhea occurred in 13 of 184 included children, 7 in the treatment group and 6 in the placebo group [40].

A large RCT, including 727 hospitalized children, demonstrated that administration of *B. animalis* subsp. *lactis* failed to prevent nosocomial infections in admitted children who were older than 12 months [41].

In summary: Although there is insufficient evidence to recommend the systematic administration of probiotics to prevent nosocomial diarrhea, all data seem to suggest benefit *for L. rhamnosus* GG ATCC53103.

3.2. Functional Gastrointestinal Disorders

3.2.1. Infantile Colic

Infant colic or excessive crying affects 10–30% of healthy infants and their families worldwide. Colic is defined by Wessel's criteria of crying or fussing for three hours or more a day, for three days or more per week, for three weeks in infants aged less than three months [42]. The natural history is believed to be self-limiting, with symptoms resolving spontaneously by three or four months after birth [43]. Meta-analyses and systematic reviews on probiotics in infantile colic are listed in Table 6.

Table 6. Meta-analyses and systematic reviews on probiotics in infantile colic.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Harb et al., 2016 [45]	NA	*L. reuteri* DSM 17938	NA	Effective against colic in breastfed infants
Schreck et al., 2017 [46]	NA	*L. reuteri* DSM 17938	10^8 CFU/d 21 to 28 days	Effective against colic in breastfed infants
Sung et al., 2018 [44]	345 (345/0/0)	*L. reuteri* DSM 17938	0.2×10^8 CFU/drop, 5 drops orally/d	Effective against colic in breastfed infants
Ong et al., 2019 Cochrane Review [43]	NA	*L. reuteri* DSM 17938	NA	Probably effective against colic in children
Simonson et al., 2020 [47]	NA	Several, but recommending *L. reuteri* DSM 17938	NA	Probably effective against colic in children
In summary: *L. reuteri* DSM 17938 reduces infant colic in breastfed infants.; however, no recommendation can be made in formula fed infants.				

Legend: NA: Not available; CFU: Colony-Forming Units; L: *Lctobacillus.*

The majority of available studies have evaluated probiotics as a therapeutic tool. Evidence suggests that in breastfed infants *L. reuteri* DSM 17938 decreases infantile colic, resulting in a mean difference of crying time per day at the age of 3 weeks of 56 min [44,45]. Five RCTs were included in an Cochrane meta-analysis. The majority of the included infants were breastfed and received 1×10^8 CFU of *L. reuteri* DSM 17938 once daily for 21 to 28 days, compared to placebo. Their results showed that probiotic supplementation led to a two-fold greater chance of an at least 50% reduction in daily crying time in colic infants [46]. A recent systematic review, including 20 trials (15 RCTs and five meta-analyses), evaluated the effect of probiotics for the management and prevention of colic. Term infants with an adequate birth weight, without recent antibiotic or probiotic treatment, without evidence of failure to thrive or signs of illness and without major congenital or acquired disorders, were included [47]. The efficacy of *L. reuteri* DSM 17938 was evaluated in 6 RCTs in breastfed infants, showing significantly decreased crying and fussing [47]. It can be concluded that there is evidence that *L. reuteri* given to breastfed infants induced over 50% reduction of duration of crying compared to placebo [44,46,48,49]. A RCT including mainly formula-fed colicky infants treated with daily 1×10^8 CFU *L. reuteri* DSM 17938 could not confirm the beneficial effect [50].

A recent Cochrane review including six RCT's comparing the prophylactic use of probiotics compared to placebo showed that probiotic supplementation made little or no difference on the occurrence of colic. They do appear to reduce crying time, with the most studied strain, *L. reuteri* DSM 17938, resulting in a reduction of approximately 45 min of daily crying time [43]. Among formula-fed infants, those who received *B. breve*

versus placebo had less crying time. Each month of treatment, the difference increased and reached a statistical significance after three months. No significant differences were seen in the breast-fed children [51]. Conversely another research group could not confirm these findings [52]. Researchers also evaluated the daily administration of drops containing *L. reuteri* and vitamin D3 compared to vitamin D3 alone. They reported a significant lower intake of anti-colic medication at the age of three months, associated with less primary care contacts. The infants included were all breastfed, confirmation of these results in formula-fed infants needs to be available before recommending routine use [53].

In summary: there is evidence that *L. reuteri* DSM 17938 effectively reduces infant colic in breastfed infants. No recommendation can be made for formula-fed infants.

3.2.2. Regurgitation

Gastroesophageal reflux (GER) is defined as the passive passage of gastric contents back into the esophagus with or without regurgitation and vomiting, caused by a transient relaxation of the lower esophageal sphincter due to postprandial gastric distension. Gastroesophageal reflux disease (GERD) occurs when GER leads to troublesome symptoms of excessive crying, feeding refusal, failure to thrive, sleep disturbance, chronic cough or opisthotonos [54]. Reflux is extremely common in infants and treatment is based on conservative measures like thickened feedings and upright position after feeding [54,55]. Some probiotic strains are shown to enhance gastric emptying [56]. RCTs on probiotics in regurgitation are listed in Table 7.

Table 7. Randomized controlled trials on probiotics in regurgitation.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Indrio et al., 2011 [59]	42 (42/0/0)	*L. reuteri* DSM 17938	10^8 CFU/d for 30 d	↓ daily regurgitation
Garofoli et al., 2014 [57]	40 (40/0/0)	*L. reuteri* DSM 17938	10^8 CFU/d = 5 drops/d for 28 d	↓ daily regurgitation
Indrio et al., 2014 [58]	589 (589/0/0)	*L. reuteri* SM 17938	10^8 CFU/d = 5 drops/d for 90 d	↓ daily regurgitation (prevention)
Vandenplas et al., 2017 [60]	280 (280/0/0)	*Bifidobqcterium lactis*	10^7 CFU/g powder	↓ daily regurgitation
In summary: Although there is some data suggesting benefit of *L. reuteri* DSM 17938, the evidence is insufficient to recommend the routine administration of this probiotic in the prevention or managament of regurgitation.				

Legend: NA: Not available; CFU: Colony-Forming Units; L: *Lactobacillus.*

L. reuteri DSM 17938 prevents regurgitation during the first month of life in breast-fed term infants. A RCT compared 40 infants who received probiotic supplementation or placebo and showed, when treated with *L. reuteri* DSM 17938, 10^8 CFU daily for four weeks, a decrease in the number of regurgitation episodes a day [57].

Another RCT in 2014 studied the impact of *L. reuteri* DSM 17938 during the first 3 months of life on the onset of colic, reflux and constipation in term children. The study showed a statistical significant difference for regurgitation episodes a day, 2.9 versus 4.6 ($p < 0.01$), in the probiotic and the placebo group, respectively [58].

In a RCT, 42 children with regurgitation were included and divided in a probiotic group and a placebo group. Patients in the probiotic group received 10^8 CFU of *L. reuteri* DSM 17938 per day for a period of 30 days. Parents noted the frequency of regurgitation at home and gastric emptying time was calculated by an ultrasound at the beginning and the end of the study. The study showed a statistically significant difference in reducing gastric distension, accelerating gastric emptying and thereby diminishing episodes of regurgitation in patients receiving the probiotic strain [59].

In 2017, the safety of a new synbiotic formula, supplemented with *B. lactis* and fructo-oligosaccharides with lactose and a protein ratio of 60% whey and 40% casein, was evaluated. 280 infants were included over a period of 3 months. Results showed a normal growth compared to exclusive breastfed infants and showed a statistical significant decrease of functional constipation (3.2%), regurgitation (10.2%) and infantile crying and

colic (10.5%) compared to median prevalence according to the literature (7.8%, 26.7% and 17.7% respectively) [60].

In summary: there is insufficient evidence to recommend a specific strain in the management of regurgitation, although there are some promising data for *L. reuteri* DSM 17938.

3.2.3. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a type of functional abdominal pain disorder (FAPD). IBS is defined by pediatric Rome IV criteria and include all of the following for at least two months prior to diagnosis: abdominal pain for at least four days per month, associated with a change in frequency of stool and a change in appearance of stool [61]. Meta-analyses and systematic reviews on probiotics in IBS are listed in Table 8. In a recent meta-analysis of the epidemiology of pediatric FAPD, including 196,472 children, a prevalence of 13.5% was reported, of which IBS was reported most frequently [62].

The effect of *L. reuteri* DSM 17938 in children with FAPD and IBS has been evaluated in several RCTs with conflicting results [63]. The most recent study compared this probiotic strain to placebo in 54 children with FAPD. It showed that both *L. reuteri* and placebo reduced the frequency and intensity of abdominal pain. However, *L. reuteri*, but not placebo, improved the normal activities of the affected children and their families [64]. Another research group, however, reported the superiority of *L. reuteri* DSM 17938 at 10^8 CFU used twice daily for four weeks, compared to the placebo [65]. Compared with placebo, *L. rhamnosus* GG ATCC53103 supplementation was associated with a significant higher rate of treatment responders, defined as no pain or a decrease in pain intensity in the overall patient population and in the IBS subgroup [66]. A total of 141 children entered a double blind, placebo controlled RCT and received either *L. rhamnosus* GG ATCC53103 or placebo for eight weeks and entered follow-up for eight weeks. *L. rhamnosus* GG ATCC53103, but not placebo, caused a significant reduction of both frequency and intensity of abdominal pain [67]. *VSL#3*® is a probiotic mixture comprising eight different strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* (*L. plantarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. acidophilus*, *B. breve*, *B. longum*, *B. infantis* and *S. salivaris* subsp. *thermophilus*). VSL#3® is now commercialized as Visbiome® in many countries. A trial compared the effect of six weeks VSL#3® versus placebo in 59 children with IBS. At the end, after a two-week washout period, participants switched to the other group for another six weeks' treatment. VSL#3® was significantly superior to placebo in the primary endpoint, the subjective assessment of relief of symptoms, as well as in three out of four secondary endpoints: abdominal pain and discomfort, abdominal bloating and gassiness and family assessment of life disruption. No significant difference in stool pattern was seen [68].

Table 8. Meta-analyses and systematic reviews on probiotics in irritable bowel syndrome.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Guandalini et al., 2010 [68]	59 (59/0/0)	VSL#3®	NA, 6 weeks	Improves relief of symptoms
Horvath et al., 2011 [66]	290 (NA)	*L. rhamnosus* GG ATCC 5 3103	1×10^9–3×10^9 2×/d for at least 4 weeks	Significant higher rate of treatment responders
Pärrty et al., 2018 [63]	NA	*L. reuteri* DSM 17938	NA	Conflicting results
In summary: There are insufficient data to recommend routine administration of probiotic strains in the management of IBS.				

Legend: NA: Not available; CFU: Colony Forming Units. VSL#3®, now called Visbiome®, is a probiotic mixture comprising eight different strains of *L. plantarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. acidophilus*, *B. breve*, *B. longum*, *B. infantis* and *S. salivaris* subsp. *thermophilus*); IBS: Irritable bowel syndrome.

In summary: given their safety profile, probiotics seem to be an attractive therapeutic option and clinicians may consider their use, especially of *L. rhamnosus* GG ATCC 53103 and VSL#3® in children with persistent symptoms. However, data are scarce and additional high-quality studies are required before probiotic administration in pediatric IBS can be recommended [69].

3.2.4. Functional Constipation

Functional constipation (FC) is a very common problem in childhood and is defined by the Rome IV criteria [70,71], including two or fewer defecations per week with at least one episode of fecal incontinence per week, a history of retentive posturing or excessive stool retention, a history of painful or hard bowel movements, the presence of a large fecal mass in the rectum and a history of large diameter stools that can obstruct the toilet. Meta-analyses and systematic reviews on probiotics in FC are listed in Table 9.

Table 9. Meta-analyses and systematic reviews on probiotics in functional constipation.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Tabbers et al., 2014 ESPGHAN & NASPGHAN guidelines [75]	NA	Several	Several	No significant effects
Koppen et al., 2016 [73]	424 (424/0/0)	Several	Several	No significant effects
Huang et al., 2017 [72]	49 (NA)	Several	Several	↑ Stool frequency
Wojtyniak et al., 2017 [74]	515 (515/0/0)	Several	Several	No significant effects
In summary: No meta-analysis or guideline recommends the administration of a specific strain in the management of functional constipation.				

Legend: NA: Not available.

Probiotics have been shown to significantly increase stool frequency in Asian children in a recent meta-analysis and systematic review. However there was significant heterogeneity between the included trials. Probiotics had no effect on improving stool consistency in children [72]. A recent systematic review on the use of pre-, pro- and synbiotics in the treatment of pediatric FC included 13 RCTs. The majority of the included studies did not demonstrate a significant effect of pre-, pro- and synbiotics on outcome measures such as defecation frequency, fecal incontinence and painful or difficult defecation [73]. Another review also reported no significant difference between the probiotic and the control groups with respect to treatment success. While some studied strains showed some effects on defecation frequency, none of the probiotics had beneficial effects on frequency of incontinence episodes and abdominal pain [74]. Evidence-based recommendations on the treatment of constipation in children developed by ESPGHAN and NASPGHAN (North American Society of Pediatric Gastroenterology, Hepatology and Nutrition) do not support the use of probiotics. This recommendation is based on the evaluation of five RCTs, in which both positive results (*L. rhamnosus* Lcr35, *B. longum* and *L. reuteri* DSM17938) as negative results (*L. rhamnosus GG*, *B. lactis* strain DN-173 010) were reported. None of the obtained findings were repeated in other trials [75].

In summary: there is insufficient evidence to recommend a specific strain in the management of functional constipation.

3.3. Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are chronic, relapsing and remitting inflammatory disorders of the gastrointestinal tract. IBD is classified into Crohn's disease (CD), ulcerative colitis (UC) or IBD-unclassified, the latter meaning that at time of diagnosis it is impossible to categorize the features as either CD or UC [76]. UC is characterized by diffuse inflammation of the colon extending from the rectum proximally. CD can affect any area of the intestinal tract, but most commonly involves the terminal ileum and colon and 20% of the affected children will have perianal involvement, including fissures, fistulas and/or abscesses [77].

3.3.1. Ulcerative Colitis (UC)

Meta-analyses and systematic reviews on probiotics in UC are listed in Table 10. In UC, varying results are described when using the probiotic *Escherichia (E.) coli* Nissle

1917 in children. An initial study in 2008 showed promising results, however this was not followed by any RCT in children [78]. In adults, several studies showed a beneficial effect of *E. coli* Nissle 1917 compared to standard treatment with mesalazine alone in maintaining remission of the disease [79]. Again, these results were not confirmed by any RCT [80]. Another frequently studied probiotic in children and adults is VSL#3®. In a RCT, VSL#3® has been shown to be effective in inducing and maintaining remission when given as an adjunct to standard therapy with mesalazine; 90% of the children treated with VSL#3® and mesalazine achieved remission compared to 36% in the control group. Moreover, VSL#3® was also considered effective in maintaining remission, because 21% of the patients treated with the probiotic relapsed over the course of one year, whereas 73% of the control group relapsed [81]. Similar results were achieved in an open-label study and VSL#3® was tolerated well by the children without any adverse effects [82]. A small study in children also evaluated the effects of *L. reuteri* ATCC 55730 when administered with oral mesalazine compared to a placebo group. The study showed an increase in remission and an improvement of clinical, endoscopic and histological scores [83]. A recent Cochrane review, including mainly adults in 12 studies, concluded on the other hand that the effectiveness of probiotics for maintenance of remission remains unclear, mainly due to the low quality of the available evidence [84]. An ESPGHAN position paper acknowledged the limited available evidence in favor of these probiotic strains as adjuvant to standard therapy in induction and maintenance of remission [76].

Table 10. Meta-analyses and systematic reviews on probiotics in ulcerative colitis.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Miele et al., 2018 [76] ESPGHAN position paper	NA	*L. reuteri* ATCC 55730 *VSL#3®*	NA	Limited evidence of benefit
Iheozor-Ejiofor et al., 2020 [84] Cochrane Review	1473 (NA, mainly adults)	Several	NA	Uncertain benefit
In summary: There is insufficient evidence to recommend probiotics in ulcerative colitis.				

Legend: NA: Not available; CFU: Colony-Forming Units, VSL#3®, now called Visbiome®, is a probiotic mixture comprising eight different strains of *L. plantarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. acidophilus*, *B. breve*, *B. longum*, *B. infantis* and *S. salivaris* subsp. *thermophilus*.

In summary: there is insufficient evidence to recommend the systematic administration of a specific strain in the management of UC, although there are data indicating that *L. reuteri* ATCC 55730 and VSL#3® may be beneficial [85].

3.3.2. Crohn's Disease

Systematic reviews on probiotics in Crohn's disease are listed in Table 11. One RCT was performed in 75 children with CD to test the applicability of *L. rhamnosus* GG ATCC53103. Unfortunately, this study showed no beneficial effect of *L. rhamnosus* GG compared to placebo in addition to standard therapy [86] and meta-analysis has shown that *L. rhamnosus* GG can even cause an increase of relapse rate in children [87]. In adults, *S. boulardii* CNCM I-745 was studied and initial studies showed a reduction in relapse rate [88]. However, in a subsequent RCT relapse occurred in 47.5% in the patients treated with *S. boulardii* CNCM I-745, compared to 53.2% in the placebo group. Hence, no statistical significant benefit for *S. boulardii* CNCM I-745 was found in this RCT [89]. The lack of evidence on the benefits of *S. boulardii* CNCM I-745 was confirmed in a recent systemic review [90]. ESPHAN concluded that, as of today, no beneficial evidence is found for the use of probiotics in pediatric Crohn's disease, hence the workgroup does not recommend the use of probiotics for both the induction or remission of pediatric CD. However, one must consider that currently and especially in children the number of RCTs is limited and further research is required [76].

Table 11. Systematic reviews on probiotics in Crohn's disease.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Sivanthan et al., 2018 [90]	NA	*S. boulardii*	NA	No significant effects
Miele et al., 2018 [76] ESPGHAN position paper	NA	Several	NA	No recommendation
In summary: There is insufficient evidence to recommend probiotics in Crohns' disease.				

Legend: NA: Not available.

In summary: there is no evidence that a specific strain may be beneficial in the management of Crohn's disease.

3.4. Helicobacter (H.) pylori

H. pylori is a highly prevalent chronic infection causally associated with a spectrum of gastrointestinal disorders, including gastritis, peptic ulcer disease and gastric cancer. The infection is most frequently acquired during childhood. However, in comparison with adults, infected children and adolescents are often asymptomatic and infrequently develop the aforementioned complications. However, spontaneous eradication of *H. pylori* is unlikely and the asymptomatic child may become the symptomatic adult [91]. Meta-analysis and systematic review on probiotics in *H. pylori* are listed in Table 12.

Table 12. Meta-analysis and systematic review on probiotics in *H. pylori*.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Li et al., 2013 [92]	508 (508/0/0)	Several	Several	↑ eradication rate ↓ side effects
Malfertheiner et al., 2016 Maastricht V consensus report [91]	NA	Several *Lactobacillus* strains *S. boulardii*	NA	↓ side effects
In summary: There is insufficient evidence to recommend probiotics in *H. pylori*.				

Legend: NA: Not available.

A 2014 meta-analysis, including seven RCTs, evaluated the efficacy of probiotic supplementation in children undergoing *H. pylori* eradication therapy. Compared with the control group, children in the probiotic group experienced a significant increased eradication rate and reduced risk of adverse effects. However there was no standardized protocol of species, dosage and duration of administration in the reviewed studies [92].

Lactobacilli as an adjunct to triple eradication therapy have been shown to increase *H. pylori* eradication rates by approximately 13% (71% in the control group versus 84% in the probiotic group). However, eradication rate was still below the recommended goal of 90%. The studied strains differed among reports and included *Lactobacillus acidophilus*, *L. rhamnosus* GG ATCC53103, *L. reuteri* DSM 17938, *L. casei* or compound *Lactobacillus* without further detailed information. Subgroup analysis showed that the eradication rates increased significantly in the high dose group >5 $\times$ 10^9 CFU per day and the long-term group >4 weeks of treatment. *Lactobacilli* supplementation also significantly reduces the risk of diarrhea [93].

The efficacy of the probiotic yeast, *S. boulardii* CNCM I-745, was evaluated in a meta-analysis including 11 RCTs with 2200 participants, among them 330 children. In the treatment group, 80% of patients experienced eradication, compared to only 71% in the control group. The addition of *S. boulardii* CNCM I-745 significantly increases eradication rate, but again the eradication rate was still below the goal. The risk of overall side effects was also significantly reduced, particularly of diarrhea and nausea [94].

The fifth edition of the Maastricht consensus on the management of *H. pylori* recommends that certain probiotics may have a beneficial effect on eradication, but the level of

evidence was low. Certain strains of the *Lactobacillus* genus and *S. boulardii* CNCM I-745 have shown promising results in reducing gastrointestinal side effects [91].

In summary: there is insufficient data to recommend the systematic administration of a specific strain in the eradication of *H. pylori*, because of the low level of evidence.

3.5. Necrotizing Enterocolitis and Late-Onset Sepsis

Necrotizing enterocolitis (NEC) is an ischemic and inflammatory necrosis of the bowel after the initiation of enteral feeding in preterm infants. The incidence of NEC ranges from 2.6% to 28%, with an associated mortality of ~25%. The early clinical presentation includes feeding intolerance, abdominal distention and discoloration and bloody stools [95,96]. The incidence of late-onset sepsis (LOS) is ~20% in very low birth weight (VLBW) infants [97]. Although the pathogenesis of NEC has not been clearly established, intestinal immaturity, insufficient barrier function and dysbiosis with a risk for translocation of pathogens are all involved. In the case of LOS, mechanisms are likely to be similar [98,99]. Meta-analyses and systematic reviews on probiotics in NEC and late onset sepsis are listed in Table 13.

Table 13. Meta-analyses and systematic reviews on probiotics in necrotizing entero-colitis and late onset sepsis.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
van den Akker et al., 2018 [100]	11,231 (11,231/0/0)	Several (see text)	Several	↓ incidence and mortality
Morgan et al., 2020 [101]	15,712 (15,712/0/0)	Several (see text)	Several	↓ NEC development
Sharif et al., 2020 Cochrane Review [102]	10,812 (10,812/0/0)	Several (see text)	Several	↓ NEC, mortality and late-onset invasive infections
In summary: Systematic administration of probiotic bacteria to prevent NEC is still debated in literature; therefore routine administration cannot be recommended.				

Legend: NA: Not available.

A network meta-analysis for the ESPGHAN Working Group on pre-and probiotics performed a strain-specific review on the available literature [100]. Meta-analyses that group all of the used strains together are already suggesting efficacy; 51 RCTs with over 11,000 preterm infants were included. Most strains or combinations were only reviewed in one or a few RCTs, further large studies with the most promising strains need to be performed to define optimal treatment strategies. Only 3/25 probiotic administrations resulted in reduced incidence of death: *B. bifidum* NCDO 1453 and *L. acidophilus* NCDO 1748 (two studies; 494 preemies); *B. bifidum* and *L. acidophilus* (one study; 186 patients) and *B. infantis*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus* and *Streptococcus thermophilus* (one study; 150 cases) [100]. According to the analysis, the following seven probiotic combinations resulted in a reduction of the incidence of NEC: *B. lactis* BB12 or B94 (five studies; 828 preemies), *L. reuteri* ATCC 55730 or DSM 17938 (four studies; 1459 patients), *L. rhamnosus* GG ATCC53103 (six studies; 1507 infants), combination of *B. infantis* ATCC 15697 and *L. acidophilus* ATCC 4356 (one study; 367 cases); *B. infantis* BB02, *B. lactis* BB12 and *Streptococcus thermophilus* TH-4 (two studies; 1244 patients) and the combination of *B. longum* 35624 and *L. rhamnosus* GG (two studies; 285 infants) [100]. Two treatments reduced late-onset sepsis, the combination of *B.bifidum*, *B. infantis*, *B. longum* and *L. acidophilus* (two trials with 247 infants) and the combination of *B. longum* R00175, *L. helveticus* R0052, *L. rhamnosus* R0011 and *S. boulardii* CNCM I-1079 (three studies with 241 infants) [100].

The ESPGHAN working group formulated a conditional recommendation (with low certainty of evidence), to administer either *L. rhamnosus* GG ATCC53103 or the combination of *B. infantis* Bb-02, *B. lactis* Bb-12, and *Streptococcus thermophilus* TH-4 in order to reduce NEC rates, if all safety issues are met [12].

The AAP concluded that because of the lack of Food and Drug Administration (FDA)-regulated pharmaceutical-grade products in the United States, conflicting data regarding safety and effectiveness, and possibility to harm in a highly vulnerable population, current evidence cannot support the routine probiotic administration to preterm, especially if birth weight is <1000 g [13].

In a 2020 American systematic review and network meta-analysis of studies to determine the effects of single-strain and multi-strain probiotic preparations on outcomes in preterm, low birth weight infants, superiority of combinations of one or more *Lactobacillus* subsp. and one or more *Bifidobacterium* subsp. was confirmed. The combinations of *Bacillus* subsp. and *Enterococcus* subsp., and one or more *Bifidobacterium* subsp. and *Streptococcus thermophilus* lead to largest reduction of NEC development [101]. In their official recommendation on the use of probiotics in gastrointestinal disorders, AGA suggests using a combination of *Lactobacillus* and *Bifidobacterium* spp., or *B. animalis lactis*, or *L. reuteri*, or *L. rhamnosus* GG ATCC53103 over no and other probiotics in preterm infants [7].

A 2020 Cochrane systematic review included 56 RCT's in which 10,812 infants participated. Probiotics may reduce the risk of NEC, 33 infants need to be treated to have one additional beneficial outcome. They probably reduce mortality and late-onset invasive infection and have little or no effect on severe neurodevelopmental impairment. Few data were available for extremely preterm (born more than 12 weeks early) or extremely low birth weight infants (less than 1000 g) and analysis did not show any effect on NEC, death or infection. The certainty of this evidence was assessed as being low, due to limitations in trial designs. Further high-quality research is needed, especially for extremely premature and low birth weight infants to provide sufficient evidence of good quality and applicability for practice in this vulnerable population [102].

Concern regarding the safety of probiotics in these fragile patients question the use of probiotics in preterm. As a consequence, daily practice differs substantially in different centers, resulting in a discrepancy in administration from 0 to 100% according to over 150 different neonatal intensive care units [103]. Many trials do not report any adverse event, but some cases of *Lactobacillus* or *Bifidobacterium* sepsis have been reported in infants receiving probiotics [104–107]. Most affected infants had severe diseases, such as short-bowel syndrome or immunodeficiency [103]. Evidence has shown that preventive measures excluding probiotic administration can result in a decrease in NEC. The risk–benefit ratio depends on the incidence of NEC in a specific neonatal care center since preventive measures excluding probiotics result in a decrease in NEC [108].

In summary: there is insufficient evidence to recommend the systematic administration of a specific strain in the prevention or management of NEC, although there are data reporting beneficial effects.

3.6. Allergic Diseases

3.6.1. Atopic Dermatitis

Allergic disease has become a major worldwide health concern. Cow's milk allergy (CMA) is one of the most common food allergies in early infancy and forms an important part of the atopic march. Atopic dermatitis or eczema, often linked to food allergies such as CMA, is the most common allergic manifestation in infants and young children. Therefore, its occurrence is often the main criterion of efficacy of clinical trials aimed at reducing the allergy burden in infancy. Eczema is an itchy, non-contagious inflamed skin condition [109].

The composition of the gut microbiota has been postulated to play a role in the development, because a balanced microbiome promotes potentially antiallergenic processes: Th1-type immunity, suppression of Th2-induced allergic inflammation and IgA production, which is an essential component of the mucosal immune defense. The increase in allergy prevalence during the last years has been attributed to changes in environmental factors, such as reduced consumption of fermented food, use of antibiotics and other drugs and increased hygiene. The so-called hygiene-hypothesis suggests that a lack of exposure to microbial stimuli in early childhood is a major factor involved in the steep increase of allergy. Vaginal birth or caesarean section, lack of breastfeeding and early use of antibiotics have a significant impact on the colonization patterns of the infant's gut. Therefore, the manipulation of the microbiota during pregnancy or after birth may have an impact on allergy prevention [110]. Meta-analyses and systematic reviews on probiotics in atopic dermatitis are listed in Table 14.

In 2015, the World Allergy Organization (WAO), published guidelines on prevention of allergic diseases and concluded that there is a possible prophylactic benefit of the use of probiotics in pregnant women at high risk for having an allergic child, or in women who breastfed infants at high risk of developing allergy, or in infants at high risk of developing allergy [3]. Risk factors for developing allergy in a child include a biological parent or sibling with existing or a history of allergic rhinitis, asthma, eczema or food allergy [111]. A systematic review, including 29 trials in which 12 different probiotics or combinations were used, concluded that there are significant benefits of probiotic supplementation in reducing the risk of eczema. Probiotic supplementation does, however, not reduce the risk of other allergic diseases in children [110]. The European Academy of Allergy and Clinical Immunology (EAACI), on the other hand, concluded based on a systematic review of RCTs that there is no sufficient evidence to support the use of probiotics in food allergy prevention [112]. An important limitation of the above mentioned reviews and guidelines is the absence of evidence for probiotic strain, dosage and start and duration of administration. A meta-analysis focusing on *L. rhamnosus* GG ATCC53103 concluded that there was no evidence that administration results in a reduction of the risk to develop atopic eczema [113]. A recent RCT evaluating the efficacy of supplementing mothers from 35 weeks gestation until six months postpartum if breastfeeding and the child until the age of two years found that maternal-only supplementation did not significantly reduce the prevalence of eczema, wheeze or atopic sensitization in the infant by one year. However, they did find a positive impact when the child was given the supplement [114]. *L. rhamnosus* GG was found to be the most researched strain with benefit in a recent systematic review and meta-analysis including 28 studies. The researchers concluded that probiotic supplementation has a positive impact on the prevention on atopic dermatitis [115].

Table 14. Meta-analyses and systematic reviews on probiotics in atopic dermatitis.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Muraro et al., 2014 EAACI guidelines [112]	NA	NA	NA	No efficacy in prevention
Cuello-Garcia et al., 2015 [110]	3447 (3447/0/0)	Several (see text)	Several	↓ risk of eczema
Makrgeorgou et al., 2018 Cochrane Review [116]	2599 (NA)	Several (see text)	Several	Little or no difference in eczema symptoms
Szajewska et al., 2018 [113]	889 (NA)	*L. rhamnosus* GG ATCC 53103	Several	No efficacy in prevention
Li et al., 2019 [115]	3595 (3595/0/0)	Several	Several	Prevention of atopic dermatitis
In summary: Insufficient evidence to recommend routine use of probiotics in the treatment of atopic dermatitis; *L. rhamnonsus* GG ATCC 53103 can be considered.				

Legend: EAACI: European Academy of Allergy and Clinical Immunology; NA: Not available.

A Cochrane review, which included 39 RCTs, evaluated the effect of probiotic supplementation for treating eczema. The probiotics included, belonged to the Lactobacillus or Bifidobacteria species, alone or in combination, and were administered for four weeks up to six months. The evaluated strains probably make little or no difference in improvement of patient-rated eczema symptoms [116].

In summary: The available evidence suggests that probiotics result in no to limited difference in reduction of atopic dermatitis. However, data suggest a reduction of the severity of eczema. *L. rhamnosus* GG ATCC 53103 is the best studied.

3.6.2. Asthma and Allergic Rhinitis

Asthma is one of the most common chronic respiratory diseases in children and adults. It is characterized by chronic airway inflammation and respiratory symptoms such as

wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation [117].

Rhinitis describes inflammation of the nasal mucosa and is clinically defined by symptoms of nasal discharge, itching, sneezing and nasal blockage or congestion. Rhinitis impacts negatively on physical, social and psychological well-being, due to the direct effect of symptoms and the indirect disturbance of sleep with consequent daily fatigue and the use of antihistamines. The commonest form is allergic rhinitis meaning symptoms caused by exposure to an allergen to which the patient is sensitized. Allergic rhinitis can be seasonal or perennial, according to the corresponding allergen [118,119]. Meta-analyses and systematic reviews on probiotics in asthma and allergic rhinitis are listed in Table 15.

A 2013 meta-analysis included 3257 children in nine trials. These trials were heterogeneous in the type and duration of probiotic supplementation and follow-up. The risk ratio of doctor-diagnosed asthma in participating children receiving probiotics was 0.99 and the risk ratio of incident wheeze was 0.97. No evidence to support a protective effect of perinatal use of probiotics in doctor-diagnosed asthma or childhood wheeze was found [120]. A recent meta-analysis, including 19 RCTs with 5717 children, confirmed that probiotic supplementation during pregnancy or early life was not associated with a lower incidence of asthma or wheeze. Subgroup analysis did however show that probiotics significantly reduce wheeze incidence among infants with atopic disease. These results need to be interpreted with caution, due to the small sample size of this subgroup [121]. In a recent animal study, intranasal administration of *L. rhamnosus* GG, but not *Lactobacillus* GR-1, suppressed airway hyperresponsiveness and reduced the counts of eosinophils and Th2-type cytokines in bronchoalveolar fluid [122]. *L. rhamnosus* GG ATCC53103 and *B. lactis* were shown to suppress several aspects of the asthmatic phenotype, such as airway hyperreactivity, antigen-specific IgE production and pulmonary eosinophilia [123].

The results of a long-term study in children were recently published, showing that *L. rhamnosus* HN001 (6×10^9 CFU) was associated with a significant reduction in cumulative wheeze prevalence and a non-significant reduction in cumulative rhinitis prevalence at age 11 years. In a second treatment group, *B. lactis* HN019 (9×10^9 CFU) showed no effect. Probiotics were taken daily from 35 weeks' gestation to six months postpartum in mothers while breastfeeding and from birth to age two years in infants [124].

Therapeutic effects of probiotics in human asthmatic patients are not well established. A systematic review on the role of probiotics in the treatment of allergic airway diseases includes 12 trials and showed no improvement of quality of life score in asthmatics. Probiotic intake, however, resulted in a longer time free from asthma exacerbations. The included studies on asthma have used only *Lactobacillus* species (*acidophilus*, *rhamnosus* and *casei*) as the probiotic strain with a minimum dose of $>10^9$ for at least one month [125]. An eight-week RCT in children with asthma and allergic rhinitis treated with *L. gasseri* A5 as a supplement to standard medications showed a significant reduction in asthma symptoms as well as improvement in objective airway function measurements [126]. Oral administration of *L. rhamnosus* GG alleviated asthma symptoms in an ovalbumine-sensitized model of mouse asthma [127]. Nevertheless, some studies have reported that oral probiotics have little or no clinical effect on allergic diseases, so the current evidence does not support the routine use of probiotics in the treatment of asthma.

In a 2014 systematic review, five RCTs that addressed the preventive role of probiotics in allergic rhinitis were evaluated. No difference in the incidence of allergic rhinitis between the probiotic and the placebo groups was seen and there was no significant difference in the prevention of allergic rhinitis [128]. Seventeen RCTs including 5264 children were included in a 2019 meta-analysis, which failed to identify a beneficial effect of probiotic supplementation during pre- and postnatal periods on prevention of allergic rhinitis [129].

There have been promising developments in the use of probiotics as an adjuvant treatment in allergic rhinitis [130]. Several systematic reviews and meta-analyses have shown the beneficial effects of probiotics in improving symptoms and quality of life in patients with allergic rhinitis. However, current evidence remains limited due to study

heterogeneity [128,131,132]. The administration of *L. acidophilus* 92 in fermented milk significantly improves nasal symptom scores in patients with perennial allergic rhinitis [133]. A placebo-controlled RCT included 60 children with allergic rhinitis of whom half were treated with an antihistamine, together with *L. paracasei*. The other patients received the antihistamine agent with placebo. The treatment group reported an significant improvement in quality of life scores and in nasal itching and sneezing scores [134]. A crossover RCT included 152 subjects between 18 and 45 years of age, with a diagnosis of moderate to severe allergic rhinitis. Subjects received a probiotic supplement Familact® (containing Lactobacilli, *acidophilus*, *casei*, *delbrueckii* subsp. *bulgaricus* and *L. rhamnosus* GG, *B. longum* and *breve* and *Streptococcus salivarius* subsp. *thermophilus*) with intranasal budesonide or intranasal budesonide with placebo for 8 weeks. The addition of probiotics significantly improved quality of life in persistent rhinitis patients [135]. Another research group evaluated the effect of *L. rhamnosus* GG and vitamin D supplementation on the effectiveness of grass-specific sublingual immunotherapy (SLIT) in children. They reported a decrease in symptom-medication score in all groups treated with SLIT, but a significant increase in CD4-, CD25- and Fox3- positive cells in the children receiving SLIT with *L. rhamnosus* GG, corresponding with a better immunologic response [136].

Table 15. Meta-analyses and systematic reviews on probiotics in asthma and allergic rhinitis.

	N° Patients (Children/Adults/NA)	Probiotic	Dose and Duration	Outcome
Ranjan et al., 2010 [131]	610 (357/253/0)	Several (see text)	Several	↑ Quality of life ↓ Episodes of rhinitis/year
Azad et al., 2013 [120]	3257 (3257/0/0)	Several (see text)	Several	No protection against asthma or childhood wheeze
Das et al., 2013 [125]	899 (571/292/36)	Several (see text)	Several	↑ time between episodes of rhinitis and asthma. No improvement in quality of life.
Peng et al., 2015 [128]	NA	Several (see text)	Several	No prevention of allergic rhinits
Du et al., 2019 [129]	5264 (5264/0/0)	Several (see text), *L. rhamnosus* GG	Several	Prevention of asthma
Wei et al., 2020 [121]	5717 (5717/0/0)	Several (see text)	Several	No protection against asthma or childhood wheeze
In summary: No evidence to recommend probiotics to prevent asthma and allergic rhinitis; none of the reviews recommends specific strains.				

Legend: NA: Not available.

In summary: there is insufficient evidence to recommend probiotic administration to prevent asthma and allergic rhinitis [137].

4. Discussion and Conclusions

The gut microbiome plays an important role in health and disease and probiotics represent a promising modality for prophylactic and therapeutic interventions. Several limitations, including the heterogeneity in study designs, small sample size, different strains, various combinations and treatment regimens, limit the evidence of efficacy of probiotics in pediatric diseases [1,7,8]. A general recommendation according to the different indications is listed in Table 16.

Table 16. Practical guide for pediatric use of probiotics.

Conditions	Strains	Dose	Recommended
Acute gastro-enteritis Treatment	*S. boulardii* CNCM I-745	250–750 mg/day, for 5–7 days	?
	L. rhamnosus GG ATCC53103	minimal dose of 10^{10} CFU/ day, for 5–7 days	?
	L. reuteri DSM 17938	1–4 × 10^8 CFU/day, for 5–7 days	?
	L. rhamnosus 19070-2 *and L. reuteri* DSM 12246	10^{10} CFU of each strain twice daily, for 5 days	?
Acute gastro-enteritis Prevention	*L. reuteri* DSM17938	10^8 CFU/day	?
Antibiotic-associated diarrhea Prevention	*L. rhamnosus* GG ATCC53103	5–40 × 10^9 CFU/day, for the duration of antibiotic treatment	+
	S. boulardii CNCM I-745	>250 mg and <500 mg	+
	L. casei	50–100 × 10^{10} CFU/day	+/−
C. difficile associated diarrhea Prevention	*S. boulardii* CNCM I-745	>250 mg and <500 mg in children	+
Nosocomial diarrhea Prevention	*L. rhamnosus* GG ATCC53103	>10^9 CFU/day, for the duration of hospital stay	+
Infantile colic Prevention and Treatment	*L. reuteri* DSM 17938	10^8 CFU/day, for 21–28 days	+ in breastfed No in formula fed
Regurgitation Prevention and Treatment	*L. reuteri* DSM 17938	10^8 CFU/day, for at least 30 days	No
Irritable Bowel Syndrome Treatment	*L. rhamnosus* GG ATCC53103	1–3 × 10^9 2×/day, for at least 4 weeks	?
	VSL#3®	NA, at least 6 weeks	No
Constipation Treatment	No significant effect of probiotics.		No
Ulcerative Colitis Treatment	*L. reuteri* ATCC 55730	NA	No
	VSL#3®	NA	No
Crohn's Disease Treatment	No significant effect of probiotics.		No
H. pylori Treatment	*Lactobacilli* (*acidophilus*, *rhamnosus* GG ATCC53103, *reuteri* DSM 17938, *L. casei*)	NA	No
	S. boulardii CNCM I-745	NA	No
NEC and Late-onset sepsis Treatment	*L. rhamnosus GG* ATCC53103	NA	?
	The combination of *B. infantis* Bb-02, *B. lactis* Bb-12 and *Streptococcus thermophilus* TH-4	NA	?
Atopic Dermatitis Prevention and Treatment	*L. rhamnosus* GG ATCC53103	NA	No
Asthma and Allergic rhinitis Prevention and Treatment	No significant effect of probiotics		No

Legend: NA: Not available. VSL#3®, now called Visbiome®, is a probiotic mixture comprising eight different strains of *L. plantarum, L. delbrueckii* subsp. bulgaricus, *L. casei, L. acidophilus, B. breve, B. longum, B. infantis* and *S. salivaris* subsp. thermophiles; Recommended: Routine administration can be recommended (yes/no/?(= debated in literature).

Despite these limitations, European guidelines [11,18] currently recommend the use of *L. rhamnosus* GG ATCC53103, *S. boulardii* CNCM I-745, *L. reuteri* DSM 17938, *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 in the treatment of AGE. They should be initiated as early as possible in the course of the disease and reduce duration of diarrhea with approximately 24 h. The use of *S. boulardii* CNCM I-745 or *L. rhamnosus* GG could be considered to prevent antibiotic-associated diarrhea [33]. In patients at risk for developing CDAD, the routine administration of probiotics can be considered, the use of *S. boulardii* CNCM I-745 is recommended by several guidelines [7,33].

The administration of *L. reuteri* DSM 17938 has been shown to reduce infant colic in breastfed infants [46,47]. Preliminary evidence suggests possible efficacy of *L. rhamnosus* GG and *VSL#3* in children with persistent symptoms of IBS [67–69].

There are insufficient data to recommend the systematic use of probiotics in UC. However, some evidence indicates that using VSL#3® or *L. reuteri* ATCC 55730 might be beneficial [81,83]. No sufficient beneficial evidence for the use of probiotic strains in Crohn's disease has been found [76].

Selected probiotics such as some strains of *lactobacilli* and *S. boulardii* CNCM I-745 may alter the eradication rate and/or risk of gastrointestinal side effects of standard H. pylori treatment, but the level of evidence was assessed as being low [91].

Data reporting beneficial effects of certain probiotic strains in reducing NEC are available and ESPGHAN and the AGA recommends some probiotic strains to reduce NEC [7,12]. ESPGHAN concludes that there was evidence for the following strains or combinations of strains: *L. rhamnosus* GG ATCC53103 or, the combination of *B. infantis* Bb-02, *B. lactis* Bb-12 and *Streptococcus thermophilus* TH-4 [12]. Due to conflicting data, the guidelines of the Committee of the Fetus and Newborn of the APP cannot support the routine administration of probiotics to reduce NEC, particularly in infants with a birthweight <1000 g [13].

There is insufficient evidence for the systematic use of probiotics to prevent or treat asthma and allergic rhinitis [137].

In summary: we conclude that benefit of selected probiotic strains for managing or preventing selected pediatric conditions has been demonstrated. The quality of the available evidence, the strain-specificity, and the efficacy depending on influencing factors such as dosage, matrix, duration, route of administration and the indication, currently limit the routine probiotic administration in the pediatric population.

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