nutrients

The Guidelines for Balanced Diet and Healthy Lifestyles during Pregnancy

The Management of Health and Morbidity in Pregnancy

Edited by

Megan E. Jensen

MDPI

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Contents

About the Editor

Megan E. Jensen

Megan E. Jensen (PhD) is a clinical researcher and Advanced Accredited Practicing Dietitian, part of the Hunter Medical Research Institute's Asthma and Breathing Program. She is a Senior Lecturer with the University of Newcastle, and recipient of the Hunter Children's Research Foundation Peggy Lang Early Career Fellowship. Her research is aimed at improving maternal and paediatric nutrition to mitigate the risk and impact of respiratory disease. Her current research includes the assessment of dietary intake and nutritional status in women with asthma during pregnancy, infant feeding, and the relationship with outcomes in their offspring.

Preface to "The Guidelines for Balanced Diet and Healthy Lifestyles during Pregnancy: The Management of Health and Morbidity in Pregnancy"

This Special Issue of Nutrients collated papers on a significant modifiable factor influencing maternal and fetal outcomes in pregnancy: nutrition. Although the major role that diet and lifestyle play in the trajectory of health outcomes in pregnancy has long been acknowledged, there is a dearth of research investigating the role that dietary patterns and individual nutrients play in health outcomes for mother and child, including the risk and management of comorbidities during pregnancy, and their influence on markers of fetal health and growth. It is necessary for researchers and clinicians to keep updated on the most recent findings in this area, which may influence dietary recommendations. This Special Issue serves as a platform to highlight recent research in the area of diet and lifestyle during pregnancy and the association with maternal and fetal health and disease.

Megan E. Jensen
Editor

Do the Dietary Intakes of Pregnant Women Attending Public Hospital Antenatal Clinics Align with Australian Guide to Healthy Eating Recommendations?

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Abstract: The maternal diet influences the long-term health status of both mother and offspring. The current study aimed to compare dietary intakes of pregnant women compared to food and nutrient recommendations in the Australian Guide to Healthy Eating (AGHE) and Nutrient Reference Values (NRVs). Usual dietary intake was assessed in a sample of women in their 3rd trimester of pregnancy attending antenatal outpatient clinics at John Hunter Hospital, Newcastle, New South Wales (NSW). Dietary intake was measured using the Australian Eating Survey, a validated, semi-quantitative 120-item food frequency questionnaire. Daily food group servings and nutrient intakes were compared to AGHE and NRV targets. Of 534 women participating, none met the AGHE recommendations for all food groups. Highest adherence was for fruit serves (38%), and lowest for breads and cereals (0.6%). Only four women met the pregnancy NRVs for folate, iron, calcium, zinc and fibre from food alone. Current dietary intakes of Australian women during pregnancy do not align with national nutrition guidelines. This highlights the importance of routine vitamin and mineral supplementation during pregnancy, as intakes from diet alone may commonly be inadequate. Future revisions of dietary guidelines and pregnancy nutrition recommendations should consider current dietary patterns. Pregnant women currently need more support to optimise food and nutrient intakes.

Keywords: pregnancy; nutrition; dietary intake; dietary guidelines; food-based guidelines

1. Introduction

Dietary intakes during pregnancy influence long-term health of both the mother and offspring [1]. Maternal diets can either enhance or compromise the mother's health status during pregnancy, impacting foetal development and influencing both maternal and offspring risk for non-communicable diseases later in life [2–5]. A woman with excessive gestational weight gain is more likely to develop hypertensive disorders and diabetes post pregnancy and transgenerational obesity in the offspring [5]. Maternal diet quality and adequacy of nutrient intake is associated with foetal development [6].

Maternal diets are often characterised by high intakes of energy-dense and nutrient-poor (EDNP) foods and are high in energy, saturated fat, added sugars and sodium and low in dietary fibre [1,7]. These dietary patterns may impact on adequacy of specific micronutrient intakes such as folate, iron, calcium and zinc, which are important in optimising reproductive health as well as foetal growth and development [1,8]. It has been reported in two systematic reviews that in high income countries the maternal diet does not align with life-stage specific national recommendations, increasing the likelihood of suboptimal micronutrient and macronutrient intakes [9,10]. Australian cross-sectional studies, all with over 600 participants, suggest that dietary intakes of pregnant women are likely to be inadequate and not meeting recommendations for macronutrients and food groups, with the exception of intakes of fruit [1,7,11–14]. Interestingly, research suggests that there are small differences between the diets of pregnant and non-pregnant women [1,15]. However, Savard et al. suggested that pregnant women have greater diet quality overall, raising the concern for overall diet quality amongst women of childbearing age [16].

Dietary guidelines, food selection guides and healthy eating messages form a core component of worldwide strategies to prevent non-communicable disease and encourage consumption of a wide variety of foods [14,17–19]. Pregnancy specific dietary guidelines have been developed, such as those included in the Australian Guide to Healthy Eating (AGHE) [18]. Pregnancy nutrition guidelines provide evidence-based recommendations for optimal maternal nutrition intake to promote maternal and infant health [14,18,20]. The AGHE describes types and amounts of foods that pregnant women should be consuming in order to meet macro- and micronutrient intakes [18].

Food group intakes recommended in the AGHE were derived by linear programming, where a modelling approach was used to create age and sex-specific "Foundation Diets" [18]. These Foundation Diets were determined using a range of combinations of amounts and types of foods that would meet all the nutrient reference values (NRVs), with suitable energy requirements of the smallest and least active people for each sub-group [18]. "Total diets" were subsequently derived from Foundation Diets, with additional food options to meet energy and nutrient needs for taller and more active individuals [18].

Current data reporting on the adequacy of maternal dietary intakes relevant to current national dietary guidelines remains limited. Studies that have compared dietary intake data to the most recent 2013 AGHE indicate that pregnant women are not meeting dietary recommendations, with less than 2% meeting daily vegetable serve recommendations, 1% meeting the grains recommendations and only 1% achieving higher than four serves of dairy per day [7,12,14,21]. However, these studies have largely used dietary intake data collected prior to the AGHE update in 2013 [1,7,12,14]. As such, there is a need to compare current eating patterns of pregnant women to the most recent 2013 AGHE. In doing this, future dietary guidelines can use current data in modelling approaches.

Therefore, the aim of the current study was to: (i) evaluate whether dietary patterns align with current AGHE food group recommendations for pregnancy in a contemporary sample of Australian pregnant women and (ii) describe food group intakes in the sub-group who meet NRVs for specific micronutrients (folate, iron, calcium, zinc and fibre) important in pregnancy.

2. Materials and Methods

2.1. Study Design and Participants

The current study is a primary data analysis of an observational study. Participants were pregnant women attending the public antenatal outpatient clinic and planning to deliver at John Hunter Hospital in Newcastle, NSW, Australia. Women were eligible to participate if they were aged over 18 years, between 28 and 36 weeks of gestation, and proficient in English. Participants were not excluded based on illnesses or known medical conditions. Ethics approval was received from the Hunter New England Human Research Ethics Committee (approval number 16/07/20/4.07) and University of Newcastle Human Research Ethics Committee (H-2017-0101).

2.2. Recruitment

Recruitment methods included a media release, social media posts on the Hunter Medical Research Institute and University of Newcastle Facebook pages, and flyers with pull-off tabs located in the pathology department and John Hunter Hospital antenatal clinic waiting rooms. Subsequently, the majority of the recruitment came through direct contact from trained research assistants, with pregnant women approached in the John Hunter Hospital Antenatal clinic waiting room. One out of five of the days at the John Hunter Hospital Antenatal clinic was a high-risk Diabetes clinic. Those interested in participating were screened for eligibility, with those eligible then choosing to provide consent to participate. Women who were interested but not yet 28 weeks' gestation were invited to provide a name and email address for the survey to be emailed when they reached 28 weeks' gestation. Those unable to complete the survey during their attendance or choosing not to complete it in the waiting room were sent a reminder email, together with either the incomplete, or partially completed survey. Data were collected, using REDcap software (Vanderbilt University, Nashville, TN, USA, version 8.11.3) [22] on iPad devices during a convenience sampling period from March 2018 to November 2018.

2.3. Data Collection

The 165-question survey comprised two parts: (1) general and maternal health and (2) dietary intake assessment.

2.3.1. General Demographics and Maternal Characteristics

Four sections, totalling 30 questions, collected demographic data including age, education level, nationality, marital status and income along with information related to maternal health for past (if relevant) and current pregnancies, such as weight gain, medications, smoking status and past and current pregnancy healthcare. Questions were in both multiple choice and short answer format and were created to be comparable to questions asked in the Australian Longitudinal Study of Women's Health (ALSWH) and the Women and their Children's Health study. The survey questions are provided in Supplementary Table S1.

2.3.2. Dietary Intake Assessment

Usual food and nutrient intakes over the previous 3–6 months were assessed using the Australian Eating Survey (AES), a validated self-administered, semi-quantitative food frequency questionnaire (FFQ), comprising of 120 food items [23]. Additional to the AES, there was a 15-question survey that contained questions relating to age, height, weight and behavioural aspects of eating and included food items such as soft serve and salads that are relevant to pregnancy due to risk of listeria exposure. Answers were assessed using a Likert Scale with response options ranging from never to '7 times per day'. The FFQ includes a comprehensive list of foods, including drinks, milk and dairy foods, breads and cereals, sweet and savoury snacks, main meals, other foods, vegetables and fruit. The AES is used to estimate usual dietary intakes of Australian adults and has been assessed for comparative validity relative to weighed food records and for fruit and vegetable intakes using plasma carotenoids [23,24]. Standard portion sizes were determined for each AES item in the survey, using data from the most recent National Nutrition Survey [25]. An example of a standard portion size of an item is a slice of bread [23]. The food and beverage weight per serving, used in the calculation of food group servings (as serves per day) is consistent with sizes specified in the AGHE (Table 2). Nutrient intakes from the AES FFQ were computed using data in the AUSNUT 2011–13 database [26].

2.4. Australian Guide to Healthy Eating (AGHE)

The AGHE is Australia's current national food selection guide. It was designed for healthy individuals, including pregnant women, and those with common health conditions but not for specific medical conditions or the frail elderly [18,27]. The AGHE encourages daily food choices from each of the five core food groups: (i) grain (cereal) foods; (ii) lean meats and poultry, fish, eggs, tofu, nuts and seeds and legumes/beans; (iii) vegetables and legumes/beans; (iv) fruit and (v) milk, yogurt, cheese and/or alternatives [18]. Additionally, the energy dense and/or nutrient poor "extras" or discretionary choices group contains foods that are not necessary for a healthy diet and only recommended for consumption in limited amounts, depending on total energy needs [27]. Women's food group intakes were compared to the AGHE food group servings. Women were said to meet a food group if their intake either met or exceeded the AGHE values, except for the "extras" category, which was reported as the percentage of total energy derived from AGHE core and discretionary food groups (Table 2).

2.5. Nutrient Reference Values (NRVs)

NRVs are specific daily nutrient intake targets developed by the National Health and Medical Research Council of Australia, associated with better health outcomes and lower risk of nutritional deficiencies [28]. The estimated average requirement (EAR), adequate intake (AI) and acceptable macronutrient distribution range (AMDR) are the most appropriate comparison values for population intakes. The EAR describes the daily nutrient target that should meet the requirements of half the healthy population at any particular life-stage and gender group [28]. An AI is used when an EAR is unable to be set and describes the average daily nutrient level that is assumed to be adequate by a group (or groups) of apparently heathy people [28]. AMDRs are recommended ranges for the percentage of daily energy intake from macronutrients [28]. Nutrient values for each participant were compared to the NRVs to determine whether pregnant women were meeting or not meeting the NRVs.

2.6. Statistical Analysis

To improve the validity of the study, energy intake mis-reporting was explored using cut-offs recommended by Meltzer et al. (2008), excluding those who reported daily energy intakes <4.5 or >20.0 MJ/d [29]. Descriptive statistics were used to summarise demographic characteristics, including age, education level, nationality, income, marital status and smoking status across the women. The main outcome measures of the study were daily servings of food groups (serves/day) and proportions of women meeting the AGHE recommendations and NRVs. Daily food group intakes in servings were calculated using the AGHE and compared with the AGHE recommendations for pregnant women aged 19–50 years. To determine the proportion of women achieving adequate intakes, macronutrients and micronutrients were compared with pregnancy specific values (EARs, AIs and AMDRs where applicable). The median daily food servings and macronutrient intakes for the subgroup who met the NRVs for calcium, zinc, iron, folate and fibre were reported. Data were tested for normality, with normally distributed data reported as mean [95% confidence interval (95% CI)] and non-normal data as the median [interquartile range (IQR)]. All data manipulation and analyses were performed using SPSS, version 22 (IBM Corp., Armonk, NY, USA).

3. Results

Of the 1115 women who expressed interest in participating and started the online screener, 169 were ineligible or did not complete screening. Of the remaining 946 women, six did not provide consent. For inclusion in the current analysis, participants needed a complete response to all 120 questions in the AES food list, resulting in a final sample of 534 women (Supplementary Figure S1). Demographic characteristics of the women with complete data are summarised in Table 1. Women self-reported their pre-pregnancy height and weight, which was used to calculate BMI and categorise their weight status. These values were also measured by midwives at the clinic for accuracy of reporting. A

total of 88 women did not report either weight and/or height. Pre-pregnancy BMI was calculated for 446 women, with 57.1% classified as overweight or obese, which is consistent with the general trend amongst women in Australia (total 59.8% overweight or obese) [30]. Where supplement use was reported, types of supplements and medication were not consistently reported and therefore excluded from this study. Additional questions related to food intolerance and food allergies were not asked as it was deemed unnecessary for the overall aim of the research.

Table 1. Socio-demographic characteristics of participating pregnant women (*n* = 534).

Variables	Value	
n = 534, unless otherwise stated [†]	Mean	CI
Age (years)	30.0	[29.5–30.4]
Gestation period (weeks) at time of survey *n* = 515	31.4	[31.2–31.7]
	n (%)	
Married/de facto status	469 (87.8)	
Aboriginal or Torres Strait Islander	34 (6.4)	
Born in Australia	481 (89.9)	
English is the only language spoken	504 (94.4)	
Above high school qualification *	350 (65.5)	
Difficulty managing available income [¶]	238 (44.6)	
Current smoker (*n* = 532)	38 (7.1)	
Current supplement user	395 (74.0)	
BMI (kg/m2) [‡] (*n* = 446) Underweight (<18.5) Normal (18.5–24.99) Overweight (25–29.99) Obese (30)	20 (4.5) 171 (38.3) 80 (17.9) 176 (39.2)	
In the past 12 months, the individual/family ran out of food or could not afford to buy more (*n* = 533)	21 (3.9)	
First pregnancy	202 (38.0)	

CI, confidence interval. [‡] BMI, body mass index. BMI determined from self-reported height and weight. * Above high school qualification = trade or apprenticeship, certificate or diploma, university degree and higher university degree. [¶] Difficulty managing available income included "It is impossible", "It is difficult all of the time" and "It is difficult some of the time". [†] Not all questions were forced, and thus, numbers who provided information on the socio-demographic characteristics vary.

Daily food consumption is summarised in Table 2. Food group intakes in daily food group servings and nutrient intakes are reported for both the total sample of women (*n* = 534) and for the sub-group least likely to have misreported total energy intake (*n* = 503). Of the 31 women identified as mis-reporters, 28 reported a total energy intake less than 4.5 MJ/day, and three reported a total energy intake over 20.0 MJ/day. The median and interquartile range (IQR) for percentage energy attributed to nutrient-dense core foods [67(58–75)] and energy-dense, nutrient-poor noncore foods [33(25–42)] are reported in the table. Fats, oils and discretionary items were included in nutrient-poor noncore foods and were not reported as an additional food group due to the set-up of the FFQ.

The percentage of women achieving the daily food group recommendations according to the AGHE is summarised in Table 3. There were no women who achieved AGHE food group servings for all five food groups. Fruit was met by the largest number of women (*n* = 204, 38.2%), whereas breads and cereals were met by the least (*n* = 3). Legumes, which are considered both a vegetable and meat alternative, were added only to the vegetables group for the purpose of this study. Eggs, tofu and nuts were considered as part of the meat and alternatives food group.

Table 2. Daily food consumption in pregnant women from the John Hunter Hospital antenatal clinic.

		All Women (*n* = 534)		Excluding Energy Intake Mis-Reporters (*n* = 503) [¶]	
		Median	IQR	Median	IQR
Food Groups					
Food Group Servings (Servings/Day) *	**AGHE (Serves/Day)**				
Breads and Cereals	8.5	2.7	1.7–3.7	2.8	1.8–3.7
Fruit	2	1.7	1.0–2.6	1.8	1.0–2.6
Vegetables and Legumes	5	3.8	2.8–5.1	3.9	2.9–5.2
Dairy	2.5	1.3	1–1.8	1.4	1.0–1.9
Meat and Alternatives	3.5	2.3	1.6–3.3	2.4	1.7–3.3
Nutrient Intakes					
	NRVs (Unit/Day)				
Macronutrients					
Energy (kJ) with Dietary Fibre	-	8079	6468–9966	8280	6718–10,004
CHO (% E)	AMDR 45–65%	45	41–49	45	41–49
Protein (% E)	AMDR 15–25%	18	16–20	18	16–20
Fat (% E)	AMDR 20–35%	37	34–40	37	34–40
Sat. Fat (% E)	<10%	14	13–16	14	13–16
Omega 3 (mg)	-	170.2	109–257	179.4	119–263
Fibre (g)	28	25.8	19–33	26.3	20–33
% Energy from Core Foods	-	67	58–75	68	59–75
% Energy from Non-Core Foods	-	33	25–42	32	25–41
Micronutrients					
Thiamin (mg)	EAR 1.2	1.5	1.1–2.0	1.5	1.1–2.0
Riboflavin (mg)	EAR 1.2	2.0	1.5–2.5	2.0	1.5–2.5
Niacin Equivalents (mg)	EAR 14	35.8	28.4–45.0	36.8	30.0–45.6
Vitamin C (mg)	EAR 40	162.8	115.1–220.4	166.2	119.5–223.0
Dietary Folate Equivalents (μg)	EAR 520	525.8	406.3–668.3	537.4	427.0–670.2
Retinol Equivalents (μg)	EAR 550	903.3	632.3–1195.3	920.9	674.7–1209.1
Magnesium (mg) 19–30 Years Old	EAR 290	359.4	293.6–446.1	366.4	303.3–447.7
Phosphorus (mg)	EAR 580	1345.4	1059.0–1665.5	1364.3	1103.0–1674.9
Calcium (mg)	EAR 840	769.5	557.5–968.5	785.0	583.4–974.2
Iron (mg)	EAR 22	10.1	7.9–12.6	10.3	8.2–12.7
Zinc (mg)	EAR 9.0	10.8	8.6–13.5	11.0	8.9–13.6
Sodium (mg)	AI 460–920	1733.7	1325.6–2190.4	1775.9	1382.8–2203.3
Iodine (ug)	EAR 160	121.8	88.3–156.4	123.4	95.1–157.9
Potassium (mg)	AI 2800	3152.5	2531.8–3892.2	3199.0	2623.2–3947.5

IQR, interquartile range; EAR, estimated average requirement; AI, average intake. [¶] Determined those who likely misreported using Meltzer et al., cut off values (<4.2 mJ or >20.0 mJ/day) [29]. * Serving size: (a) Breads and cereals: Bread 40 g, cereal 30 g, cooked porridge 120 g, muesli 30 g, cooked rice/pasta/noodles/barley/quinoa 70–120g, dry biscuits 40 g; (b) Fruit: Whole fruit (including canned) 150 g, fruit juice 125 mL, dried fruit 30 g; (c) Vegetables: Cooked or fresh vegetables 75 g; (d) Dairy and alternatives: Milk 250 mL, hard cheese 40 g, soft cheese (ricotta) 120 g, yogurt 200 g; (e) Meat and alternatives: Lean (cooked) beef/veal/lamb/pork/65 g, poultry (cooked) 80 g, fish (cooked)100 g, eggs 120 g, nuts/seeds/nut butters 30 g, tofu 170 g, cooked or canned legumes 150 g; (f) Extras: Sweet biscuit 35 g, sweet pastries/cakes/pies 40 g, savoury pies/pastries 60 g, pizza 60 g, hamburger 60 g, chocolate 35 g, processed meats 110 g, sausage 50–60 g, potato crisps/corn chips 30 g, jam/honey 45 g, ice-cream 75 g, fat spread 20 g, sugar 40 g, light beer 600 mL, full strength beer 400 mL, wine (including sparkling) 200 mL, spirits/liqueurs 60 mL, fortified wine 60 mL.

The percentage of pregnant women meeting NRVs important in pregnancy are reported in Table 4. The AMDR for protein (*n* = 455, 85.2%), EAR for vitamin C (*n* = 525, 98.5%) and EAR for phosphorus (*n* = 516, 96.6%) was met by the largest number of women, whereas 39 (7.3%) women achieved less than 10% daily energy from saturated fat. Sodium had the lowest rate of adherence, with *n* = 32 (6%) within the AI of 460–920 mg. The number of women exceeding 920 mg/day of sodium was 493 (94.4%), and 3 women (0.6%) consumed under 460 mg/day. This analysis was re-run by BMI categories, using the 446 participants who self-reported height and weight. This data can be viewed in Supplementary Tables S2 and S3.

Table 3. Percentage of pregnant women achieving Australian Guide to Healthy Eating (AGHE) daily food group recommendations.

Meeting Recommendations *	AGHE (Serves/Day)	All (*n* = 534)		Excluding Energy Intake Mis-Reporters (*n* = 503)	
		n	%	n	%
Breads and cereals	8.5	3	0.6	3	0.6
Fruit	2	204	38.2	202	40.2
Vegetables, including legumes	5	142	26.6	139	27.6
Dairy	2.5	72	13.5	70	13.9
Meat and alternatives	3.5	97	18.2	95	18.9

* Defined by the Australian Guide to Healthy Eating food group recommendations for pregnant women.

Table 4. Estimated proportion of pregnant women whose intakes met Nutrient Reference Values (NRVs).

	NRVs (Units/Day)	All (*n* = 534)		Excluding Energy Intake Mis-Reporters (*n* = 503)	
		n	%	n	%
Macronutrients					
CHO (% E)	AMDR 45–65%	287	53.7	272	54.1
Protein (% E)	AMDR 15–25%	455	85.2	433	86.1
Fat (% E)	AMDR 20–35%	177	33.1	165	32.8
Sat. Fat (% E)	<10%	39	7.3	33	6.6
Fibre (g)	28	211	39.5	208	41.4
Micronutrients					
Thiamin (mg)	EAR 1.2	363	68	356	70.8
Riboflavin (mg)	EAR 1.2	455	85.2	448	89.1
Niacin Equivalents	EAR 14	523	97.9	502	99.8
Vitamin C (mg)	EAR 40	526	98.5	501	99.6
Dietary folate equivalents (µg)	EAR 520	272	50.9	270	53.7
Retinol Equivalents (µg)	EAR 550	440	82.4	429	85.3
Magnesium (mg)	EAR 290	410	76.8	406	80.7
	EAR 300	387	72.5	383	76.1
Phosphorus	EAR 580	516	96.6	501	99.6
Calcium (mg)	EAR 840	215	40.3	212	42.1
Iron (mg)	EAR 22	7	1.3	5	1.0
Zinc (mg)	EAR 9.0	378	70.8	374	74.4
Sodium (mg)	AI 460–920	32	6	13	2.6
Iodine (µg)	EAR 160	121	22.7	119	23.7
Potassium (mg)	AI 2800	349	65.4	346	68.8
Meeting key pregnancy nutrients iron, folate, zinc, calcium and dietary fibre	-	4	0.75	3	0.56

EAR, estimated energy requirement; AI, average intake.

Median (IQR) food group intakes (servings/day) for those who achieved the pregnancy NRVs for folate, calcium, zinc, fibre and iron were 7.5 (5.2–8.3) (breads and cereals), 2.3 (1.2–3.4) (fruit), 5.5 (2.4–8.0) (vegetables), 0.9–2.1 (1.5–2.7) (dairy), and 4.3 (1.7–9.2) (meat and alternatives). Out of 534 women, four women met all five pregnancy NRVs; however, one of these women misreported. Out of the three women who met the NRVs and were not classified as mis-reporters, one did not meet any of the AGHE food group targets; one only met the dairy food group (3.1 serves/day); and one met the food group intake for meat and alternatives (6.7 serves/day), vegetables (7.1 serves/day), fruit (3.3 serves/day) and breads and cereals (8.79 serves/day). None of the three women met all of

the AGHE food group targets for pregnancy. Only four women met the NRVs for all five nutrients important in pregnancy; therefore, little value can be gained from further analysis of this aim.

4. Discussion

The principal aim of the current analysis was to evaluate whether Australian pregnant women were eating in accordance with the current AGHE and to report food group intakes of the subgroup of women who met the NRVs for folate, calcium, iron, zinc and fibre, from food intake alone. Results indicate that pregnant women may not be able to meet AGHE recommendations for all food groups nor achieve national recommendations for key micronutrients from food intake alone. The few women (*n* = 4) who met all five of the key pregnancy NRVs had food group intakes that differed to those recommended in the AGHE; however, one of these women classified as a mis-reporter. Pregnancy is a time where women have increased motivation to make dietary improvements [31]. Despite this motivation, within a contemporary sample of pregnant women, they are still not meeting the recommendations. It is also important to acknowledge the large increases in food group recommendations between pregnant and non-pregnant women. Additionally, pregnant women are at increased risk of digestive complications such as nausea and vomiting [32], constipation [33] and reflux [34], potentially hindering their ability to meet the large amount of food recommended in the AGHE.

Nationally representative Australian studies have compared the dietary patterns of pregnant women to both previous and current AGHE versions. These dietary patterns were primarily drawn from studies published between 2011–2018, with their data mostly collected prior to 2013 [1,7,12,14]. This study uses current eating patterns, collected in 2018 for comparison to the most recent 2013 AGHE. Australian dietary patterns have evolved over time as foods available also change, necessitating more current data to compare to national recommendations [35].

Similar to previous studies, the current analysis demonstrates that pregnant women are not meeting nutrition guidelines [1,7,12,14]. More women met the guideline for fruit than any other food group. In a cross-sectional study by Blumfield et al., an FFQ was used to assess intakes in a cohort of 606 pregnant women from the ALSWH [1]. With data collected in 2003, the median fruit intake was 2.2 serves per day, with 55.4% meeting the recommendations. This compares to the current study where the median intake was 1.71 serves of fruit per day, with only 38.2% meeting the target of two serves per day (*n* = 204) [1]. On the other hand, vegetable intake was greater compared to previous studies, with 26.6% of women from the current study meeting the guideline of five serves per day, which may have been an artefact of using a different FFQ compared to the other studies. Additionally, using an FFQ as a measure of dietary assessment may miss food items that are not included in the food list, or food items that are consumed in larger portion sizes than those in the FFQ. Australian cross-sectional studies by Lee et al. (*n* = 1570) and Malek et al. (*n* = 857) saw 10% and 10.3% meeting five serves of vegetables per day, respectively [7,12]. According to the AGHE, legumes are considered part of both the vegetables and meat alternatives food groups [18]. The current study analysed legumes as part of the vegetable food group, potentially contributing to the greater adherence to the AGHE recommendation for vegetables, than seen in previous studies.

Interestingly, a small number of women met the dairy and meat/vegetarian alternatives food groups, 13.5% (*n* = 72) and 18.2% (*n* = 97), respectively. In contrast, a cross-sectional study by Forbes et al. demonstrated that 50% of women increase their milk consumption during pregnancy but reduce their intake of meat, contrary to recommendations [36]. A possible reason for this finding may be that pregnant women commonly consume dairy foods, specifically cow's milk, to relieve heartburn, a common symptom reported amongst pregnant women [37]. Additionally, a cross-sectional study of 148 pregnant and 130 non-pregnant women reported that pregnant women consumed larger amounts of dairy and beef than non-pregnant women [15]. If only a small number of pregnant women are meeting recommendations for intakes of these food groups, this may suggest that intake of dairy and beef for non-pregnant women may be problematic as well. This contradicts findings from the study by Blumfield et al., where there were no significant differences between the intakes of dairy and meat

between pregnant and non-pregnant women trying to conceive, respectively [1]. However, this may not factor the higher pregnancy NRVs, which are not reflected as more nutrient dense diets in pregnant women verses non-pregnant women [1].

Consistent with previous findings, the recommended servings of the breads and cereals group was met by the least number of women (*n* = 3, 0.6%). Studies by Lee et al. and Malek et al., have reported 1.8% and 4% of pregnant women respectively meeting the 2013 AGHE for breads and cereals [7,12]. The lack of consumption of grains is consistent with low fibre intakes, where 211 (39.5%) of participants in the current study met the NRV of 28 g of fibre per day. The low grain intake observed also resulted in suboptimal carbohydrate intake, where the median intake of carbohydrates was on the low end of the AMDR at 45% of overall energy intake. However, low grain intakes may be associated with recruitment from high risk Gestational Diabetes clinics as patients were all on insulin and seeing a Dietitian once a fortnight. Low fibre intake can increase the likelihood of constipation, a problem commonly reported by women during pregnancy [7]. These results are consistent with previous studies and highlight the question as to whether the AGHE is achievable for pregnant women. Additional modelling may be required to inform contemporary and achievable diet recommendations. Alternatively, there may be a need for Australian practitioners to provide more support mechanisms to help pregnant women achieve these guidelines. The challenge is to optimise macronutrient intakes, given the trade-off between total carbohydrate and fat intake, and to avoid reliance of foods high in sodium [38]. The majority of women in the current study were exceeding the upper end of the recommended sodium intake (460–920 mg/day) and had a high total fat and saturated fat intake. There are potential health consequences associated with a high fat, high sodium diet, particularly in highly processed foods during pregnancy, including altered placental function and predisposition to metabolic disease in the offspring [38].

Only seven women met the pregnancy NRV for iron (22 mg/day) from food intake alone. This is consistent with the AGHE Food Modelling evidence, where all dietary models for Foundation and Total diets were unable to provide sufficient iron to meet the EAR in pregnant women [27]. The document suggests that iron supplements may be necessary and are commonly prescribed during pregnancy; however, it does not formally recommend iron supplementation in pregnancy [27]. A Canadian 2017 cross-sectional study in Quebec demonstrated that 97% of pregnant women had dietary intakes of iron below the proposed EAR; however, with supplementation, only 10% of women had inadequate iron intakes [39]. Iron deficiency during pregnancy poses serious health problems for the offspring and mother, such as preterm delivery, low birthweight and maternal depression [40,41]. The prevalence of postpartum iron-deficiency anaemia varies between 4–27% worldwide [42]. In a recent US cross-sectional study, in a sample of 102 non-anaemic pregnant women, 42% were observed to be deficient in iron [43]. As such, it is likely that the pregnancy iron NRV cannot be met by food alone, which reinforces the need for supplementing the diet with additional nutrients, for example through the fortification of cereal products and the recommendation of a prenatal or iron supplement.

Only four women (0.75%) met the NRVs for all five key nutrients important in pregnancy (fibre, calcium, iron, folate and zinc). These data indicate a low conformance with NRVs amongst the population. A systematic review by Hillier et al. stated that there is emerging evidence that women should have healthier eating patterns prior to pregnancy, although there is a lack of knowledge about the importance of nutritional intake during pregnancy [31]. However, they may not be aware of the need to increase important nutrients [36]. Aside from iron, calcium and fibre also had low adherence (40.3% and 39.5%, respectively). Additionally, folic acid, a nutrient widely known for its role in the prevention of neural tube defects, only had a 50% adherence rate [44]. There is a low proportion of pregnant women meeting NRVs, potentially due to impractical targets or a need for widespread dietary improvement amongst the population.

Results from the current study indicate the importance of additional vitamin and mineral supplementation during pregnancy, particularly for iron. The NRV for iron was met by the least number of women, and with iron removed from the analysis, 118 women met the NRVs for folate,

calcium, zinc and fibre. Supplements were consistently used by 74% of women; however, we cannot determine their micronutrient contribution, due to lack of detail reported on brand and dosage. For a supplement to provide the EAR value of iron, it would need to provide approximately 12 mg, this value being the difference between the median iron intake in this analysis (10.1 mg) and the EAR (22 mg). For the context of this study, the aim was to determine whether pregnant women are meeting AGHE and NRV targets from food intake alone. However, understanding type and amount of supplementation routinely used by pregnant women is important in determining their micronutrient contribution during pregnancy. This is necessary to guide future revisions of dietary recommendations, as well as the advice from health professionals. Health professionals need to tailor diet and supplementation advice for iron to patients, to prevent intakes exceeding the upper limit of 45 mg per day. Higher dose supplementation with or without sufficient dietary iron can result in unpleasant side effects in the gastrointestinal tract, such as constipation, nausea and vomiting [45]. Median intakes were above their respective EAR values, at 525.75 µg (vs. 520 µg) for folate and 10.76 mg (vs. 9.0 mg) for zinc. A US cross-sectional study, comparing the usual dietary intakes of 1003 pregnant women, observed the mean folate intake from food alone at 630 µg/day vs. 1451 µg/day with supplementation [46]. Whilst adequate folic acid is widely known for its role in the prevention of birth defects, health professionals need to ensure their recommendations do not lead women to exceed the upper limits of these nutrients during pregnancy [46]. Supplementation in pregnancy has shown to be an effective and cost-effective mechanism for improving maternal nutrient intake compliance with NRVs and reducing the risk of adverse health outcomes in infants [47]. However, the changes in maternal hormones lead to adaptions in the utilisation of maternal nutrients, in order to ensure the foetus receives a continual supply of nutrients for growth and development [48]. These adaptive responses support women in meeting increased demands for nutrients despite the nutritional intake and status of the mother [48].

The food group intakes of the four women who met the NRVs for fibre, calcium, iron, folate and zinc differed to those recommended in the AGHE. These findings, although a small sample, are consistent with a 2011 study by Blumfield et al., where it has been shown that women can achieve nutrient targets without adhering to food group targets [1]. The three women in this subgroup who did not misreport had higher conformance to the AGHE than demonstrated by the entire cohort. Nevertheless, no member of this sub-group met all of the AGHE recommendations.

The findings from the current study suggest that the AGHE and NRVs may not take into account current dietary intake patterns. More diversity in food group recommendations that better align with the eating patterns of Australian pregnant women is needed. Barriers to healthy eating during pregnancy, such as food cravings, morning sickness and constipation, may contribute to non-conformance with the nutrition guidelines [49]. The lack of knowledge of the contents, or even the existence of these nutrition guidelines may also play a role [49]. Identifying the areas of the maternal diet that differ to national recommendations and understanding these dietary patterns can assist in informing future revisions of guidelines and targeting nutrition interventions accordingly. Additionally, understanding the dietary intake patterns of women preconception and postpartum will allow interventions to target women of childbearing age, which may be an effective way to improve nutrient intake and ultimately pregnancy outcomes. Data that uses current eating patterns should be considered in future modelling and food selection guide revisions, to develop nutrition guidelines that women of reproductive age can follow. Perhaps there is a need to analyse the diet using more than one dietary assessment method, to limit the respective biases associated with each methodology. Additionally, further examination into supplementation, together with food, will be highly beneficial as evidence for nutrition recommendations. Future research should observe dietary and lifestyle behaviours, food group intake and supplementation, to provide health professionals with the knowledge and evidence to deliver dietary advice.

A strength of the current study is that it uses dietary data collected post 2014 to compare intakes of Australian pregnant women to the current AGHE. A limitation is that it only captures data from

one urban area in NSW, albeit using a large sample size, the John Hunter Hospital being the main referral hospital for the Hunter area. However, the data collection did not collect demographic data on other ethnicities, which is a potential limitation in terms of understanding dietary intakes in relation to recommendations. The John Hunter Hospital antenatal outpatient clinic services both medium- and high-risk patients requiring ongoing management of Gestational Diabetes Mellitus, pre-eclampsia or those who have had previous adverse outcomes, women with babies in breech position or those who are attending the clinic drug and alcohol services or Indigenous health services. As such, the dietary intake of participants may be influenced by socio-economic status or a medium- to high-risk health conditions. For example, carbohydrate and fibre intake of participants may be influenced by the presence of gestational diabetes. This study uses the AES FFQ, previously shown to be valid and reliable in assessing usual intake up to six months; however, it has not specifically been validated for pregnant women. Data was captured during the 3rd trimester only (28–36 weeks' gestation) and does not take into account differences in intake over the trimesters. However, previous studies indicate little change in dietary patterns over the course of the pregnancy [50,51]. FFQs have a low participant burden compared to other forms of dietary assessment, although self-reported dietary data poses the risk of misreporting. This limitation has been addressed using energy cut off points by Meltzer et al. [29]. Further, the AES FFQ, although previously used in pregnancy [51], has not been validated in this population group, and therefore, the findings of this study need to be interpreted in this context. The current study also examined nutrient intakes from dietary data alone, noting that 74% of women were taking supplements. Due to inconclusive data reporting on supplement usage and branding, the analysis could not determine the micronutrient contribution from these supplements. As a result, only a small sample of women met the NRVs for iron, folate, calcium, fibre and zinc from food intake alone. However, the aim was to determine whether pregnant women are meeting AGHE and NRV targets from food intake alone.

5. Conclusions

Dietary patterns indicate that in this sample of pregnant women attending a major public antenatal hospital, intakes are not aligned with national recommendations for pregnancy. This highlights that pregnant women need more support to improve their dietary patterns in order to optimise micronutrient intakes. Those who met pregnancy specific NRVs had dietary patterns more closely aligned to AGHE targets, although they still did not meet these recommendations. This suggests either that women may not be aware of the guidelines or that targets are not achievable. Ideally, all women should be provided with evidence-based nutrition advice during pregnancy. There is a need to raise awareness among antenatal healthcare providers of the low adherence to national recommendations and ensure that all women receive accurate information about dietary intake and vitamin and mineral supplementation, as a strategy to optimise maternal and infant health. In addition, future revisions of the AGHE should take into account the current eating patterns of pregnant women and consider the recommendation of supplementation for those who are unable to meet targets.

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Article

Maternal Diet Quality, Body Mass Index and Resource Use in the Perinatal Period: An Observational Study

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Abstract: The impact of pre-pregnancy obesity and maternal diet quality on the use of healthcare resources during the perinatal period is underexplored. We assessed the effects of body mass index (BMI) and diet quality on the use of healthcare resources, to identify whether maternal diet quality may be effectively targeted to reduce antenatal heath care resource use, independent of women's BMI. Cross-sectional data and inpatient medical records were gathered from pregnant women attending publicly funded antenatal outpatient clinics in Newcastle, Australia. Dietary intake was self-reported, using the Australian Eating Survey (AES) food frequency questionnaire, and diet quality was quantified from the AES subscale, the Australian Recommended Food Score (ARFS). Mean pre-pregnancy BMI was 28.8 kg/m^2 (range: 14.7 kg/m^2–64 kg/m^2). Mean ARFS was 28.8 (SD = 13.1). Higher BMI was associated with increased odds of caesarean delivery; women in obese class II (35.0–39.9 kg/m^2) had significantly higher odds of caesarean delivery compared to women of normal weight, (OR = 2.13, 95% CI 1.03 to 4.39; $p = 0.04$). Using Australian Refined Diagnosis Related Group categories for birth admission, the average cost of the birth admission was \$1348 more for women in the obese class II, and \$1952 more for women in the obese class III, compared to women in a normal BMI weight class. Higher ARFS was associated with a small statistically significant reduction in maternal length of stay (RR = 1.24, 95% CI 1.00, 1.54; $p = 0.05$). There was no evidence of an association between ARFS and mode of delivery or "midwifery-in-the-home-visits".

Keywords: dietary assessment; pregnancy; nutrition; economic evaluation; directed acyclic graphs (DAGs); maternal and infant

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1. Introduction

Obesity in pregnancy has become a major challenge for obstetric care in high-income countries [1]. Approximately 50% of women who become pregnant have overweight (body mass index (BMI) > 25 kg/m^2–30 kg /m^2) or obesity (BMI > 30 kg/m^2) [1], and the prevalence of obesity is rising [2]. High pre-pregnancy BMI has been strongly associated with excessive gestational weight gain [3], incidence of gestational diabetes mellitus, pre-eclampsia, pre-term delivery [3], large-for-gestational-age infants, caesarean delivery [4], miscarriage, antepartum stillbirth, complications at delivery and increased postpartum weight retention [1,3,5]. Given the elevated risk to the mother and infant, obstetric and

midwifery clinical practice guidelines recommend that healthcare facilities have well-defined pathways for the care of women with obesity, with increased care and monitoring relative to the antenatal care pathways of non-obese women [6,7]. This has resource use implications for the healthcare system. Clinical practice guidelines also provide "healthy eating in pregnancy" recommendations to address knowledge related to risk of diet-related conditions such as obesity [6]. However, there are no routine implementation interventions ensuring that clinical practice guideline recommendations for healthy eating in pregnancy are translated into practice [6]. This is a concern, as many Australian women fail to meet nationally recommended nutrient targets and do not appear to improve their diet quality when planning to become pregnant, or during pregnancy [8,9]. The economic implications of poor maternal nutrition, and its relationship with BMI and the use of healthcare resources (henceforth referred to as healthcare-resource use) is underexplored [10].

A recent World Health Organisation report, titled *Promoting Health and Preventing Disease: An Economic Case*, identified that improved maternal nutrition was as a potentially cost-effective target for health-promotion strategies aiming to improve maternal and infant health outcomes [11]. The volume of services and total expenditure on the delivery of maternity services means that relatively minor improvements in the cost per maternity patient could generate significant cost savings to public hospitals [12]. In particular, antenatal nutrition and gestational weight gain were identified as targets for health-promotion interventions aiming to improve maternal weight status and reduce demand on the healthcare system [13]. A recent study of infants born to mothers with overweight or obesity in the United Kingdom found that the usage rate for all healthcare services was significantly greater in infants born to mothers with obesity than infants born to mothers with healthy weight [14]. Infants born to mothers with obesity experienced a 39% higher rate of inpatient admissions and a 55% longer duration of inpatient stays, utilising, on average, 72% more resource costs [14]. Similarly, a cross-sectional comparative study of the short- and long-term effects of gestational diabetes mellitus (GDM) on healthcare costs found GDM was independently associated with an average additional cost of €817.60 (€2012) during pregnancy, due to additional delivery and neonatal care costs and an additional €680.50 in annual infant healthcare costs two to five years post-pregnancy [15]. A modelled economic evaluation exploring the short-term costs of maternal overweight, gestational diabetes and related macrosomia was conducted by Lenoir-Wijnkoop et al. [16] and found the average total additional costs for overweight was estimated to be $18,290 (USD) per pregnancy/delivery, which consists of an additional $13,047 for mothers with overweight and $5243 for their infants. Maternal diabetes was associated with an additional $15,593 per pregnancy/delivery, while foetal macrosomia was a significant risk factor for the development of obesity in childhood [16]. While overweight and obesity in women of child-bearing age and their offspring are of international concern, less attention has been paid to the economic consequences. At present, the cost of nutrition related perinatal health outcomes is unknown [10]. The range of potential targets for antenatal health promotion interventions, including nutrition interventions, is extensive, and healthcare-decision makers face growing pressure to optimize value, as well as quality, of healthcare [17].

Ensuring evidence-based healthcare is effective, as well as efficient and equitable, is critical if governments are to succeed in realising improved population health outcomes and contained per capita healthcare expenditure [18]. To identify technologies, interventions and models of care that provide the greatest value, healthcare providers are increasingly using health economic analyses to inform evidence-based decision-making [19]. Applied health economic evaluation informs evidence-based decision making by assisting healthcare-decision makers "identify, measure, and value activities with the necessary impact, scalability, and sustainability to optimize population health" [20]. High-quality cost and effectiveness data are a prerequisite for evidence-based decision-making. The highest cost of routine maternity care is incurred during the admission for birth (76%), followed by the non-admitted healthcare provided during the antenatal (17%) and postnatal (6%) periods [12]. However, the breakdown of these costs by population group is unknown. There is also insufficient evidence of the cost of nutrition interventions in pregnancy [10]. Given this absence of evidence, data on maternal dietary intake,

obesity and their relationship with healthcare-resource use is needed to inform research, guidelines and decision makers of the economic impacts of current antenatal health promotion and clinical practice [21]. To address these evidence gaps, a cross-sectional population-based study was designed to quantify specific perinatal-healthcare-resource use associated with maternal weight status and diet quality in a sample of pregnant women attending a public hospital in New South Wales, Australia. The hypothesis was that high BMI and low diet quality would be associated with increased healthcare-resource use, with diet quality potentially having a direct effect, independent of BMI. The aims of this study were as follows:

i.   Assess the diet quality of pregnant Australian women attending a public hospital antenatal clinic;
ii.   Estimate the total effect of BMI, adjusted for diet quality, on healthcare-resource use during the delivery admission, including mode of delivery, length of stay, admission to intensive care and midwifery-in-the-home service;
iii. Estimate the total effect of maternal diet quality on healthcare-resource use during the delivery admission;
iv.   Estimate the direct effect of maternal diet quality on healthcare-resource use during the delivery admission.

2. Materials and Methods

2.1. The Study

The study was an observational cross-sectional study in which patients attended public hospital antenatal outpatient clinics for routine antenatal care and were managed according to current clinical practice. The target sample size was 600 women with complete diet-quality scores, which were informed by investigator experience and feasibility. The study was advertised in the local newspaper and disseminated across university social media. Posters and fliers advertising the study were placed in the antenatal clinic, satellite clinics and birthing packs. Patients were also invited to complete the survey whilst in the waiting room, prior to their antenatal appointment, by trained volunteers.

All subjects gave their informed consent for inclusion before they participated in the study. The study was approved by the University of Newcastle Human Research Ethics Committee, Australia, study reference number H-2017-0101. Hunter Area Research Ethics Committee in August 2016 reference number HREC/16/HNE/189. The reporting adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

2.1.1. Study Population and Setting

Pregnant women aged 18 years or older, at 28–36 weeks of gestation (third trimester), and planning to deliver at the John Hunter Hospital were eligible to participate in the study. The time period of 28–36 weeks of gestation was selected, since the tool selected to measure diet, the Australia Eating Survey (AES), assesses intake over the previous three to six month, and we had previously shown significant correlations between dietary intake in early and late pregnancy [9]. The John Hunter Hospital, located in the Hunter New England Local Health District, New South Wales, Australia, is a large (550 bed) tertiary referral hospital, delivering around 4000 babies each year [22]. Participants were not excluded based on illnesses or known medical conditions.

2.1.2. The Survey

Self-reported demographic, health and diet quality data were collected at baseline (recruitment), and medical records data for the delivery admission were collected after discharge of mother and infant. The baseline survey consisted of four components: (1) consent and participant information statement; (2) participant information; (3) demographic data; and (4) the AES and could be completed in about 25–35 min. All subjects gave their informed consent for inclusion before they participated in

the study. Study data were collected and managed, using REDCap electronic data capture tools hosted at The University of Newcastle [23,24].

2.1.3. Study Recruitment

Trained volunteer research personnel (University students enrolled in the final years of a Bachelor of Nutrition and Dietetics) recruited study participants from the clinic between March 2018 and November 2018. All personnel undertook a mandatory workshop and further in-clinic training alongside a project officer. A brief and informative script was used by research personnel, to verbally screen women for eligibility, inform women of the survey content and purpose, and invite women to participate. Consenting participants then completed the survey on a tablet via the REDCap offline mobile application. Women at less than 28 weeks of gestation were invited via email to complete the survey when they reached 28 weeks' gestation. Women unable to complete the survey in the clinic due to fatigue, distractions (e.g., other children or feeling unwell) or being called to their appointment were emailed the remainder of their survey for later completion. An automated reminder email was sent seven days later, to all participants who had not finished the survey. All study participants had given birth by January 2019.

2.2. Statistical and Economic Analyses

The economic analysis took a healthcare provider's perspective to identify, measure and value outcomes associated with the provision of routine healthcare in the delivery period. The analysis excluded costs to patients and society. Since the time horizon for inclusion of relevant healthcare-resource use is set at less than 12 months, conversion or discounting of costs was not required [25].

2.2.1. Identification and Measurement of Exposure and Outcomes

Diet quality was quantified, using the previously validated Australian Recommended Food Score (ARFS) [26–28], derived from a subset of questions from the AES food frequency questionnaire for adults [26]. The AES is a 120-item semi-quantitative food-frequency questionnaire that was designed to assess usual dietary intake of individuals aged 18 years or older, based on a list of foods most commonly eaten by Australians. The AES has undergone comprehensive evaluation for validity and reliability, reported elsewhere [26]. The total ARFS score is calculated by summing the points for foods that are aligned with the core foods in the Australian Guide to Healthy Eating consumed at least weekly, with a total score ranging from 0 to 73 [26–28]. A higher score reflects greater alignment with recommendation in the Australian Dietary Guidelines.

Maternal clinical outcomes and healthcare-resource use from the delivery admission and associated home healthcare, Maternity Home Services, was collected from hospital databases using individual patient medical record numbers (MRN). Specific healthcare-resource use required for the management of maternal obesity was identified from the literature and reviewed by content experts (see Appendix A: Table A1). For the purpose of the current analyses, healthcare-resource use is defined as follows:

i.   Mode of delivery: caesarean versus vaginal (natural, instrumental, breech, compound).
ii. Maternal length of stay: (count in days).
iii. Maternal admission to intensive care: (yes or no).
iv. Midwifery-in-the-home service utilisation: total number of follow-up care visits associated with maternal discharge post-delivery (count).

Establishing associations between an intervention target and an outcome is a mandatory precursor to economic evaluation [19]. For the current study, if associations between BMI or diet quality and mode of delivery or admission to intensive care were established, healthcare-resource use was then defined and costed, using the Australian Refined Diagnosis Related Group (AR-DRG) classification system for admitted acute episodes of care in Australian public and private hospitals. The AR-DRG codes classify units of hospital output and group inpatient stays into clinically meaningful categories at similar levels

of complexity (outputs) and consuming similar resources (inputs) [29]. Independent Hospital Pricing Authority national weighted activity unit (NWAU) calculators are used to estimate cost of care based on AR-DRG classifications. All costs were reported in 2020, Australian dollars ($AUD).

Mode of delivery and admission to intensive care have specific AR-DRG classifications. However, length of stay and midwifery-in-the-home care visits are non-clinical variables that do not have a diagnostic criterion. As such, length of stay and midwifery-in-the-home were reported in clinically relevant natural units, days and total number of visits, respectively.

2.2.2. Development and Use of Causal Diagrams

Many nutrition research studies aim to identify and quantify causal relationships between nutrition and health outcomes [30]. The limitations of traditional methods for assessing associations in observational studies and inferring causality are widely recognised [31]. However, the use of experimental design in the antenatal period needs careful ethical and practical consideration [31]. In order to investigate causality, observational data must be interrogated carefully, with attention to the potential for known and unknown confounders and other biases [31]. Incorrect casual inferences are more likely to occur in observational studies than clinical trials, due to confounding bias [31]. A common way to control for confounding bias in an observational study is to include confounders as covariates in a regression model; however, careful consideration of which variables should be adjusted for is required [30]. Adjustment is needed to ensure that the effect estimate for the exposure of interest is unconfounded. It is commonly believed that it is necessary to control for all potential confounders and that adjusting for more confounders cannot worsen causal inference; however, the inclusion of unnecessary covariates, or over-adjustment, carries the risk of introducing unintended bias and reducing statistical power [32].

For the current study, there exist complex preconception processes influence maternal and infant health outcomes and healthcare-resource use, and these may also influence diet quality (see Appendix A: Table A1). To depict the presumed causal relationships between the exposure, outcome and potential confounding variables related to the exposure and/or outcome, directed acyclic graphs (DAGs) were developed, using existing evidence (listed in Appendix A: Table A1) and expert opinion. DAGitty, a browser-based environment for creating, editing and analysing causal diagrams (DAGs) [33], was used to create a DAG, to visually depict the direct or total effects of interest for each aim: Aims (ii), (iii) and (iv). The three DAGs are included in the Supplementary Materials, along with the DAGitty code to reproduce them and the potential minimum adjustment sets that were identified.

A facility of DAGitty is its analysis of the DAG and provision of candidate "minimum adjustment sets" for estimating unconfounded effects of interest. Each adjustment set is minimal in the sense that it is sufficient to remove confounding bias for the effect of interest and includes no unnecessary variables. The inclusion of unnecessary covariates can reduce efficiency or introduce unintended bias. For a given effect of interest, there are potentially multiple minimum adjustment sets, any one of which could be used. The identified adjustment sets for each aim are listed below:

- Aim (ii) adjustment set: maternal age, maternal education, parity and ARFS.
- Aim (iii) adjustment set: maternal education,
- Aim (iv) adjustment set: maternal age, maternal education and BMI.

2.2.3. Statistical Methods

For Aim (i), the diet quality of pregnant Australian women attending a public hospital antenatal outpatient clinic was measured by using a diet-quality index and reported as total ARFS score, using descriptive statistics (mean with standard deviation or median with range for continuous variables, and frequency with percent for categorical variables). For estimating the effects in Aims (ii), (iii) and (iv), regression models were fitted within a generalized linear modelling (GLM) framework, with response distribution and link function as appropriate for each response.

Caesarean delivery was modelled by using logistic regression, assuming a binomial response distribution (for caesarean versus vaginal birth), and using the logit link function. Logistic models were estimated with Firth's penalised Maximum Likelihood [34], to reduce bias in parameter estimates due to data sparsity involving some response/explanatory variable combinations. Maternal length of stay and number of midwifery in the home visits were modelled as count responses, using Poisson regression with the log link function. Overdispersion was assessed by using hypothesis tests for the dispersion parameter. The proportion of participants admitted to higher-level care (0.6%) was too rare to perform regression analyses for this outcome.

During the modelling process, fit statistics were examined, to assess whether categorical variables could be simplified by combining categories. Based on the Akaike Information Criterion (AIC) and Likelihood Ratio Test (LRT), maternal education was reduced from seven categories to a binary variable (university versus not), and parity was reduced from a count variable to a binary variable indicating primiparous (parity = 0) versus not (parity > 0). We also considered reducing the number of BMI categories; however, based on an increased AIC and significant LRT, the six-level variable was retained. ARFS was rescaled (into quintiles) to aid interpretation of effect estimates. The validity of using ARFS quintiles has been reported elsewhere [28].

Results are reported as exponentiated parameter estimates with 95% Wald confidence intervals accompanied by *p*-values from Wald tests. Statistical significance was declared at the conventional 0.05 level, to two decimal places for all analyses. Data manipulation and statistical analyses were performed by using SAS 9.4 (SAS Institute, Cary, NC, USA)™ software.

3. Results

3.1. Study Recruitment

A total of 1117 individuals commenced the survey (see Figure 1). Of these, two withdrew during survey completion. A total of 148 did not consent to participate or partially completed the consent questions, and 61 participants did not meet the eligibility criteria. A total of 670 consenting participants were eligible to participate and were linked to medical records data.
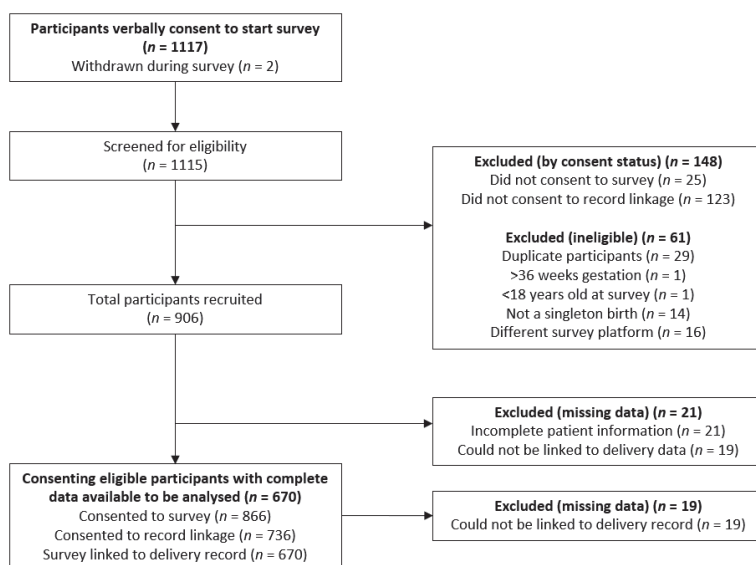
Figure 1. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) flow diagram of participant inclusion and exclusion.

3.2. Participant Demographics

The mean age of participants was 30 years (range: 18.4–53.0), and the mean gestation length at time of survey was 32 weeks. Most participants were born in Australia (90%) and spoke English at home (93%). A total of 6.8% of participants identified as Aboriginal or Torres Strait Islander. A total of 85% of participants were married or in a de facto relationship, 11% were single mothers and 3.3% were divorced or separated. The most frequent level of educational attainment was ≤year 12 (or equivalent) level of education (37.1%). The most frequent annual household income category was ≥$104,000 (27%), and a further 25% of participants reported incomes of $65,000 to $104,000. The mean pre-pregnancy BMI was 28.8 kg/m^2 (range: 14.7 kg/m^2–64 kg/m^2), with 59% of participants having overweight or obesity, 37% having normal weight and 4.5% having underweight. Just over half (54%) of participants said they had received pregnancy diet advice from a health professional during the current pregnancy. Table 1 summarises study participant demographic and health data.

Table 1. Summary of study participant demographic and health data.

Participant Demographic and Health Data		
Characteristic	**Statistic or Class**	**Total (*N* = 670)**
Age at survey	mean (SD)	30.3 (5.5)
	median (min, max)	30.1 (18.4, 53.0)
Aboriginal or Torres Strait Islander	No	600 (93%)
	Yes	44 (6.8%)
Born in Australia	No	62 (9.6%)
	Yes	581 (90%)
Marital status	Married/de facto	548 (85%)
	Divorced/separated	21 (3.3%)
	Single	73 (11%)
Language spoken at home	English only	598 (93%)
	Other	44 (6.9%)
Highest educational qualification	No formal qualifications	20 (3.1%)
	Year 10 or equivalent	107 (17%)
	Year 12 or equivalent	111 (17%)
	Trade/Apprenticeship	29 (4.5%)
	Certificate/Diploma	176 (27%)
	University undergraduate	151 (23%)
	University postgraduate	50 (7.8%)
Annual household income	Less than $20,800	32 (5.1%)
	$20,800 to less than $41,600	44 (7.0%)
	$41,600 to less than $65,000	68 (11%)
	$65,000 to less than $104,000	158 (25%)
	$104,000 or more	172 (27%)
	Not provided	153 (24%)
Weeks of gestation at survey	mean (SD)	32 (3)
	median (min, max)	31 (28, 36)

Table 1. *Cont.*

Participant Demographic and Health Data		
Characteristic	**Statistic or Class**	**Total (N = 670)**
Received pregnancy diet advice from health professional	Yes	325 (54%)
	No	263 (44%)
	Unsure	15 (2.5%)
Pre-pregnancy body mass index (BMI) measured	mean (SD)	28.8 (8.3)
	median (min, max)	26.8 (14.7, 64.0)
	Underweight (<18.5 kg/m^2)	30 (4.5%)
	Normal (18.5–24.9 kg/m^2)	247 (37%)
	Overweight (25.0–29.9 kg/m^2)	139 (21%)
	Obese Class I (30.0–34.9 kg/m^2)	116 (17%)
	Obese Class II (35.0–39.9 kg/m^2)	64 (9.6%)
	Obese class III (≥40 kg/m^2)	74 (11%)
Number ANC visits	mean (SD)	12.1 (5.3)
	median (min, max)	11.0 (1.0, 40.0)
Alcohol risk score	mean (SD)	0.1 (0.5)
	median (min, max)	0.0 (0.0, 9.0)
Number term pregnancies	mean (SD)	1.3 (1.1)
	median (min, max)	1.0 (0.0, 8.0)
Number preterm pregnancies	mean (SD)	0.1 (0.4)
	median (min, max)	0.0 (0.0, 3.0)
Number living children	mean (SD)	1.3 (1.1)
	median (min, max)	1.0 (0.0, 10.0)
History of endocrine disease	No	534 (80%)
	Yes	136 (20%)
History of hypertension	No	606 (90%)
	Yes	64 (9.6%)
Maternal risk factor—diabetes	No	488 (73%)
	Yes	182 (27%)
Maternal risk factor—hypertension	No	607 (91%)
	Yes	63 (9.4%)
Maternal risk factor—anaemia	No	448 (67%)
	Yes	222 (33%)
Maternal risk factor—smoke during pregnancy	No	568 (85%)
	Yes	102 (15%)

3.3. Aim (i): Diet Quality of Pregnant Women

Diet quality was assessed using the ARFS, with a mean ARFS of 28.8 (SD 13.1) points. The mean ARFS for those with a pre-pregnancy BMI in the normal weight category was 31.2 (SD 13.1). The mean ARFS was lower for women outside the normal BMI category, and ranged from 27.1 to 28.3 points (Table 2).

Table 2. Maternal-diet quality, measured using the Australian Recommended Food Score (ARFS), and specific healthcare-resource use by BMI category ($N = 670$).

Characteristic	Statistic or Class	Underweight ($n = 30$)	Normal ($n = 247$)	Overweight ($n = 139$)	Obese Class I ($n = 116$)	Obese Class II ($n = 64$)	Obese Class III ($n = 74$)
Diet quality (ARFS)	mean (SD)	27.2 (13.8)	31.2 (13.1)	27.2 (14.3)	27.1 (12.7)	28.3 (9.8)	28.2 (12.9)
	median (min, max)	28.0 (4.0, 56.0)	34.0 (1.0, 54.0)	30.0 (1.0, 50.0)	29.0 (2.0, 52.0)	29.5 (9.0, 46.0)	29.0 (1.0, 51.0)
Maternal length of stay (days)	mean (SD)	1.6 (1.5)	1.9 (1.6)	2.1 (1.6)	2.2 (1.5)	2.2 (1.6)	2.2 (1.6)
	median (min, max)	1.0 (0.0, 5.0)	2.0 (0.0, 9.0)	2.0 (0.0, 7.0)	2.0 (0.0, 8.0)	2.0 (0.0, 9.0)	2.0 (0.0, 7.0)
Number of "midwifery-in-the-home" visits	mean (SD)	1.8 (1.0)	1.6 (0.9)	1.6 (0.9)	1.6 (0.9)	1.6 (0.7)	1.5 (0.8)
	median (min, max)	2.0 (0.0, 5.0)	2.0 (0.0, 6.0)	2.0 (0.0, 4.0)	2.0 (0.0, 4.0)	2.0 (0.0, 3.0)	2.0 (0.0, 3.0)
Delivery mode	Vaginal birth	21 (70%)	167 (68%)	79 (57%)	65 (56%)	33 (52%)	36 (49%)
	Caesarean section	9 (30%)	80 (32%)	60 (43%)	51 (44%)	31 (48%)	38 (51%)

3.4. Aim (ii): Estimate of the Total Effect of BMI on Healthcare-Resource Use

Results from the analyses investigating the total effect of BMI on specific healthcare-resource use are shown in Table 4. The mean gestational age at birth was 38.4 weeks (SD 1.4 weeks), and 93% of infants were delivered at term (>37 weeks). The most common birth type was normal vaginal birth (50%), a further 40% of the babies were delivered via caesarean section and 10% had an abnormal vaginal birth (including instrumental, breech and compound birth). Four women required higher level care or were admitted to intensive care (refer to Table 3).

Table 3. Participant demographics and healthcare-resource use summary statistics.

Characteristic	Statistic or Class	Total ($N = 670$)
Infant birthweight (grams)	mean (SD)	3359.4 (515.1)
	median (min, max)	3390.0 (1450.0, 4830.0)
Gestational age at birth (weeks)	mean (SD)	38.4 (1.4)
	median (min, max)	38.0 (31.0, 41.0)
Maternal length of stay (days)	mean (SD)	2.1 (1.6)
	median (min, max)	2.0 (0.0, 9.0)
Mode of delivery	Normal vaginal birth	334 (50%)
	Caesarean section	269 (40%)
	Abnormal vaginal birth	67 (10%)
Pre-term birth (<37 weeks)	No	626 (93%)
	Yes	44 (6.6%)
Gender of infant	Male	326 (49%)
	Female	344 (51%)
Birthweight category	Low birth weight (<2500 g)	35 (5.2%)
	Normal range	568 (85%)
	Macrosomia (>4000 g)	67 (10%)
Midwifery-in-the-home care visits	mean (SD)	1.6 (0.9)
	median (min, max)	2.0 (0.0, 6.0)
Maternal admission to higher level care (intensive care)	No	664 (99%)
	Yes	4 (0.6%)

The mean maternal postnatal length of stay was 2.1 (SD 1.6) days, and the median was 2.0 (range: 0.0, 9.0), inclusive of the four women admitted to intensive care. The mean length of stay for women in the normal BMI weight class was 1.9 (SD 1.6) days and was slightly lower in the underweight BMI category, at 1.6 (SD 1.5) days. Amongst overweight and obese women, the mean length of stay was 2.1 (SD 1.6) in the overweight class and 2.2 (SD 1.6) for women in the BMI category obese class III. The mean number of midwifery-in-the-home care visits was 1.6 (SD 0.9), and the median was 2.0 (range: 0.0, 6.0). The mean number of midwifery-in-the-home care visits for women in the overweight category was 1.6 (SD 0.9), and the range did not vary substantially across pre-pregnancy BMI categories obese class I–III (1.55–1.5).

Women in the overweight and obese categories had increased odds of caesarean delivery, relative to women in the normal BMI category. The magnitude of this effect increased with increasing BMI category. The association for obese class II (35.0–39.9 kg/m^2) reached 0.05 significance (OR = 2.13, 95% CI 1.03 to 4.39; p = 0.04), indicating that women in obese class II had about double the odds of caesarean delivery, compared to women with normal BMI (Table 4).

Table 4. Estimates of the effect of diet quality and pre-pregnancy BMI on healthcare-resource use.

	Caesarean Delivery		Maternal Length of Stay		MITH Visits	
	Odds Ratio (95% CI)	*p*-Value	Rate Ratio (95% CI)	*p*-Value	Rate Ratio (95% CI)	*p*-Value
Aim (ii)—total effect of BMI *						
Underweight	0.58 (0.16 to 2.08)	0.40	0.78 (0.49 to 1.23)	0.28	0.95 (0.63 to 1.44)	0.82
Normal	(ref)		(ref)		(ref)	
Overweight	1.57 (0.91 to 2.71)	0.11	1.04 (0.86 to 1.26)	0.71	0.95 (0.78 to 1.18)	0.66
Obese Class I	1.18 (0.65 to 2.16)	0.58	1.07 (0.87 to 1.32)	0.51	0.89 (0.70 to 1.12)	0.31
Obese Class II	2.13 (1.03 to 4.39)	0.04	1.11 (0.87 to 1.42)	0.41	0.99 (0.75 to 1.30)	0.92
Obese class III	1.92 (0.98 to 3.73)	0.06	1.10 (0.87 to 1.39)	0.41	0.90 (0.69 to 1.16)	0.41
Aim (iii)—total effect of ARFS **						
Quintile 1	1.08 (0.64 to 1.85)	0.77	1.20 (1.00 to 1.44)	0.05	1.02 (0.83 to 1.26)	0.85
Quintile 2	1.16 (0.69 to 1.96)	0.58	1.10 (0.91 to 1.32)	0.33	1.09 (0.89 to 1.34)	0.41
Quintile 3	0.72 (0.42 to 1.24)	0.24	1.05 (0.87 to 1.27)	0.60	1.01 (0.82 to 1.25)	0.91
Quintile 4	0.93 (0.54 to 1.62)	0.80	1.12 (0.92 to 1.35)	0.26	0.99 (0.79 to 1.22)	0.90
Quintile 5	(ref)		(ref)		(ref)	
Aim (iv)—direct effect of ARFS ***						
Quintile 1	1.23 (0.71 to 2.16)	0.46	1.27 (1.05 to 1.53)	0.01	1.00 (0.81 to 1.24)	0.99
Quintile 2	1.25 (0.72 to 2.17)	0.43	1.14 (0.94 to 1.37)	0.19	1.07 (0.87 to 1.32)	0.52
Quintile 3	0.74 (0.42 to 1.29)	0.29	1.07 (0.88 to 1.29)	0.50	1.00 (0.81 to 1.24)	0.96
Quintile 4	0.97 (0.55 to 1.71)	0.92	1.13 (0.93 to 1.37)	0.21	0.98 (0.78 to 1.21)	0.83
Quintile 1	(ref)	.	(ref)	.	(ref)	.

* Adjusted for ARFS, maternal age, maternal university education (yes versus no) and primiparous (yes versus no). ** Adjusted for maternal university education (yes versus no). *** Adjusted for BMI, maternal age and maternal university education (yes versus no). (ref): reference category used.

The association was very similar for women in obese class III, who also had about a two-fold higher odds of caesarean delivery (OR 1.92; 95% CI 0.98 to 3.73; p = 0.056).

A total of 666 patients had AR-DRG classification for their birth admission. Of these, there were 242 (99%) women in the normal weight category and 62 (98%) women in obese class II and III.

The AR-DRG cost for birth admission did not vary by length of stay or maternal age. In general, there was a higher rate of complex deliveries among women with obesity. Among women in the normal BMI category, 32% had a caesarean delivery and 11% had a birth classified as having "major complexity". Rates were higher for women in obese class II, with 50% having caesarean delivery and 18% having a "major complexity" birth. Rates were slightly higher again for women in obese class III, with 53% having caesarean delivery and 25% having a "major complexity" birth.

We have reported and compared costs of delivery for women with normal BMI (reference) and women in obese class II and obese class III, due to high similarity of effect estimates and likely clinical importance of results for both obesity classes (see Table 5). The average cost per patient for women in normal weight was $7962. The average cost per patient for women in obese class II was $9309. The incremental difference in admitted patient cost was $1348. That is, in this sample, the birth admission for women in BMI category Obese class II cost $1348 more than women in normal weight class. The average cost of the delivery admission for women in obese class III was $9914, which was $1952 more than for women in the normal weight class and $605 more than women in obese class II.

Table 5. Maternal birth admission Australian Refined Diagnosis Related Group (AR-DRG) classification (with description and price ($AUD, 2020) and mean cost per patient for study participants in BMI categories normal and obese class II and III.

AR-DRG			Normal		Obese Class II		Obese Class III	
Code	Description	NWAU Cost	n * = 242	Cost ($) **	n = 62	Cost ($) **	n = 72	Cost ($) **
O01A	Caesarean delivery, major complexity	$17,170	5	$85,850	2	$34,340	10	$171,700
O01B	Caesarean delivery, intermediate complexity	$12,310	39	$480,090	14	$172,340	15	$184,650
O01C	Caesarean delivery, minor complexity	$10,074	34	$342,516	15	$151,110	13	$130,962
O02A	Vaginal delivery with operating room procedures, major complexity	$12,691	3	$38,073	0	$0	0	$0
O02B	Vaginal delivery with operating room procedures, minor complexity	$9119	6	$54,714	3	$27,357	0	$0
O60A	Vaginal delivery, major complexity	$8967	19	$170,373	9	$80,703	8	$71,736
O60B	Vaginal delivery, intermediate complexity	$6206	82	$508,892	15	$93,090	22	$136,532
O60C	Vaginal delivery, minor complexity	$4560	54	$246,240	4	$18,240	4	$18,240
Cost per patient ***				**$7962**		**$9309**		**$9914**

* Number of participants with AR-DRG available. ** Cost ($) = number of participants × NWAU cost (by AR-DRG classification). *** Cost ($) per total number of patients, by BMI category. NWAU: National Weighted Activity Unit.

3.5. Aim (iii): Estimate of the Total Effect of Maternal Diet Quality on Healthcare-Resource Use

Results from the analyses investigating the total effect of maternal diet quality on healthcare-resource use during the delivery admission are shown in Table 4. There were no significant effects of ARFS on mode of delivery or the number of midwifery-in-the-home visits a patient required. Women in ARFS Quintile 1 had a 20% increase in the mean length of stay relative to Quintile 5 (RR 1.20; 95% CI 1.00 to 1.44; $p = 0.05$). That is, women with poor diet quality had an increase in average length of stay, when compared to women with the highest level of diet quality.

3.6. Aim (iv): Estimate of the Direct Effect of Maternal Diet Quality on Healthcare-Resource Use

Results from the regression analyses investigating the direct effect of maternal diet quality on healthcare-resource use during the delivery admission are shown in Table 4. Women in ARFS Quintile 1 had a 27% increase in the mean length of stay relative to Quintile 5 (RR 1.27; 95% CI 1.05 to 1.53; $p = 0.01$). That is, independent of a woman's BMI, those with an ARFS score in Quintile 1 (lowest diet quality) had a 27% increase in average length of stay when compared to women with an ARFS score in Quintile 5 (highest diet quality). There was no significant direct effect of ARFS on caesarean delivery or midwifery in the home visits.

Given there was no statistically significant association between ARFS and mode of delivery, admission to intensive care and midwifery in the home visits, analysis of the economic impact of ARFS on these outcomes was not conducted.

4. Discussion

This observational study sought to quantify specific perinatal-healthcare-resource use associated with maternal weight status and diet quality, in a sample of pregnant women attending a public hospital in NSW, Australia. It was hypothesized that high BMI and low diet quality would be associated with increased healthcare-resource use, with diet quality potentially having a direct effect, independent of BMI. This study found the odds of caesarean delivery was about two-fold higher for women in obese class II than for women of normal weight. In this sample, the effect size for the association between BMI category obese class III was very similar, but did not quite reach the nominal 0.05 significance threshold (OR 1.92; 95% CI 0.98 to 3.73; p = 0.056). With consideration for the real-world impacts of BMI on healthcare-resource use, given similarity of the effect sizes, in a larger sample size, both obese class II and III would likely have achieved statistical significance. Based on these findings and evidence-based guideline recommendations for increased routine monitoring for women classified as obese [6], the impact of both obese class II and obese class III on caesarean delivery is expected to be clinically important. As such, the impact of both obese class II and III on average inpatient cost was explored.

AR-DRG classifications include an estimate of case complexity, which is a classification system within the AR-DRG classifications, to "better explain the variation in costs occurring in the admitted patient data within the ARDRG classification" [35]. There were higher rates of caesarean delivery and cases with "major complexities" for women in BMI category obese class II and III, relative to women with a BMI in the normal-weight category. The birth admission for women in BMI category obese class II cost $1348 more than women in normal-weight class. The average cost of the birth admission for women in obese class III was $1952 more than for women in the normal-weight class and $605 more than for women in obese class II. Within the perinatal period alone, small improvements in maternal pre-pregnancy BMI could deliver substantive economic benefits to the healthcare system and community. Aside from the economic impact, obesity and increased case complexity have procedural complications for clinicians and the healthcare system. For example, complications from anaesthesia are higher in obese patients compared to normal weight patients [36]. There is increased risk of incorrectly placing an epidural in obese patients as the distance to the epidural space is greater with increased BMI [36,37], risk of difficult intubation is increased in obese patients, monitoring and positioning obese patients under anaesthesia can also pose specific challenges [36]. Obesity is also associated with an increased risk of maternal mortality and anaesthesia-related maternal mortality [37]. From a midwife's perspective, a 2011 study of midwives and other health professionals caring for obese childbearing women in NSW, Australia, found midwives were concerned about the rapid impact of the obesity epidemic on maternity services and that study participants felt increased pressure in the management of obese pregnant women and the complications associated with their BMI [38]. Pre-pregnancy public health interventions to reduce maternal pre-pregnancy BMI may prevent the onset or mitigate complications in the delivery period and reduce the obesity related risks to mothers, clinicians and the healthcare system.

This study also found that diet quality had a direct effect on maternal length of stay, independent of BMI. Women in ARFS Quintile 1 had a 27% increase in the mean length of stay relative to Quintile 5 (RR 1.27; 95% CI 1.05 to 1.53; p = 0.01). That is, independent of a woman's BMI, those with an ARFS score in Quintile 1 (lowest diet quality) had a 27% increase in average length of stay when compared to women with an ARFS score in Quintile 5 (highest diet quality). The method in which diet quality acts on length of stay is also unknown. The investment required to improve maternal diet quality is unknown [10]. Further investigation is required, given that poor dietary patterns are common among this population [39] and that current systematic review indicate interventions to improve maternal

BMI and pregnancy outcome show inconsistent finding in regard to cost-effectiveness [40,41]. This study found no significant association between pre-pregnancy BMI and maternal length of stay or midwifery-in-the-home care visits. Analyses also showed that maternal diet quality had no direct effect on caesarean delivery or midwifery-in-the-home care visits. Greater understanding of the economic impact of maternal-health behaviours and specific dietary components on healthcare-resource use and health outcomes is warranted [10].

Strengths and Limitations

The limitations of traditional methods for assessing associations in observational studies and inferring causality are widely recognised [31]. In order to investigate causality, observational data must be interrogated carefully, with attention to the potential for known and unknown confounders and other biases [31]. Use of DAGs in observational nutrition research allows for stronger causal inferences, as compared to conventional statistical adjustments alone. A strength of the current study was the extensive DAG development process informed by the relevant literature, and expert opinion to inform assumptions underpinning the statistical and economic models.

This observational study was conducted in the John Hunter Hospital, NSW, where admitted inpatient-cost data are not stored in administrative hospital datasets and, hence, were outside the data available for this analysis. In the absence of individual patient-cost data, the AR-DRG classification was used as a proxy for admitted-inpatient costs [29]. For the purpose of future research, individual patient-cost data may provide greater specificity regarding the association between patient outcomes, resource use and cost. The John Hunter Hospital antenatal outpatient clinic services high-risk patients requiring ongoing management of GDM, pre-eclampsia, those who have had previous adverse outcomes, women with babies in breech position or those who are attending the clinic drug and alcohol services or Indigenous health services. A limitation of the current study is that the sample of women is expected to have worse health outcomes and thus higher healthcare-resource use compared to the broader population of pregnant Australian women. The current analysis also did not allow for data linkage across all service providers. The John Hunter Hospital has five satellite antenatal clinics that patients can attend, but radiology and pathology can be performed at the hospital, in public or private clinics, and patients may attend private general practitioners, specialists and care providers throughout the antenatal period. Data for health service provision prior to delivery were also unavailable. The inherent recall bias associated with retrospective self-report surveys is recognised as a limitation. Further, the AES food frequency questionnaire, although previously used in pregnancy [42], has not been validated in this population group, and, therefore, the findings of this study need to be interpreted in this context.

5. Conclusions

The current study aimed to quantify specific perinatal-healthcare-resource use associated with maternal weight status and diet quality, in a sample of pregnant women attending a public hospital in New South Wales, Australia. This study found that the odds of caesarean delivery more than doubled for those in obese class II relative to normal weight women, with pre-pregnancy BMI positively associated with an increased risk of caesarean delivery. On average, the birth admission for women in BMI category obese class II costs $1348 more than women in normal weight class, and women in obese class III cost $1952 more than women in the normal weight class. Both obese classes II and III had a higher incidence of caesarean section and complex cases, compared to women in the normal weight class. Our analyses showed that, independent of a woman's BMI, those with an ARFS score in Quintile 1 (lowest diet quality) had a 27% increase in average length of stay when compared to women with an ARFS score in Quintile 5 (highest diet quality). Maternal-diet quality had no direct effect on caesarean delivery or midwifery-in-the-home care visits. Poor dietary patterns are common during pregnancy [39]; thus, interventions to improve maternal BMI and diet quality could deliver substantive economic benefits to the healthcare system and community.

Appendix A

Table A1. List of characteristics collected via patient medical records and evidence of association on preconception process influencing health outcomes and healthcare-resource use.

Study Data	Description	Preconception Process
Demographics		
Maternal age	Age in years	- Increased incidence of maternal hypertension and gestational diabetes mellitus, non-elective caesarean delivery and instrumental delivery and preterm delivery and neonatal intensive care admission among mothers of advanced maternal age [43–45]. - Mothers of advanced maternal age have increased pregnancy risk when compared to younger mothers [43,46].
Education	Maternal education level acquired: high school, TAFE, tertiary education, post-graduate	- Lower-educated women are more likely to smoke, have passive smoking exposure, have low health control beliefs, and not attend antenatal classes or take supplements [47]. - Low educational attainment has been associated with higher rates of pre-pregnancy obesity [48].
Partner status	Relationship status: single, married, de facto, divorced	- Partner (marital) status has been used previously in antenatal diet quality studies [8,49], serving as a participant specific metric for socioeconomic advantage and disadvantage [50].
Insurance status	Health insurance status: no insurance, private health insurance, private insurance without obstetrics	- The inter-sector difference in obstetric practice [51,52] and maternal and infant health outcomes are well documented [53,54]. - Studies have shown increased rates of caesarean section and pre-labour caesarean section amongst patients with private health insurance [51,55].
Lifestyle		
Cigarette smoking	Did the mother smoke nicotine during this pregnancy?	- Antenatal smoking is strongly correlated with preterm birth, low birth weight and adverse infant outcomes [56].
Alcohol consumption	AUDIT-C score	- Antenatal alcohol and substance use are strongly correlated with adverse maternal and infant health outcomes [57,58]. - Older women were significantly more likely than younger women to report drinking while pregnant, but equally likely to reduce their consumption when they became pregnant as their younger counterparts [59].
Diet Quality	Maternal ARFS during current pregnancy	- Suboptimal eating patterns during pregnancy contribute to EGWG, gestational hypertension, pre-eclampsia, GDM, pre-term birth, low and high birth weight, birth defects and still birth [8,9].

Table A1. *Cont.*

Study Data	Description		Preconception Process
Previous Medical History			
Body mass index	At booking visit (20 weeks gestation)	-	Clinical practice guidelines recommend that healthcare facilities have well-defined pathways for the care of pregnant obese women, with increased monitoring and management in comparison to the pathways for the care of healthy-weight women [7].
		-	High pre-pregnancy BMI has been shown to be strongly associated with EGWG [3] and increased resource use in the antenatal period [3,60,61].
		-	There is a linear trend between maternal pre-pregnancy BMI and risk for both elective and unplanned caesarean section [62,63].
Previous mode of delivery	Total number of previous caesarean sections	-	Attempting vaginal birth after a previous caesarean section, or repeat elective caesarean section, carries additional risks to the mother and baby [64].
Parity	Number of previous pregnancies	-	Parity has been associated with advanced maternal age, sociodemographic status and educational attainment [45].
Assisted reproductive therapy required? (ART)	Did the mother require ART or IVF to conceive this pregnancy?	-	Perinatal risks that may be associated with assisted reproductive technology (ART) and ovulation induction include multifetal gestations, prematurity, low birth weight, small for gestational age, perinatal mortality, caesarean delivery, placenta previa, abruptio placentae, preeclampsia and birth defects [65].
Diabetes	Has the mother been diagnosed with type I or type II diabetes?	-	Patients with pre-gestational diabetes (types 1 and 2) are more prone to higher rates of pre-eclampsia, prematurity and caesarean section.
		-	Pregnancy may accelerate maternal and infant complications of diabetes [66].
Recent Antenatal Period			
Weight change	Did the patient gain an appropriate amount of weight during pregnancy?	-	EGWG are risk factors for GDM, pregnancy-induced hypertension and pre-eclampsia, venous thrombo-embolism, labour induction and caesarean delivery [67].
Hypertensive disorders	Was the mother diagnosed with hypertensive disorders?	-	High maternal-diet quality may reduce the risk of gestational hypertension for the mother [49].
		-	Current clinical guidelines for management of hypertensive disorders in pregnancy recommend diagnosis of hypertensive disorders "should lead to increased observation and vigilance" [68].

Table A1. *Cont.*

Study Data	Description	Preconception Process
Gestational diabetes:	Was the mother diagnosed with GDM?	- Prevalence is affected by maternal factors such as history of previous GDM, ethnicity, advanced maternal age, family history of diabetes, pre-pregnancy weight and EGWG [66]. - Potential maternal complications during pregnancy and delivery include pre-eclampsia and higher rates of caesarean delivery, birth injury and postpartum haemorrhage [66]. - For the neonate, complications can include macrosomia (large for gestational age) growth restriction, birth injuries, respiratory distress, hypoglycaemia and jaundice [66]. - GDM is diagnosed at any time throughout the pregnancy, and management includes a prescriptive diet which is expected to be different from the mother's diet pre-GDM diagnosis [69].
Plurality	Number of infants born (2, 3, 4, . . . , x)	- Guidelines advise of additional care that should be offered to women with twin and triplet pregnancies above that are routinely offered to all women during pregnancy [70].
Gestation	Number of weeks at delivery	- Gestational age at birth is an important predictor or infant mortality and length of stay [71].
Infant birth weight	Infant birth weight in grams	- Birthweight is a key indicator of infant health and a principal determinant of infant mortality [50]. - Factors that contribute to low birthweight include extremes of maternal age, illness during pregnancy, low socioeconomic position, multiple pregnancy, maternal history of spontaneous abortion, harmful behaviours such as smoking or excessive alcohol consumption, poor nutrition during pregnancy and poor antenatal care [50]. - Low birth weight is a risk factor for inadequate foetal development and amplified risk of chronic disease throughout life [50].
Mode of delivery	Caesarean; surgical intervention (including internal manoeuvres); vaginal birth	- It is considered self-evident that the cost of caesarean delivery is more expensive than natural birth. - Even amongst similar cases, the charges associated with mode of delivery vary widely [72]
Mother length of stay	Mothers length of stay in days	- It is considered self-evident that length of stay and admission to intensive care accrues higher healthcare-resource use and total cost of admission than those without.
Infant length of stay	Infant length of stay in days	- Neonatal service levels range from no planned service, Level 1 to Level 6 [73]. Level 6 neonatal care is provided in specialist children's hospitals where neonatal surgery and complex genetic and metabolic services are located. It is considered self-evident that Level 6 care will accrue higher costs than Level 1 care, due to the complexity of care provided.
Infant admission to nursery	Neonatal intensive care admission	

ART, Assistive reproductive therapy; AUDIT-C, Alcohol Use Disorders Identification Test; EGWG, excessive gestational weight gain; GDM, gestational diabetes mellitus; TAFE, Technical and Further Education.

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Brief Report

Fetal Head Growth during Early to Mid-Gestation Associated with Weight Gain in Mothers with Hyperemesis Gravidarum: A Retrospective Cohort Study

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Abstract: The epigenetic impact of malnutrition in mothers with hyperemesis gravidarum (HG) on their offspring has not been fully elucidated. Recently, several reports have demonstrated that children born to mothers with HG were small for gestational age and had low birth weight, reduced insulin sensitivity, and neurodevelopmental delays during childhood. Therefore, we examined the relationship between fetal growth and changes in the maternal body weight in HG cases. A total of 34 patients with HG were hospitalized and delivered at term between 2009 and 2012. The records of 69 cases of pregnant women without a history of HG were extracted after matching their maternal age, parity, pregestational body mass index (BMI), gestational age, and fetal sex ratio with those of the HG group for comparison. The maternal weight gain at term was less in the HG than in the control group. There was no statistical difference in birth weight, placental weight, and ultrasonic fetometric parameters expressed in standard deviation (SD) scores, including biparietal diameter, abdominal circumference, and femur length, between the HG and the control group. Whereas fetal head growth in the HG group was positively associated with maternal weight gain at 20 weeks of gestation only, this association was not observed in the control group. We herein demonstrate that maternal weight gain from the nadir is associated with fetal head growth at mid-gestation. Thus, maternal undernutrition in the first trimester of pregnancy could affect fetal brain growth and development, leading to an increased risk of neurodevelopmental delays in later life.

Keywords: fetal growth; hyperemesis gravidarum; neurodevelopment

1. Introduction

Hyperemesis gravidarum (HG) involves persistent severe nausea and vomiting in pregnant women, affecting both the mother and the fetus [1]. The incidence of HG varies from country to country, ranging from 0.3% in Sweden to 1.2% in the United States and 3.6% in Japan [2–4]. The adverse effects caused by HG include dehydration, vitamin deficiency, and electrolyte imbalance in pregnant women [5] and an increased risk of low birth weight and small size for gestational age in the fetus [6]. In addition, HG may affect maternal acceptance of pregnancy as well as acceptance of motherhood and later quality of life [7]. With regard to the etiology of HG, a genetic study revealed a variance in the gene encoding an intracellular calcium release channel involved in vomiting and cyclic vomiting syndrome in families with possible inheritance of HG [8]. Moreover, through a genome-wide association study in humans, the placenta and appetite genes *GDF15* and *IGFBP7* were shown to be associated with

HG [9,10]. Since the concept of the developmental origin of adult diseases has been introduced by accumulating epidemiological research, undernutrition during pregnancy, particularly during the first trimester, is known to be related to the future development of adult-onset disorders, such as obesity, cardiovascular disease, and diabetes in the early pregnancy [11]. Grooten et al. pointed out the relationship between early pregnancy, severe maternal weight loss, and elevation of blood pressure in the offspring as early as at 5–6 years of age [12]. Moreover, HG may result in reduced insulin sensitivity and neurodevelopmental delays at school age in offspring of mothers who experienced HG during pregnancy [13]. The adverse clinical effects of maternal undernutrition on neurodevelopmental growth, including those resulting from HG, have been reported. Fejzo et al. report that children born to mothers with HG are more likely to have neurodevelopmental problems, including attention disorders, learning delays, sensory disorders, and speech/language development delays [14]. Offspring born to mothers experiencing HG are also at an increased risk of behavioral and emotional disorders when they become adults [15].

To explore specific growth patterns of the fetus in pregnant mothers with HG, we investigated the association between fetal growth parameters and maternal body weight by analyzing ultrasonographic fetometric measurements.

2. Materials and Methods

Between 2009 and 2012, 34 women having HG with singleton pregnancies, who required hospitalization and delivered at full term (37 weeks 0 day to 41 weeks 6 days of gestation), were identified from 2778 recorded deliveries in the hospital records. This study received approval from the Institutional Review Board of Tokyo Women's Medical University (REC no. 2208, June 2011). We diagnosed HG in the women if two of the following criteria were present: weight loss greater than 5% in the first trimester, ketone bodies in the urine, or the inability for food and fluid intake [16]. Prior to hospitalization, all patients were instructed by midwives and obstetricians to have frequent balanced meals, including low-fat and high-protein foods, and separate liquids and solids. On hospitalization, most patients were managed by peripheral intravenous administration of fluids, electrolytes, glucose, and vitamins in addition to oral food intake. The control group consisted of 69 cases who were matched by age, parity, pre-pregnancy body mass index (BMI), gestational age at delivery, and neonatal gender ratio. Maternal body weight was measured upon every perinatal visit without shoes and heavy clothing. The weight gain from the nadir was defined as the difference between the body weight at 20 weeks of gestation and the lowest body weight before admission. Ultrasonographic fetometry was performed on a GE Voluson S8 (GE Healthcare Japan, Tokyo). The clinical background characteristics of the participants in both groups are shown in Table 1. Fetal growth parameters including biparietal diameter (BPD), abdominal circumference (AC), femur length (FL), and estimated fetal body weight (EFW) were measured at approximately 20, 30, and 36 weeks of gestation and expressed as standard deviation (SD) scores. The mean values were subtracted from each measured value and divided by the given SD in each group. These values were compared statistically using JMP software, version 11, by Wilcoxon/Kruskal–Wallis and regression analyses. The statistical significance was set at $p < 0.05$.

Table 1. Clinical background of the HG and control groups.

Group	HG (*n* = 34)	Control (*n* = 69)	*P*-Value
Ethnicity	Japanese 33, Burmese 1	Japanese 69	0.716
Smoking before pregnancy	1	0	0.716
Smoking during pregnancy	0	0	
Maternal age (years old)	33.0 ± 5.0 (23–42)	33.0 ± 4.0 (23–41)	0.88
Parity	0.50 ± 0.70 (0–3)	0.60 ± 0.80 (0–3)	0.94
Pre-gestational BMI (Kg/m^2)	21.0 ± 3.4 (15.8–34)	21.0 ± 3.7 (16–32)	0.71
Gestational age at birth (weeks)	39.0 ± 1.1 (37–41)	39.0 ± 1.0 (37–41)	0.13
Birth weight (g)	3148.9 ± 348.0 (2536–3948)	3070.9 ± 316.0 (2592–4440)	0.98
Sex ratio (male/female)	1:1	1:1	0.61
Placenta weight (g)	569.9 ± 103.1 (410–820)	582.0 ± 107 (205–1544)	0.71
Weight ratio of fetus to placenta	0.18 ± 0.030 (0.13–0.26)	0.1 9 ± 0.030 (0.11–0.52)	0.73
Weight gain at 20 weeks of gestation	−0.29 ± 2.8 (−6.9–−4.7)	3.2 ± 2.5 (−4.8–9.0)	0.0001
Net weight gain (Kg)	8.7 ± 3.5 (0–15.6)	10.0 ± 3.3 (3.3–19.4)	0.011
Weight gain from the nadir (Kg)	15.0 ± 4.9 (6–25.6)		

HG, hyperemesis gravidarum; BMI, body mass index; Values are expressed as mean ± standard deviation (SD) (minimum–maximum).

3. Results

The clinical background characteristics of the participants in both groups are shown in Table 1. Ethnicity, smoking history, maternal age, parity, pre-gestational BMI, gestational age at delivery (weeks), birth weight, gender ratio, placental weight, and the weight ratio of the neonate to the placenta were not statistically significant. Maternal weight gain, measured at 20 weeks of gestation and at term, was significantly smaller in the HG group than in the control group. However, the birth weight was not statistically different between the two groups.

The clinical background of mothers with HG is shown in Table 2. Ultrasonographic fetometric data are shown in Table 3. BPD, AC, FL, and EFW of the HG group and control group were not significantly different at 20 weeks, 30 weeks, and 36 weeks of gestation.

With regard to the association between maternal weight gain and fetal growth parameters in each trimester of gestation, the SD score of the BPD and EFW at 20 weeks of gestation were positively associated with the weight gain from the nadir (Figure 1) and the weight gain from the pre-pregnancy period (Figure 2) in the HG group, while the maternal weight gain was positively associated only with the AC at 30 to 36 weeks of gestation in the control group (Table 4).

Table 2. Clinical background of mothers with HG.

Group	HG (*n* = 34)
Onset of HG (gestational age in weeks)	9.0 ± 2.0 (6–15)
Weight loss (Kg)	6.3 ± 2.8 (0–14)
Weight loss ratio (%)	8.5 ± 3.8 (4.0–18.2)
Duration of admission (days)	27.0 ± 18.0 (8–90)

Values are expressed in mean ± SD (minimum–maximum).

Table 3. Values of the fetal growth parameters in the HG group and control group.

Fetal Growth Parameters	Gestational Age (Weeks)	HG (*n* = 34)	Control (*n* = 69)	*P*-Value
BPD (SD score)	20	0.33 ± 0.8	0.09 ± 0.69	0.12
	30	0.26 ± 1.1	0.35 ± 0.82	0.63
	36	0.17 ± 0.89	0.33 ± 0.74	0.35
AC (SD score)	20	0.33 ± 1.1	0.47 ± 0.96	0.53
	30	0.15 ± 1.1	0.32 ± 0.97	0.44
	36	0.22 ± 0.67	0.40 ± 0.86	0.30
FL (SD score)	20	−0.24 ± 0.7	−0.02 ± 0.8	0.17
	30	−0.11 ± 0.84	−0.003 ± 0.86	0.55
	36	−0.2 ± 0.91	−0.045 ± 1.1	0.48
EFW (SD score)	20	0.17 ± 0.85	0.10 ± 0.69	0.67
	30	0.09 ± 0.86	0.05 ± 0.75	0.79
	36	−0.19 ± 0.75	−0.16 ± 0.73	0.81

BPD, biparietal diameter; AC, abdominal circumference; FL, femur length; EFW, estimated fetal bodyweight. Values are expressed in mean ± SD.
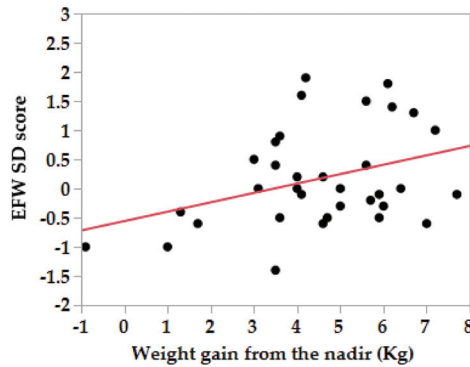
Figure 1. Correlation between SD scores of the estimated fetal body weight at 20 weeks of gestation in mothers with hyperemesis and maternal weight gain from the lowest body weight to the weight at 20 weeks of gestation. EFW SD score = −0.56 + 0.16 × Weight gain from the nadir (Kg). EFW, estimated fetal body weight.
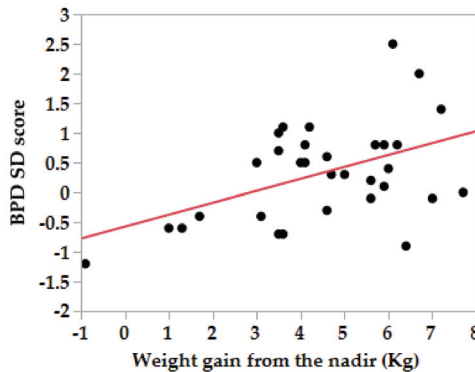

Figure 2. Correlation between SD scores of the biparietal diameter at 20 weeks of gestation in mothers with hyperemesis gravidarum and maternal weight gain from the lowest body weight to the weight at 20 weeks of gestation. BPD SD score = −0.57 + 0.20 × Weight gain from the nadir (Kg). BPD; biparietal diameter.

Table 4. Association between maternal weight gain and fetal growth parameters at prespecified stages.

GW	Correlation Coefficient = r (*p* Value)	HG Group Weight Gain (*n* = 34)				Control Group Weight Gain (*n* = 69)		
		Nadir 20 Weeks	Pre-Gest 20 Weeks	20–30 Weeks	30–36 Weeks	Pre-Gest 20 Weeks	20–30 Weeks	30–36 Weeks
20	BPD	0.47 (0.0048)	0.38 (0.02)			0.037 (0.76)		
	AC	0.18 (0.30)	0.17 (0.42)			0.024 (0.85)		
	FL	0.23 (0.18)	0.26 (0.12)			0.128 (0.88)		
	EFW	0.36 (0.037)	0.03 (0.86)			0.026 (0.83)		
30	BPD			0.04 (0.98)			0.23 (0.06)	
	AC			0.10 (0.58)			0.006 (0.96)	
	FL			0.18 (0.31)			0.21 (0.82)	
	EFW			0.049 (0.79)			0.18 (0.15)	
36	BPD				0.07 (0.70)			0.049 (0.68)
	AC				0.25 (0.17)			0.34 (0.0045)
	FL				0.08 (0.67)			0.13 (0.0045)
	EFW				0.06 (0.75)			0.27 (0.027)

GW, gestational weeks; Pre-gest: Pre-gestational body weight.

4. Discussion

Main Findings

This retrospective study reveals that fetal head growth evaluated with ultrasound fetometry is positively correlated with maternal weight gain from both the pre-pregnancy period and the nadir in the first trimester of gestation in women with hyperemesis gravidarum. The same association was not observed in the matched control group.

4.1. Strengths and Limitations

The strengths of our study include the comparisons made with the matched control group. Although our sample was small, both the correlation coefficient and the statistical significance were high. As this study is based on data collected retrospectively during routine clinical services, we measured ultrasound fetometric data as SD scores to compare the outcomes of the two groups. The limitations of this study are the accuracy and reliability of the pre-pregnancy body weight values, as they were self-reported, and the lack of details of the nutritional support before and during hospitalization. However, our findings show that weight gain from the nadir and ultrasound fetometric fetal growth parameters are still highly associated.

4.2. Interpretation

Our study is the first to indicate an association between the growth of the fetal head and maternal body weight gain from pre-pregnancy and from the nadir in pregnant mothers with HG at mid-gestation. The same association between fetal head growth and the increase in maternal body weight was not observed in the matched control group. Why the effect of maternal undernutrition was only observed in the HG group has not been elucidated. However, it has been suggested that there may be a nutritional threshold that needs to be met to satisfy the nutritional demand for brain growth and development of the fetus.

Only a few reports discussed the relationship between maternal nutrition and fetal brain growth in humans. Baker et al. reported that the head circumference of a neonate is positively associated with the ponderal index when maternal nutrition during pregnancy is adequate [17]. However, this association was not demonstrated in a group with a heavier placenta in which maternal nutrition was supposedly poor during pregnancy. Hinkle et al. reported similar findings to ours, demonstrating that fetal BPD and femur length were positively associated with maternal weight gain only at 17 weeks of gestation in women having a high risk of developing small for gestational age baby, including smoking during pregnancy, history of prior low birth weight delivery, low pre-pregnancy body weight, and complications of hypertension and chronic renal diseases [18]. It is understood that in situations where the feto–maternal nutritional transport is restricted, brain growth would be secured in the first instance. The effects of maternal nutritional restriction on the fetal brain have been shown previously in an animal model. Ma et al. report that in early to mid-gestational nutrient-restricted ewes, the brain weight of the fetus was transiently reduced at mid-gestation and returned to normal levels at term [19]. With regard to the qualitative effect of malnutrition on the brain, Edlow et al. showed that differences in the relative deficiency in micronutrients including minerals, vitamins, and amino acids provoked differential expression of more than 1000 genes in the mice embryonic brain, such as brain-derived neurotrophic factor (BDNF) and Kruppel-like factor 3 (KLF3), a transcription regulator linked to various neurological disorders [20]. In addition, protein restriction during the perinatal period is known to impair hippocampal development, leading to reduced BDNF levels. These animal studies suggested that the mechanism by which brain development is affected by under- or malnutrition is not related to caloric intake but to the intake of proteins and micronutrients including vitamins.

The intake of micronutrients, vitamin B_{12}, and folate in early pregnancy is demonstrated to be associated with cardiometabolic risk in the offspring at the age of 5–6 years in humans [21]. Although evidence of the effects of nutritional restriction on the human fetal brain is lacking, adverse clinical effects of maternal undernutrition on neurodevelopmental growth, including those resulting from HG, have been reported. Fejzo et al. reported that children born to mothers with HG are more likely to have neurodevelopmental problems, including attention disorders, learning delays, sensory disorders, and speech/language development delays [14]. Offspring born to mothers experiencing HG are also at an increased risk of behavioral and emotional disorders when they become adults [15]. Vitamin deficiency as an etiology of neurodevelopmental problems in HG remains to be elucidated, because in most HG cases, vitamin B group, vitamin C, and folate are routinely administered. If that is the case, we can speculate that another deficiency, such as amino acid deficiency, may be a candidate for the neurodevelopmental problems in HG cases.

Embryologically, the early to mid-gestation period is a critical period for brain growth, with the earliest synapses in the spinal cord developing during early gestation at 6 to 7 weeks [22]. Furthermore, subcortical structures have been shown to develop around mid-gestation at 12 to 22 weeks [23]. Nonetheless, the brain metabolism of the fetus has not been studied. Expanding on the existing body of evidence, our observation of a significant correlation between fetal head size and weight gain in mothers experiencing HG may be a sign of a morphological change in the fetal brain in response to maternal undernutrition. Our observation that fetal head growth was temporarily associated with maternal nutritional status may only be the coincidental sign of this morphological change in the fetal brain.

In a rat model, adverse effects of malnutrition on the fetus, other than brain development issues, include an increased risk of prostate disorders in later life [24]. Although such effects have not been demonstrated in humans, in cows, restricting nutrition shortly before conception until the end of the first trimester of pregnancy results in a decreased number of postnatal follicles, eventually leading to subfertility [25].

5. Conclusions

In this study, we demonstrated that maternal weight gain from the nadir was associated with fetal head growth at mid-gestation. Moreover, maternal undernutrition in the first trimester of pregnancy could affect fetal brain growth and development, leading to an increased risk of neurodevelopmental delays in later life. Further studies, including experiments using animal models, are needed to clarify the relationship between undernutrition in the first trimester of pregnancy and neurodevelopmental problems in later life.

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

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Epidemiology and (Patho)Physiology of Folic Acid Supplement Use in Obese Women before and during Pregnancy

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Abstract: Preconception folic acid supplement use is a well-known method of primary prevention of neural tube defects (NTDs). Obese women are at a higher risk for having a child with a NTD. As different international recommendations on folic acid supplement use for obese women before and during pregnancy exist, this narrative review provides an overview of epidemiology of folate deficiency in obese (pre)pregnant women, elaborates on potential mechanisms underlying folate deficiency, and discusses considerations for the usage of higher doses of folic acid supplements. Women with obesity more often suffer from an absolute folate deficiency, as they are less compliant to periconceptional folic acid supplement use recommendations. In addition, their dietary folate intake is limited due to an unbalanced diet (relative malnutrition). The association of obesity and NTDs also seems to be independent of folate intake, with studies suggesting an increased need of folate (relative deficiency) due to derangements involved in other pathways. The relative folate deficiency, as a result of an increased metabolic need for folate in obese women, can be due to: (1) low-grade chronic inflammation (2) insulin resistance, (3) inositol, and (4) dysbiotic gut microbiome, which plays a role in folate production and uptake. In all these pathways, the folate-dependent one-carbon metabolism is involved. In conclusion, scientific evidence of the involvement of several folate-related pathways implies to increase the recommended folic acid supplementation in obese women. However, the physiological uptake of synthetic folic acid is limited and side-effects of unmetabolized folic acid in mothers and offspring, in particular variations in epigenetic (re)programming with long-term health effects, cannot be excluded. Therefore, we emphasize on the urgent need for further research and preconception personalized counseling on folate status, lifestyle, and medical conditions.

Keywords: obesity; folic acid supplement use; neural tube defects

1. Rationale

In order to prevent neural tube defects (NTDs) in offsprings, women are advised to take a 0.4 mg folic acid supplement from the moment they wish to get pregnant up until the first trimester of pregnancy [1]. This advice applies to all women, except for women with a history of a previous child with a NTD, who are advised to take a higher dose of 4–5 mg folic acid supplement [1].

A growing number of women is obese when trying to get pregnant, with an increased risk of having a child with a NTD [2,3]. Meta-analyses showed a dose-response association between maternal Body Mass Index (BMI) and NTDs, and the risk rapidly increased in women with a BMI $\geq$ 30 kg/m^2 (Table 1) [4–6]. In addition, a BMI $\geq$ 30 kg/m^2, defined as maternal obesity, is also associated with the severity of the NTD in the offspring [7,8].

Table 1. Overview of three meta-analyses on the association between maternal obesity and NTD in offsprings.

	Years Included	Number of Studies	Design	Results (OR (95% CI))			
				Normal Weight	Overweight	Obese	Severely Obese
Rasmussen et al. 2008 [4]	January 2000–January 2007	12	Cohort and case-control studies	1 (ref)	1.22 (0.99–1.49)	1.70 (1.34–2.15)	3.11 (1.75–5.46)
Stothard et al. 2009 [5]	January 1966–May 2008	18	Cohort and case-control studies	1 (ref)		1.87 (1.62–2.15)	
Huang et al. 2017 [6]	up to 15 December 2015	22	Case-control studies	1 (ref)	1.20 (1.04–1.38)	1.68 (1.51–1.87)	

Given the known association between inadequate maternal folate intake and NTD in offsprings, and the increased risk of NTDs in obese women, the question arises whether obese women more often have a folate deficiency [9]. There might be an absolute folate deficiency from diet (folate) due to a suboptimal intake that is associated with obesity, combined with the fact that obese women may be less compliant in taking supplements (folic acid) [10–12]. On the other hand, obese women can have a relative folate deficient status, caused by a state of chronic low-grade inflammation, which results in an increased metabolic need of folate. Importantly, studies have shown that obese women had an increased risk of NTDs, regardless of their folate intake [13,14]. There are no studies that have assessed whether a high dose of folic acid results in less NTD pregnancies in obese women. Therefore, the rationale to prescribe higher doses of folic acid supplementation has to come from indirect evidence. Several underlying mechanisms have been suggested as determinants in the causal pathway of a relative folate deficiency in obese women, such as chronic inflammation and hyperinsulinemia [15]. However, an overview of causes of folate deficiency in obese women, potential underlying (patho)physiological mechanisms and how they might contribute to a higher risk of NTDs is lacking.

Moreover, different international recommendations on folic acid supplement use for obese women before and during pregnancy are used [16,17]. Therefore, we provide an overview of the epidemiology of folate deficiency in obese (pre)pregnant women, elaborate on potential mechanisms underlying folate deficiency, and discuss considerations for advising higher doses of folic acid supplements. Moreover, we propose suggestions for clinical practice making use of the current evidence, and suggest some areas for further research.

2. Epidemiology of Folate Deficiency in Obese (pre)Pregnant Women

2.1. Absolute Deficiency

Studies have shown that women with obesity have a lower intake of folate (Table 2). Women with obesity are less likely to use preconceptional folic acid supplement compared to normal weight women, 45.2% versus 60.4%, respectively [12]. They are also less likely to use folic acid supplements on a daily base, 26% versus 33%, respectively [10]. Moreover, women with obesity are less likely to receive enough folate through their diet than lean individuals, i.e., relative malnutrition [18,19]. Both a lower intake of folic acid supplements and a lower dietary intake of folate accounts for lower folate levels in serum, red blood cells, and body fluids. Moreover, decreased folic acid intake is often due to unplanned pregnancies and failed contraceptive methods prevalent in obese women [10].

Though it is clear that obese women have a lower intake of folate, obesity is associated with other factors that are subsequently determinants of a lower intake of folate. Earlier studies indicated that smoking, lifestyle, age, parity, educational level, income level, and whether the pregnancy was planned were determinants of folate intake [20,21]. In a multivariable model, maternal weight status was independently associated with adequate use of folic acid, even after excluding women with an unplanned pregnancy [20].

Table 2. Intake of folate and folic acid supplements in women, per weight category.

	Study Design	Population	Sample Size	Outcome	Results (% or Mean ± SD)			
					Normal Weight	Overweight	Obese	*p*-Value
Masho et al. 2016 [10]	Cohort study	Women with singleton pregnancy living in USA	104.211	Daily intake of folic acid supplement	33%	29%	26%	<0.0001
Farah et al. 2013 [12]	Cohort study	White European women with a singleton pregnancy	288	Use of folic acid supplement	60%	60%	45%	0.029
Bird et al. 2015 [18]	Cohort study	Non-pregnant women aged ≥19 years living in the USA	538	Folate intake through diet (µg/L)	559 ± 12.7	557 ± 14.5	517 ± 10.5	0.002

2.2. Relative Deficiency

Obese women had lower serum folate levels even after controlling for folate intake through supplements and diet (β = −0.26, 95% CI: −0.54, 0.02); *p* = 0.07) [22]. When comparing non-obese and obese women with a similar folate intake, serum levels in obese women tend to be lower than in non-obese women, suggesting the current recommendations of folic acid supplement use could be subjected to review.

An increased need for folate is suggested to be caused by altered metabolic processes and chronic low-grade inflammation that could eventually underlie the increased risk for women with obesity on NTDs. Moreover, in women of higher weight categories, an adequate intake of folic acid of 0.4 mg/day did not lower the risk of NTDs [13]. A similar finding was reported by Parker et al., where women with obesity were at increased risk of NTDs, irrespective of adequacy of folic acid intake following the current standard 'one-fits-all' dosing regimens [14].

3. Theoretical Background

3.1. One-Carbon Metabolism

One-carbon metabolism is a complex of interlinking metabolic pathways that are fundamental for molecular biological processes involved in cell multiplication, differentiation, and programming [23]. It provides essential one-carbon moieties used as substrate or cofactor of the linked folate and methionine pathways, as displayed in Figure 1 [24]. We focus on these pathways, however, one carbon metabolism comprises of a series of metabolic pathways [25]. The main substrate of the folate pathway is tetrahydrofolate (THF), which is converted into 5-methyltetrahydrofolate (5-MTHF). Together with homocysteine, it is converted into methionine by methionine synthase (MS) using vitamin B12 as cofactor [26]. The methionine pathway is essential for the provision of methyl groups after transmethylation into S-adenosylmethionine (SAM), the most important methyl donor in the cell [27].

One of the main products of one-carbon metabolism is the contribution to the biosynthesis of nucleotides and epigenetic programming. Interruption of molecular biological processes involved in neural tube development and dependent on one-carbon metabolism, such as cell multiplication, differentiation, apoptosis and programming, can impair the closure of the neural tube. In order to facilitate rapid DNA replication of the tissues involved in the formation of the neural tube, a large pool of nucleotides is required for DNA synthesis and methyl groups for epigenetic programming for neuroepithelial cells. Inadequate supply of nucleotides and methyl groups blocks cellular replication, increases DNA damage, and impairs epigenetic programming and as such the proper development of the neural folds [28]. Given its central role in one-carbon metabolism, folate plays a key role in the molecular biological processes involved in the development of NTDs.
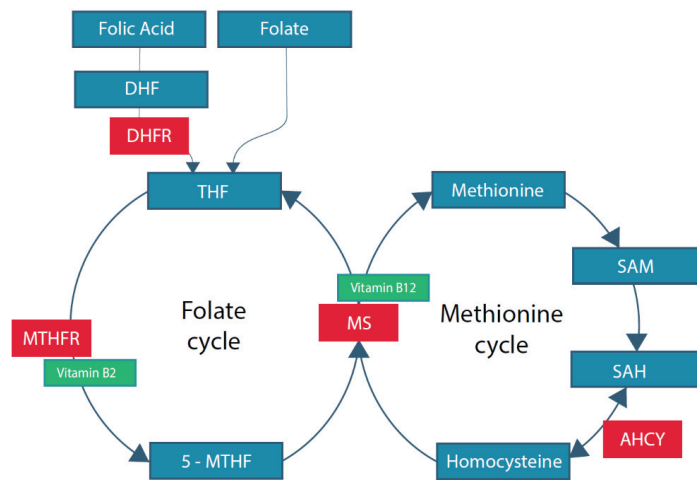
Figure 1. Folate related one-carbon metabolism. DHF: dihydrofolate; DHFR: dihydrofolate reductase; THF: tetrahydrofolate; MTHFR: methylene tetrahydrofolate reductase; 5-MTHF: 5-methyltetrahydrofolaat; MS: methionine synthase; SAM: S-adenosyl-methionine; SAH: S-adenosyl-homocysteine; AHCY: S-adenosylhomocysteine hydrolase.

3.2. Folate

The most important dietary substrates and cofactors involved in one-carbon metabolism include methionine and choline, together with the B vitamins, cobalamin, and folate. Folate is an essential water-soluble B-vitamin and naturally occurs in fruits and vegetables. Folate-rich foods include in particular leafy green vegetables, lentils, beans, and citrus fruits. In general, the term folate refers to the natural forms in foods and body fluids, while the term folic acid applies to the more stable but synthetic supplemental form. Folate is a crucial mediator in the one-carbon metabolism, where it acts as a dietary methyl donor together with methionine, betaine, and choline [23]. Folate derived from food needs to be hydrolysed from polyglutamates to monoglutamates, before absorption takes place in the jejunum [29]. This process leads to a lower bioavailability that varies between 30% and 98% [30,31]. Another source is synthetic folic acid, present in fortified foods and in various supplements. The bioavailability of this form is commonly estimated at 85% [32]. Synthetic folic acid is a monoglutamate and needs to be converted by dihydrofolate reductase (DHFR) to be taken up in its the active form, THF, in the intestinal cells.

3.3. Epigenetics

Generally, epigenetics is defined as the alterations in the gene expression profile of a cell that are not caused by changes in the DNA sequence [33]. Epigenetics is critical to normal genome regulation and development. One-carbon metabolism is essential for epigenetic modifications by providing methyl groups for the methylation of DNA and associated (histone) proteins as well as RNA, for which an adequate folate supply is important. With one-carbon metabolism being essential, it is plausible that folic acid plays a role in epigenetics and its related plasticity of gene methylation. Indeed, periconceptional folic acid supplement use has been shown to be associated with epigenetic changes [34]. Although, maternal intake of folic acid supplements and dietary folate are positively associated with long interspersed nuclear elements (LINE-1) methylation, a surrogate marker of global DNA methylation, transgenerational effects could not be demonstrated in cord blood [35–37].

Such epigenetic modifications, particularly where DNA methylation is involved, have been proposed as plausible mechanisms underlying associations between folate and various disease outcomes, such NTDs, cardiovascular diseases, and cancer [38].

4. Pathophysiology of Relative Deficiency of Folate in Obese Women

4.1. Impaired One-Carbon Metabolism

Hyperhomocysteinemia, conventionally described as a serum level above 15 micromol/L, is a sensitive marker of an impaired one-carbon metabolism [39]. Considering the pathways within the one-carbon metabolism, a folate deficiency and as such less supply of methyl groups, contributes to higher levels of homocysteine, and higher levels of homocysteine lead to a higher demand for folate used for remethylation of homocysteine [40]. Moreover, hyperhomocysteinemia is a risk factor for several poor health outcomes, including, among others, neurological disorders, vascular diseases and reproductive disorders [23,41,42]. Pregnancy complications such as preeclampsia, intra-uterine growth restriction, and prematurity are associated with high maternal levels of homocysteine [23,43,44]. Hyperhomocysteinemia is more common in women with obesity, compared to non-obese individuals: two studies reported statistically significant differences in homocysteine levels between obese and non-obese women; 12.76 ± 5.30 μM/L versus 10.67 ± 2.50 μM/L, respectively, and 10.2 μM/L [4.6–26.3] versus 8.9 [4.4–25.8] respectively [45,46]. Suggested folate-related pathways that could underlie this finding are discussed below. In addition, an overview of potential underlying (patho)physiological pathways of folate deficiency and NTDs in obese women is displayed in Figure 2.
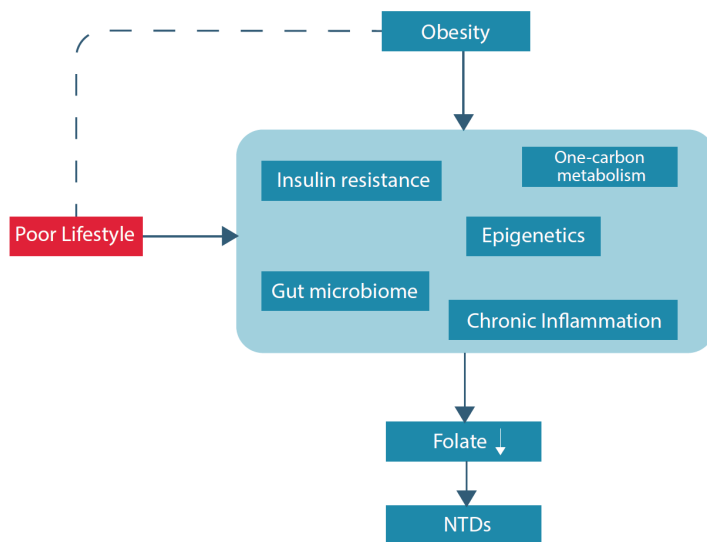
Figure 2. Overview potential underlying (patho)physiological pathways of folate deficiency and NTDs in obese women.

4.2. Physiology of Adipocytes

Adipose tissue is traditionally categorized into white and brown adipose tissue. Brown adipose tissue is specialized in energy expenditure and thermogenesis [47,48]. White adipose tissue is responsible for storing and releasing energy in the human body by controlling lipogenesis and lipolysis, respectively. During the process of lipogenesis, free fatty acids and glycerol are taken up from the blood stream and are stored as triglycerides in adipocytes [49]. On the contrary, lipolysis is the mechanism by which triglycerides are

catabolized into free fatty acids and glycerol that are released into the bloodstream where they act as an energy source for other organs [50].

Obesity is characterized as an excessive growth of adipose tissue [51]. Furthermore, obesity is known to cause both hypertrophy as hyperplasia of the adipocyte [52]. These processes are associated with an infiltration of macrophages into the adipose tissue. This promotes inflammation and introduces TNFα into the tissue [53]. Moreover, the expansion of adipose tissue in obesity is linked to an inappropriate supply with oxygen and hypoxia development [54]. Subsequent inflammatory reactions inhibit preadipocyte differentiation and initiate adipose tissue fibrosis [55]. Not all obese individuals develop adipose tissue fibrosis followed by inflammation; however, obesity-related hypertrophic adipocytes may induce inflammation by producing pro-inflammatory adipokines [56].

4.3. Pro-Inflammatory State

The obesity-related low-grade chronic inflammation is generated by the production of pro-inflammatory cytokines, as IL-6 and TNF-α, and adipokines, as leptin [57]. Consumption of excess energy may as well acutely induce inflammatory responses [58,59]. Hence, it is thought that excess energy by overfeeding is another starting signal of inflammation, causing overactivation of tissues involved in metabolism, like adipose tissue, liver, and muscle, which in reaction to this stimulus provokes the inflammatory response [60,61]. Thus, besides continuous, low-grade chronic inflammation, there also might be additional, acutely induced inflammatory responses caused by excess supply of food. The inflammation-related collateral tissue damage activates tissue repair responses, requiring one-carbon moieties for synthesis of adequate amounts of proteins, lipids, nucleotides, and others. Since the folate dependent one-carbon metabolism supports cell proliferation at the detriment of B-vitamins, obesity-induced inflammation is associated with hyperhomocysteinemia and, thereby, folate deficiency. In addition, hyperhomocysteinemia is not only a result of inflammation, but hyperhomocysteinemia will again promote inflammation due to the excessive oxidative stress generated from high homocysteine levels [62].

4.4. Insulin Resistance

Adipose tissue regulates energy storage and release by lipogenesis and lipolysis. Obesity is associated with an increased basal lipolysis, which might be caused by an impaired sensitivity of adipocytes to insulin signaling, overexpression of the leptin gene in adipocytes, and increased circulating levels of leptin [63]. By the increased rate of lipolysis, higher amounts of fatty acids and glycerol are catabolized and enter the bloodstream. Increased serum levels of fatty acids, non-esterified fatty acids (NEFAs) in particular, are considered to be the most critical factor in inducing insulin resistance [50]. This is a pathological condition in which the capacity of cells to respond to normal levels of insulin is reduced. Increased NEFA levels are observed in persons with obesity and are associated with insulin resistance. Moreover, insulin resistance establishes within hours after an acute increase in plasma NEFA levels [64]. Beside the lipolysis-derived factors, the increased release of inflammatory cytokines influences the development of insulin resistance as well [65,66]. Especially, TNF-α and IL-6 cause an upregulation of potential mediators of inflammation that contribute to insulin resistance.

Additionally, chronic inflammation in general is not only associated with hyperhomocysteinemia and folate deficiency, but also with insulin resistance [67]. Although the exact working mechanism is not unravelled yet, it is suggested that insulin resistance influences activity of key enzymes in the folate dependent one-carbon metabolism, including 5,10-methylenetetrahydrofolate reductase (MTHFR) and cystathione b-synthase (CBS) [68,69]. Furthermore, it has been demonstrated that insulin signaling is affected by high levels of homocysteine, which is a condition associated with obesity [70,71]. Insulin signaling is an essential process in glucose homeostasis, since it increases the uptake of glucose into muscle and fat cells and reduces the synthesis of glucose in the liver. GLUT4 is one of the most important insulin-regulated glucose transporters responsible for decreasing blood

glucose concentrations by facilitating glucose uptake into muscle and adipose tissue [72]. In the absence of insulin, the majority of GLUT4 is sequestered in intracellular vesicles in muscle and fat cells. When insulin levels increase, translocation of GLUT4 to the plasma membrane is induced and diffusion of circulating glucose down its concentration gradient into muscle and fat cells is facilitated. Homocysteine is one of the factors known to disrupt insulin signaling by impeding the GLUT4 translocation or recruitment on the plasma membrane and therefore reducing glucose uptake, which results in higher levels of glucose in the blood plasma [67].

4.5. Hyperglycaemia

Insulin resistance forces the pancreatic β-cells to produce more insulin to be able to prevent hyperglycaemia. However, when the compensatory insulin production is no longer sufficient, excessive amounts of glucose circulate in the blood plasma. This condition is referred to as hyperglycaemia, which is a defining characteristic of diabetes mellitus [73]. Besides maternal obesity, diabetes mellitus is a known risk factor for NTDs. Both obesity and diabetes mellitus are features of the metabolic syndrome [15]. The metabolic syndrome is further characterized by other metabolic risk factors including dyslipidemia, chronic hypertension, proinflammatory state, and prothrombotic state [74]. In the presence of 1 or 2 features of the metabolic syndrome, the fetus is on a 2-fold and 6-fold higher risk for NTD, respectively [75]. While the increased risk of NTDs associated with obesity appears to be independent of diabetes, a possible mechanism might be hyperglycemia due to insulin resistance in obese women [15].

Glucose levels are monitored and regulated by the islets of Langerhans in the pancreas and glucose is an essential factor for aerobic metabolism. Evidence suggests that the early developing embryo is dependent on maternal glucose metabolism, with detrimental effects in case of disbalance and hyperglycemia [76]. Thus, at the time of neural tube closure (around the fourth week of gestation), mothers with poorly regulated glucose levels are likely to have an suboptimal in utero environment, causing abnormal organogenesis [43,77,78]. To date, the exact working mechanism has not been elucidated yet. Only a few studies have reported evidence for this explanation, mostly focusing on the genetic susceptibility related to hyperglycemia as a risk factor for NTDs. Previous animal studies investigating molecular causes of NTDs in the embryos of diabetic mothers, demonstrated that in mouse embryos, expression of Pax3 is suppressed beginning on embryonic day 8.5 and subsequently, neuroepithelial cells undergo apoptosis and NTDs occur at increased frequency compared to embryos from nondiabetic pregnancies [79]. Moreover, in an embryos mouse model, which demonstrates a homozygous loss of function mutation in the Pax3 gene, NTDs can be rescued by either folic acid or thymidine supplementation [80,81]. This finding suggests that folic acid prevents NTDs by ensuring sufficient biosynthesis of factors for cell proliferation. Furthermore, a recent review of randomized controlled trials indicated that folic acid supplementation in non-pregnant populations, including women and men, had potential benefits on insulin resistance and glycemic control [82]. The mechanisms by which folic acid supplements lowers glucose levels and insulin resistance are still unclear. One of the suggested explanations is that hyperhomocysteinemia increases vascular oxidative stress, which could relate to insulin resistance and impaired insulin secretion during hyperglycemia [83,84]. As such, folate or folic acid supplements might decrease oxidative stress and, thereby, could prevent hyperglycemia and its detrimental effects.

4.6. Inositol

Inositol has been the focus of a large number of studies and is also involved in both folate uptake and glucose metabolism. Myo-inositol and D-chiro inositol are inositol isomers. Myo-inositol is the predominant form, which can be produced by the human body from D-glucose and is naturally present in foods, such as cereals, legumes, and meat [85]. Both isomeric forms of inositol were found to have insulin-like properties, acting as second messengers in the insulin intracellular pathway. Furthermore, both of

these molecules are involved in increasing insulin sensitivity of different tissues, and thereby, improving health outcomes associated with insulin resistant, such as diabetes mellitus and reproductive disorders [86–88]. A randomized controlled trial showed that myo-inositol supplementation, started in the first trimester, in obese pregnant women reduced the incidence of gestational diabetes mellitus in the myo-inositol group compared with the control group, 14.0% compared with 33.6%, respectively (p = 0.001; odds ratio 0.34, 95% confidence interval 0.17–0.68) [89]. This reduction was achieved by improving insulin sensitivity.

Besides the insulin-like properties, an animal study demonstrated that myo-inositol is capable of significantly reducing the incidence of spinal NTDs in curly tail mice, a genetic model of folate-resistant NTDs [90]. Furthermore, in humans, significantly lower inositol concentrations have been reported in the blood of mothers carrying NTD fetuses compared with normal pregnancies, and mothers with low blood levels of inositol showed a 2.6-fold increased risk of an affected offspring [91].

Moreover, inositol is suggested to have preventive effects on NTD occurrence in curly tail mutant mouse [90]. Protection against diabetes-induced NTDs has been observed as well in other rodent models [92]. Hence, the animal data support a distinct inositol-dependent metabolic pathway that, when stimulated, can prevent NTDs.

4.7. Role of the Gut Microbiome

The gut microbiome can directly influence the folate status and via the cofactors vitamin B12 en B2, which contribute to a relative folate deficiency. The gut microbiome is the entirety of microorganisms, bacteria, viruses, protozoa, and fungi, and their collective genetic material present in the gastrointestinal tract [93]. For this overview, we focus on the bacterial microbiome. Gut bacterial microbiota are involved in a variety of essential processes, including the fermentation of indigestible food components into absorbable metabolites, the synthesis of essential vitamins, such as folate and vitamin B12, the removal of toxic compounds, the strengthening of the intestinal barrier, and the stimulation and regulation of the immune system [94–96]. Diversity is of great importance to a healthy intestinal microbiome, since it ensures redundancy, with multiple microbes competent to perform similar functions [97]. An imbalance in microbial populations, called dysbiosis, is associated with several poor health outcomes, including, among others, inflammatory bowel disease, neurological diseases, and diabetes [98,99]. Moreover, there is increasing evidence, mainly from animal studies, that alterations in the intestinal microbiome lead to metabolic and weight changes in the host [100,101].

An animal study found in genetically obese mice a 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes [102]. Moreover, it is noted that changes affect the metabolic potential of the mouse gut microbiota. Previous research indicated that the obese microbiome has an increased capacity to harvest energy from the diet [101]. Furthermore, this trait is transmissible: colonization of germ-free mice with an 'obese microbiota' results in a significantly greater increase in total body fat than colonization with a 'lean microbiota'. Besides the role of the gut microbiota as a contributing factor to the pathophysiology of obesity, it is also recognized as a source of B vitamins, in particular of folate and vitamin B12. It is produced by the colonic microbiota, mainly as the monoglutamate form of folate, the form that is absorbed at the highest rate. Thus, intestinal bacteria are a source of folate [103]. Even though absorption of folate occurs primarily in the duodenum and upper jejunum, the colon represents another depot of folate potentially affecting the general folate status of the host.

Moreover, the composition of the intestinal microbiome contributes to the regulation of intestinal permeability [104]. Short-chain fatty acids have been suggested as a mediator via which intestinal microbiota might promote the integrity of the intestinal mucosa. A higher intestinal permeability has been associated with obesity, leading to a 'leaky gut' with suboptimal uptake of micronutrients [105]. Hypothetically, there might be a derangement in the absorption of folate as well.

5. Considerations for Advising Higher Doses of Folic Acid Supplements

Positive effects of folic acid supplement use on NTD birth prevalence rates in the general population are shown in doses ranging from 0.36 mg (NTD occurrences) to 4 mg (NTD recurrences) per day. However, after these randomized controlled trials, further investigation into an optimal dose for preventive effects could not be performed anymore due to ethical considerations [9,106–108].

The presence of unmetabolized folic acid, which accumulates in serum above doses of 0.2 mg per day, is generally regarded as a marker of dihydrofolate reductase (DHFR) saturation in its capacity to convert folic acid to tetrahydrofolate (THF) [109–112].

Various animal experiments showed that folic acid, especially when applied directly into the brain, possess powerful excitatory and convulsive properties by unknown mechanisms, although evidence suggests that unmetabolized folic acid might induce neurotoxicity [113–115].

An observational study reported an increased risk of impaired psychomotor development with the use of 5 mg of folic acid per day [116]. Daily intakes of 800 μg to 5 mg of folic acid from supplements have been associated with an increased risk of cancer development and mortality perinatally and later in life [117]. Since folate is an important methyldonor for periconceptional epigenetic programming, high doses of folic acid can induce variations in the epigenome of the offspring [34,118]. Until now, there is no conclusive evidence which dose of folic acid supplement use causes adverse effects in either the pregnant woman or the fetus [119].

There is only indirect evidence that obese women could benefit from an increased dose of folic acid in the prevention of NTDs in the offspring, as discussed in the previous sections. Hence, until the possible alterations in folate metabolism and corresponding requirements of folic acid supplement use in obese women are clarified, an increased folic acid supplementation dosage is only justified when harmful effects are ruled out.

6. Current Guidelines

In the previous sections, we described plausible folate-related pathways underlying the increased risk of NTDs in the offspring of obese women. No study has performed a trial where obese women are randomized to a high dosage versus a normal low dosage, and are followed-up until birth outcomes, including NTDs. As both a relative folic acid deficiency and insulin resistance are plausible mechanisms, direct evidence that an increased dosage of folic acid prevents NTDs in obese women is lacking [120]. Therefore, current guidelines are based on indirect evidence, which may explain the differences in these guidelines. British and Australian guidelines recommend 5 mg/day of folic acid in obese women, while American and Canadian guidelines do not mention special recommendations for folic acid supplement use in obese women [16,17]. These differences in recommended folic acid supplement use for obese women might be related to national folic acid food fortification programs. In the United States and Canada, folic acid fortification of most cereal grains is mandatory, while in the United Kingdom and Australia, this is only applied to wheat flour. New guidelines should not only be based on substantial scientific evidence. Local or national circumstances or customs, such as folic acid food fortification programs, should also be taken into account.

7. Recommendations

7.1. Recommendations for Practice

Although there is insufficient evidence that it is effective and safe to increase the recommended dose of folic acid supplement use for obese (pre)pregnant women in the prevention of neural tube defects, we formulated the following recommendations for clinical practice to improve absolute folate deficiency, either through supplement use or dietary intake:

- Be aware of a suboptimal absolute folate intake in obese women, both as a result of a lack of compliance to folic acid supplement use as well as of a relative malnutrition due

to a folate deficient diet, as discussed in Section 2. More than half of pregnant women reported to start using folic acid supplements after a positive pregnancy test, which is on average after 5.5 weeks of gestation [121,122]. Since the closing of the neural tube occurs between week 4 and 6 of pregnancy, the majority of pregnant women start using folic acid supplements too late for the prevention of NTDs (Figure 3). Therefore, the preconception period is the window of opportunity to determine and treat folate deficiency or hyperhomocysteinemia in women with obesity and provide lifestyle counseling to improve dietary folate intake and stimulate weight loss [123]. Additionally, parameters of chronic inflammation and glucose metabolism could be measured as a risk analysis. Face-to-face lifestyle counseling could be combined with an online program, for example the evidence-based eHealth platform 'Smarter Pregnancy'. This eHealth intervention showed improvements in lifestyle behaviors, including folic acid supplement use and nutritional intake, in the total study population as well as in the subgroup of overweight and obese women [124]. Since unplanned pregnancies and failed contraceptive methods are prevalent in obese women, this group is less likely to attend preconception care. As presented in Figure 3, folic acid supplement use in general should start before conception to have its full potential. Therefore, the general practitioner could inform women, independent of their BMI, who, for example, stop taking their contraceptives.

- Obese women can be monitored by assessment of serum folate and red blood cell folate during the periconceptional period, as well as plasma total homocysteine status. Based on these parameters, folate status, one-carbon metabolism, and related pathways can be improved by supplements or lifestyle counseling, the latter being preferred because of no concerns about safety.
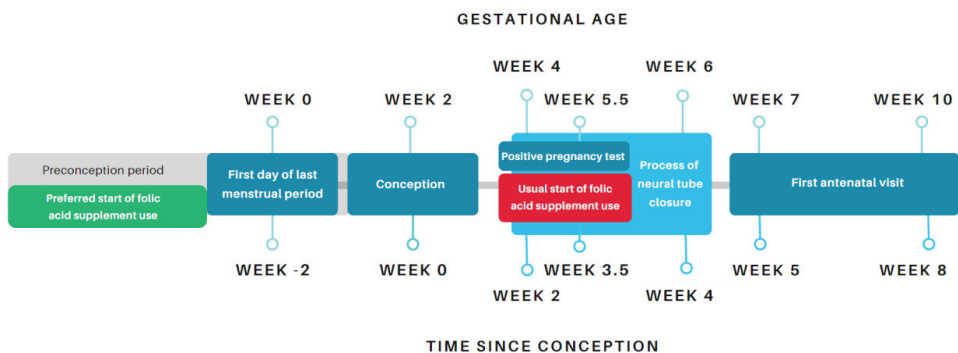
Figure 3. Illustration of the gap between recommended period of folic acid supplement use, and window of opportunity for the health care provider to advice on folic acid supplement use.

7.2. Recommendations for Future Research

- A preconceptional initiated intervention study to explore the etiology of insulin resistance and chronic inflammation in obese women and the effects of increased folic acid supplement use.
- Modification of the intestinal microbiota to maintain intestinal permeability and adequate uptake and production of essential nutrients is worth further research.
- Further research should focus on the implementation of interventions to target absolute folate deficiencies. Lifestyle programs have the potential to increase dietary folate intake, folic acid supplement use, and overall lifestyle improvement among obese women [124]. Wide implementation and evaluation of such interventions could provide a powerful preventive measure.

8. Conclusions

Women with obesity are at an increased risk of NTDs in their offspring and there is substantial evidence that folate deficiency plays a significant role. However, clinical trials to show the optimal dose of folic acid supplement use are lacking. Scientific evidence of the involvement of several folate-related pathways implies to increase the recommended folic acid supplement use in obese women. However, the physiological uptake of synthetic folic acid is limited and side-effects in mothers and offspring, in particular variations in epigenetic (re)programming with long-term health effects, cannot be excluded. Therefore, we emphasize the urgent need for preconception personalized counseling on folate status, lifestyle and medical conditions, in particular for women with obesity. Targets for further research to substantiate folic acid recommendations in women with obesity are directed towards homocysteine, glycemic control, and the microbiome. We recommend that folic acid supplement use guidelines should be reconsidered when more scientific evidence is available.

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Review

The Impact of Maternal Body Composition and Dietary Fat Consumption upon Placental Lipid Processing and Offspring Metabolic Health

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Abstract: The proportion of women of reproductive age who are overweight or obese is increasing globally. Gestational obesity is strongly associated in both human studies and animal models with early-onset development of adult-associated metabolic diseases including metabolic syndrome in the exposed offspring. However, animal model studies have suggested that gestational diet in obese pregnancies is an independent but underappreciated mediator of offspring risk for later life metabolic disease, and human diet consumption data have highlighted that many women do not follow nutritional guidelines prior to and during pregnancy. Thus, this review will highlight how maternal diet independent from maternal body composition impacts the risk for later-life metabolic disease in obesity-exposed offspring. A poor maternal diet, in combination with the obese metabolic state, are understood to facilitate pathological in utero programming, specifically through changes in lipid handling processes in the villous trophoblast layer of the placenta that promote an environment associated with the development of metabolic disease in the offspring. This review will additionally highlight how maternal obesity modulates villous trophoblast lipid processing functions including fatty acid transport, esterification and beta-oxidation. Further, this review will discuss how altering maternal gestational diet may ameliorate these functional changes in lipid metabolic processes in the obese placenta.

1. Introduction

Throughout the gestational period, maternal nutrient handling must adapt to the increasing needs of the growing fetal-placental unit to ensure developmental processes continue in a healthy and physiological manner. For example, maternal insulin sensitivity diminishes, and fasting serum lipid levels rise late in gestation to preserve necessary macronutrients for trans-placental transport into fetal circulation [1–3]. However, there is a fine balance within these physiological metabolic alterations that, when disrupted by environmental influences, can shift the course of in utero programming to promote the early life development of metabolic disorders in the offspring. Maternal gestational obesity is one such environmental influence that has been well associated with poor health outcomes in exposed offspring. Importantly, recent animal models have highlighted that, in addition to maternal obesity, a maternal diet high in fat is an important independent regulator of offspring lifelong metabolic

health. Thus, this review will primarily discuss how maternal gestational dietary composition in obese pregnancies influences future offspring health independent from maternal body composition.

Furthermore, alterations in lipid processing functions of the placenta—including fatty acid (FA) transport, lipid esterification and FA beta-oxidation—have been thought to modulate materno-fetal lipid transport and the resulting changes to fetal lipid exposures may underlie metabolic disease programming. This review will additionally highlight how maternal obesity modulates these lipid handling processes in the placenta and discuss how maternal diet may program these placental processes independently from increased maternal adiposity.

2. Maternal Obesity and Offspring Metabolic Health

The study of the impacts of maternal gestational environment on fetal growth and development is encompassed within the field of research known as The Developmental Origins of Health and Disease (DOHaD) [4,5]. This field of study evolved from the observations of Anders Forsdahl and David Barker in the 1970s and 80s whereby Forsdahl originally described an increased risk of death by coronary heart disease in those who were relatively impoverished during childhood, but later experienced prosperity [6]. Barker expanded these observations to include gestational influences and reported that low birthweight babies were at a greater risk for developing metabolic complications such as obesity, type 2 diabetes mellitus (insulin resistance) and metabolic syndrome in adulthood [4,5]. This field of study has since expanded to include the observed increased risk of later life non-communicable diseases associated with metabolic syndrome in offspring born in an environment of maternal diet-induced obesity [7,8].

The World Health Organization (WHO) categorizes healthy bodyweight in both adults and children via body mass index (BMI, kg/m^2), whereby a BMI > 25 is overweight and a BMI > 30 is obese [9]. The effects of an increased maternal body mass and associated adiposity during the gestational period on offspring later life health has been extensively documented in humans via population studies and meta-analyses [10–15]. In line with the DOHaD concept, obesity-exposed offspring have been found to be at a greater risk for later-life metabolic health issues due in part to an increased prevalence of having a birthweight that is not appropriate for their gestational age (AGA) [10,13]. While maternal gestational obesity has largely been associated with infants being born Large for their Gestational Age (LGA), there has also been a link between maternal obesity and greater risk of the offspring being born Small for their Gestational Age (SGA) [10,11,14]. Independent from maternal factors, LGA and SGA offspring are at an increased risk for developing non-communicable "adult-associated" metabolic disorders as early as four years of age [12,13]. Concerningly, however, there are reports that children born to obese women are more likely to develop metabolic disorders regardless of their birthweight, suggesting that maternal body composition during pregnancy influences offspring metabolic health simply beyond alterations in birthweight [14]. Indeed, recent studies have suggested that maternal factors including pre-pregnancy BMI may better predict the development of offspring health complications than birthweight alone [14,15].

The negative influence that maternal adiposity has on offspring metabolic health has additionally been reported in numerous animal models that attempt to elucidate the mechanisms that lead to early-life metabolic diseases in obesity-exposed offspring [16,17]. While maternal diet-induced obesity has been well associated with poor fetal metabolic outcomes in these models, it is important to note that variations are present in the dietary fat contents and periods of exposure used in these studies (Table 1). Rodent models in particular have been heavily utilized and the development of metabolic disorders in the offspring born to high-fat diet (HFD)-induced obese dams has been described the result of pathological in utero programming [18,19]. The high-fat-exposed rodent offspring have been found to exhibit an abnormal lipid profiles including hepatic steatosis that ultimately leads to Non-Alcoholic Fatty Liver Disease (NAFLD) and fibrosis at early life stages [20]. Altered glucose homeostasis is also prevalent in these obesity-exposed rodent offspring and is manifested as insulin resistance and an eventual development of type 2 diabetes mellitus (T2DM) during adolescence [21,22]. The altered

glucose and liver lipid metabolism observed in these offspring has been thought to be a precursor to the ultimate development of metabolic syndrome in gestational obesity-exposed adolescents [23,24].

Larger mammal species, including sheep, have also been used to study maternal overfeeding and obesity and its subsequent effects on offspring health and disease. As observed in human meta-analyses and rodent experiments, sheep offspring exhibit metabolic dysfunction both neonatally and into adulthood —including increased prevalence of obesity and aberrant lipid and glucose metabolism—in response to maternal obesity during gestation [25–27]. Additionally, the non-human primate (NHP) model has been well utilized and describes dysregulated fetal hepatic lipid and glucose metabolism as an underlying pathology of maternal obesity mediated offspring metabolic disease development [28,29].

Together, these human meta-analyses and animal models demonstrate that maternal obesity during the gestational period primes the exposed offspring for dysregulated lipid and glucose metabolism that ultimately results in metabolic disease development early in life.

3. Is Maternal BMI an Accurate Predictor of Offspring Metabolic Health?

The reports from these human and animal studies that link maternal obesity to offspring metabolic disease are of increasing importance to healthcare systems as the prevalence of obesity worldwide has reached unprecedented rates over the last several decades [30]. The WHO estimates that about 40% of men and women over the age of 18 were overweight or obese in 2016, and that proportion continues to rise [30]. More specific to pregnancy outcomes and in line with data from most industrialized nations, Health Canada reported in 2012–13 that 24% of Canadian women between 20–39 years of age (child-bearing age) were obese, and 44% had a waist circumference that was predictive of high risk for the development of health complications [31]. These reports suggest that the prevalence of early-onset metabolic syndrome in offspring will only continue to increase alongside the rising rates of maternal obesity.

Recent animal models utilizing dietary interventions in obese pregnancies have highlighted that body composition metrics may not be the most accurate predictors of offspring future metabolic health and that maternal gestational diet is an important influence (Table 1). For example, in sheep models of gestational overfeeding-induced obesity a maternal dietary intervention early in gestation resulted in lowered circulating plasma triglyceride levels (improved lipid metabolic function) as well as decreased plasma insulin levels (improved glucose metabolism) in fetuses from obese pregnancies at both mid and late gestation [27]. Additionally, NHP data suggest that there are vast differences in the metabolic health of fetuses from obese mothers that consume different diets during gestation [28,29,32]. McCurdy et al. (2009) identified that a diet reversal to a control diet in obese pregnant Japanese macaques was sufficient to improve liver steatosis in third trimester fetuses, suggestive of a decreased risk of postnatal NAFLD. Subsequent studies described reductions in maternal and fetal dyslipidemia and oxidative stress in diet-reversed obese pregnancies leading to benefits in fetal liver development during the third trimester [32]. Additionally, improved third trimester pancreatic islet vascularization has been reported and highlights that these offspring would be less susceptible to later-life development of type 2 diabetes mellitus [29]. These NHP studies highlight that maternal gestational obesity alone may not best predict offspring metabolic health and suggest that gestational diet is important in determining metabolic health risk in the obesity-exposed offspring.

Rodent models of obese pregnancy have also demonstrated the benefits of gestational diet reversals (Table 1). For example, the male offspring of obese rats given a dietary intervention during the gestational period have been found to have improved metabolic outcomes including improved insulin sensitivity both neonatally and into adulthood [33]. However, additional rodent studies highlight that a diet-reversal during pregnancy may not be sufficient to reverse the effects of maternal pre-pregnancy obesity, as observed in sheep and NHP models. For example, mouse embryos transferred at the 2-cell stage from high-fat-fed dams to control fed dams displayed poor in utero growth and neonatal catch-up growth, as well as an altered expression of imprinted genes that have been associated with obesity development suggesting that oocytes may be primed for adverse development as a direct result of poor maternal diet pre-conception [34]. These findings are supported by other rodent models that report poor liver and skeletal muscle mitochondrial health at post-natal day 35 in offspring exposed to maternal pre-pregnancy obesity [35,36]. Specifically, hepatic tissue of rat offspring born to obese dams displayed a marked decrease in the protein expression of markers of mitochondrial health and biogenesis despite both control and obese dams being fed a control diet during the gestational period [36].

The presence of the conflicting data between rodent and larger mammal (sheep and NHP) models may simply arise from physiological differences between these species. For example, the longer gestational period of sheep and NHP, and the fact that these species, like humans, have largely prenatal developmental processes potentially underlies the differential impacts of a gestational diet reversal intervention on fetal growth and development [37,38]. Further studies must be conducted to fully understand whether dietary changes during human pregnancy are sufficient to reverse insults from a poor maternal diet as in the NHP model and some rodent models or if human oocytes are 'primed' for metabolic disease with pre-gestational obesity exposure. Overall, these NHP and rodent studies demonstrate that maternal diet prior to conception and during pregnancy has a profound impact of the metabolic health of the offspring.

Nutrients **2020**, 12, 3031

Table 1. Summary of diet fat or feeding treatments utilized in animal models of maternal diet-induced gestational obesity and gestational high-fat exposure with and without diet reversal.

Animal Model	Dietary Fat (% Caloric Intake)	Pre-Gestational Obesity	Pre-Conception Diet Exposure	Gestational Diet Exposure	Maternal Diet Reversal	Offspring Weaning	Reference
C57/B6 mice	60% High fat diet (HFD) 25% fat control diet	HFD-induced obesity	10–12-week HFD exposure before pregnancy	HFD maintained through pregnancy and lactation	Yes—2-cell stage embryo transfer	Weaned onto control diet	Sasson [34]
C57/B6 mice	45% HFD 10% fat control diet	HFD-induced obesity	Diet commenced at 4 weeks; breeding at 10 weeks	HFD through pregnancy	No	Randomly assigned HFD or control diet	Elahi [20]
C57/BL6 mice	32% HFD 11% fat control diet	HFD-induced obesity	8-week pre-conception HFD-exposure	HFD through pregnancy	No	Fetal collections	Jones [17]
C57/B6 mice	16% HFD control diet 3% fat	Diet-induced obesity	6-week diet exposure pre-conception	HFD maintained through pregnancy and weaning	No	Pups weaned onto standard chow	Samuelsson [22]
C57/B6 mice	High trans-fat diet (6% partially hydrogenated vegetable oil + 1% soybean oil) 7% soybean oil control diet	No pre-pregnancy obesity	No HFD exposure pre-conception	High trans-fat diet through pregnancy and weaning only	No	Weaned onto control diet	de Velasco [39]
Sprague-Dawley Rats	60% HFD 24% fat control diet	HFD-induced obesity	HFD commenced Postnatal day (PND) 24; breeding PDN 120	HFD throughout pregnancy	No	Weaned onto control diet	Srinivasan [18]
Sprague-Dawley Rats	140% overfeeding model	Overfeeding-induced obesity	3-week overfeeding prior to conception	Overfeeding discontinued during pregnancy	Yes—dams switched to control feeding through pregnancy and lactation	Randomly weaned onto control (17% fat) or HFD (45% fat)	Borengasser [35]
Sprague-Dawley Rats	140% overfeeding model	Overfeeding-induced obesity	3-week overfeeding prior to conception	Overfeeding discontinued during pregnancy	Yes—dams switched to control feeding through pregnancy and lactation	Randomly weaned onto control (17% fat) or HFD (45% fat)	Borengasser [36]
Wistar Rats	45% HFD 18% fat control diet	HFD-induced obesity with pre-gestational HFD exposure	Pre-conception HFD—commenced PND 22; breeding at PND 120 Pregnancy and lactation HFD—commenced at breeding and maintained through lactation)	HFD through pregnancy	No	Randomly assigned HFD or control diet	Howie [21]
Wistar Rats	38% HFD-diets 15% fat control diet	No pre-pregnancy obesity	No HFD exposure pre-conception	HFD during pregnancy only; cross-fostered to lean dams during lactation	No	Weaned onto control diet; HFD exposure at 8 weeks	Dong [40]

Table 1. *Cont.*

Animal Model	Dietary Fat (% Caloric Intake)	Pre-Gestational Obesity	Pre-Conception Diet Exposure	Gestational Diet Exposure	Maternal Diet Reversal	Offspring Weaning	Reference
Wistar Rats	20% lard supplement in HFD 5% fat control diet	HFD-induced obesity	HFD exposure from PND 21 to breeding at PND 120	HFD maintained through pregnancy and lactation	Yes—diet intervention back to control diet at PND 90	Not specified	Zambrano [33]
Sheep	155% overfeeding model	No pre-gestational obesity	Overfeeding commenced gestational day 115	Overfeeding from gestational day 115 to gestation (~day 150)	No	Control diet during lactation and weaning	Philip [26]
Sheep	150% overfeeding model	Overfeeding-induced obesity	60-day overfeeding exposure before mating	Overfeeding through gestation, control diet during lactation	No	control diet	Long [25]
Sheep	150% overfeeding model	Overfeeding-induced obesity	60-day overfeeding exposure before mating	Overfeeding until fetal collection	No	Fetal collection	Zhu [41]
Sheep	150% overfeeding model	Overfeeding-induced obesity	60-day overfeeding exposure before mating	Overfeeding continued through pregnancy (with no intervention)	Yes—150% overfeeding until gestational day 28 (with obesity intervention)	Fetal collection	Tuersunjiang [27]
Japanese Macaque	36% HFD 14% fat control diet	HFD-induced obesity	4–7-year HFD exposure pre-conception	HFD maintained through to fetal collections at gestational day 130	Yes—diet reversal 3 months prior to breeding	Fetal collection	Salati [42]
Japanese Macaque	32% HFD 14% fat control diet	HFD-induced obesity	2–4-year pre-gestational HFD induced obesity	HFD, or diet-reversal through pregnancy	Yes—pre-conception diet reversal on subsequent pregnancy	Weaned onto mothers gestational diet	McCurdy [28]
Japanese Macaque	32% HFD 14% fat control diet	HFD-induced obesity	4–7-year pre-gestational HFD exposure	HFD, or diet reversal through pregnancy	Yes—switched back to control diet in 5th breeding season	Weaned onto in utero or reverse diet	Pound [29]
Japanese Macaque	32% HFD 14% fat control diet	HFD-induced obesity	2–9-year pre-conception HFD exposure	HFD, or diet reversal through pregnancy	Yes—switched back to control diet in 9th breeding season	Fetal collections	Wesolowski [32]

4. Maternal Dietary Fat Consumption and Offspring Metabolic Health

Human population data have suggested that circulating maternal free fatty acids levels are predictive of offspring metabolic health risks independent from measures of maternal body composition, highlighting the importance of dietary lipids during gestation [43]. Additionally, in animal-based studies, dietary fat components are altered in obese pregnancy dietary interventions further highlighting that fats themselves are important in promoting the development of metabolic disorders in exposed offspring.

Different FA species have varying impacts on metabolic health based on the length of the FA chain (short-, medium-, long or very long-chain FA) as well as on the degree of saturation of the FA [44]. For example, a diet rich in cis-monounsaturated FA species (MUFAs) and polyunsaturated fats (PUFAs) has been associated with increased levels of High-Density Lipoprotein (HDL), the "good cholesterol", and thus a healthier lipid metabolic profile [45]. More importantly, omega-3 PUFAs have also been linked to improvements in metabolic health and function and may be an important factor in preventing insulin resistance and type 2 diabetes in obese populations [46,47]. In contrast, a high consumption of trans-unsaturated FA species has been found to lower serum levels of HDL and promote a less healthy metabolic profile [45]. Additionally, a high consumption of saturated FA species has been associated with poor metabolic profiles including increased serum levels of triglycerides, free cholesterol and low-density lipoprotein (LDL), the "bad cholesterol" [48].

More importantly, consumption of certain FA species during pregnancy has been suggested to promote the development of metabolic disorders in the offspring. For example, studies in rodent model systems have highlighted that maternal diets comprised of different saturated FA chain lengths have varying impacts on offspring later-life metabolic health [40]. Specifically, gestational diets that were overabundant in medium chain length FA species from coconut oil (55% of FA species C14:0 or shorter) resulted in decreased offspring obesity development compared to offspring exposed to a maternal overconsumption of longer-chain FA species from soybean oil (all FA C16:0 or longer) [40]. Additional rodent models have demonstrated that maternal diets rich in trans-unsaturated FA species adversely affect offspring liver mitochondrial oxidative function, as well as increase circulating levels of triglycerides, highlighting an overall dysregulation of hepatic lipid handling [39]. These studies further highlight that maternal dietary fats are an important independent factor in offspring risk for metabolic disease development.

To determine the impact of maternal dietary fat content upon fetal health outcomes in human populations, it is important to fully understand the diet consumption patterns of pregnant women. More importantly, it is necessary to understand how these maternal diets deviate from the recommendations of government health agencies to provide insight into possible dietary interventions that can reduce offspring metabolic health complications. Canada's food guide for example, recommends that pregnant women only consume a small amount (1–3 tbsp) of saturated fat each day. In addition to limiting saturated fat intake, it is also suggested that these less healthy FAs should be replaced with more omega-3 and -6 PUFAs. Specifically, for pregnant women, Health Canada guidelines suggest consumption of at least 200 mg of Docosahexaenoic acid (DHA) (an omega-3 PUFA), as this FA is necessary for proper fetal brain development [49]. However, despite these guidelines, analysis of dietary consumption patterns suggests that a majority of pregnant women consume diets that greatly deviate from food guide recommendations [50]. It is estimated that, on average, one-third of total caloric intake in pregnant women is from lipid sources, and while this total fat intake does not always exceed recommendations, the specific FAs that constitute total lipid intake in these women is not ideal [50–52]. Specifically, these women have been found to consume diets that are calorie-dense but low in nutrients, overabundant in long-chain saturated FA and lacking in important unsaturated FA species such as DHA [52–54].

Overall, an increased maternal consumption of saturated FA and limited intake of omega-3 PUFAs during pregnancy may be an important in utero insult that predisposes the offspring to metabolic complications early in life.

5. The Impact of Diet and Obesity upon the Placenta

The placenta is a transient organ composed of a heterogeneous population of cells that facilitates hormone production, fetal immunity and all gaseous, nutrient and waste transport between maternal and fetal circulation. It consists of two distinct but important populations of trophoblast cells, extravillous trophoblasts (EVTs) and villous trophoblasts that arise from the outer trophectoderm layer of the pre-implantation blastocyst. EVTs invade into the uterine wall to establish the maternofetal blood connection and anchor chorionic villi to the uterine wall, while the villous trophoblast cells of the chorionic villi act as a transport layer and comprise the barrier between maternal and fetal blood supplies. The villous trophoblast layer is comprised of two unique cell population: underlying progenitor cytotrophoblast (CT) cells and fused multi-nucleated syncytiotrophoblast (SCT) cells [55].

The CT and SCT cells of the villous trophoblast layer have been identified as the most metabolically active within the placenta, and importantly maternal gestational obesity has also been identified to negatively impact these cells [55–59]. Specifically, maternal obesity is often associated with increased inflammation in placental tissues highlighted by increased pro-inflammatory cytokine abundance and macrophage accumulation that can be detected as early as midgestation [41,60,61]. Additionally, maternal gestational obesity has been linked with a decreased expression of markers of mitochondrial replication, and an overall reduction in electron transport chain activity (oxidative function) leading to reduced placental ATP levels [36,56,62]. Impairments in placental functional processes are thought underlie the aberrant fetal programming that primes obesity-exposed offspring for metabolic dysfunction and ultimately metabolic disease early in life [63]. For example, NHP models have demonstrated reduced placental vascular function and increased placental inflammation with maternal obesity that can be improved with maternal diet reversal [42]. In turn, these diet reversal-induced improvements in placental function may underlie the previously observed alterations to offspring lipid and glucose metabolism [28,29,32,42]. Understanding specifically how maternal dietary fat consumption may modulate placental lipid processing functions—including lipid transport, esterification and oxidation—and what these changes mean for the developing fetus, will provide a better understanding of the mechanisms underlying early-onset metabolic disease.

In vitro cell-based analysis of the placentamay allow for such insight into the effects of maternal dietary intervention onlipid processing functions For example, CT cells have been cultured from term human placentae following planned, non-laboring Caesarian-section births and utilized to examine placental metabolic function in obese pregnancies with and without a dietary intervention [64,65]. The isolated effects of individual lipid species on placental lipid processes, independent from maternal body composition and maternal gestational diet can also be examined through the use of immortalized villous trophoblast cell lines that are available for commercial purchase. One such cell line is the BeWo cell line, which has been demonstrated as a model of placental barrier function and has been extensively utilized to examine the isolated effects that individual PUFA species have on placental lipid transport [66,67].

6. Regulation of Placental Lipid Transport in Obesity and the Impact of Dietary Fats

The human placenta has an extensive ability to uptake lipid species and shuttle them and their metabolic byproducts into fetal circulation. Proteomic analysis of term primary human trophoblast (PHTs) has revealed that the placenta expresses lipid transport proteins on both the apical microvillous (maternal-facing) and basolateral (fetal-facing) membranes [68]. Specifically, Fatty Acid Transport Proteins 1, 2 and 4 (FATP1, FATP2, FATP4); Fatty Acid Binding proteins 1 and 3 (FABP1, FABP3) as well as Fatty Acid Translocase (FAT/CD36) are expressed in the human placenta [68–71]. In addition, isolated PHTs have demonstrated activity of Lipoprotein Lipase (LPL) indicating that lipid species packaged as triglycerides in lipoproteins (HDL and LDL) can be processedby the placenta [72,73].

The FATPs as well as FAT/CD36 are localized on both the basolateral and apical placental membranes and are involved in transporting a wide range of FA species across the placenta [68,74]. The presence of these transporters on both membranes suggests a bidirectional transfer of NEFAs

can occur to respond to the changing nutrient demands of both mother and developing fetus [68,74]. In contrast, FABP transporters that demonstrate preferential binding for PUFA species are largely localized to the maternal-facing apical membranes of the placenta [41,64]. This suggests that PUFA species are transported unidirectionally across the placenta into the fetal circulation in order to support and prioritize proper fetal brain development [68,75]. Similar to PHTs, the BeWo cell line has demonstrated the ability to uptake and transport dietary NEFAs [76]. Specifically, this cell line has been shown to express the lipid transporters: FATP1, FATP4, FAT/CD36 as well as FABP1 and FABP3 [76,77]. As BeWo cells express the same lipid transport proteins as PHTs, they may represent a viable model for studying placental barrier function and lipid transport, although caution must be taken with interpretation of data from immortalized cell lines.

Maternal obesity during pregnancy has been associated with an altered expression and activity of the lipid transporters in the placenta. Specifically, an increase in the activity of LPL and mRNA expression of FAT/CD36 in conjunction with diminished mRNA levels of FATP1, FATP4 and FABP3 as well as reduced protein expression of FABP3 have been observed with increased maternal adiposity [72,73] (Figure 1). The observed increases in the activity and expression of placental LPL and FAT/CD36 may facilitate increased lipid transport into fetal circulation and could potentially explain the increased prevalence of LGA offspring in obese pregnancies. In contrast, the specific reduction in the expression of FATP and FABP transporters may simply reflect that the placenta is attempting to modulate lipid transportto the developing fetus under conditions of lipid overload. The notion that the placenta is able to modulate materno-fetal lipid transport in response to nutritional state is supported by recent NHP experiments that identified increased protein expression of FATP and FABP transporters under conditions of maternal nutrient restriction [78].

The relative influences that individual dietary FAs have on obesity-mediated altered placenta lipid transport must be understood to predict how maternal diet interventions may impact fetal metabolic disease. While almost one-third of the total lipid consumption of pregnant women is saturated fats, current research into the effects of individual NEFA supplementation on placental lipid transport has largely emphasized the effects of dietary PUFAs. Cell culture experiments conducted with the BeWo cell line have found that a 24-h exposure to 100-µM concentrations of individual unsaturated NEFAs (Oleate, DHA, and Arachidonic Acid (AA)) has no influence on placental FATP expression [76]. Similarly, there were no significant alterations in PHT FATP expression from women who took DHA supplements during the third trimester [79]. PUFAs may in contrast, have an ability to alter the expression of FABP transporters within the placenta and specifically AA has been found to increase the expression of FABP3 in BeWo cells following after 24 h in culture [77] (Figure 1). These specific increases in the expression of FABP3 in AA-treated BeWo cells may simply be reflective of the preferential transport of PUFA species by placental FABPs, [41,64].

Future placental research must increasingly focus on the effects of dietary saturated fats to elucidate if a maternal saturated fat overconsumption independent of body composition leads to increased materno-fetal lipid transport via LPL and FAT/CD36 mediated transport. Furthermore, understanding the molecular mechanisms that potentially regulate this increased materno-fetal lipid transport could lead to the development of pharmacological inhibitors to better modulate in utero growth.
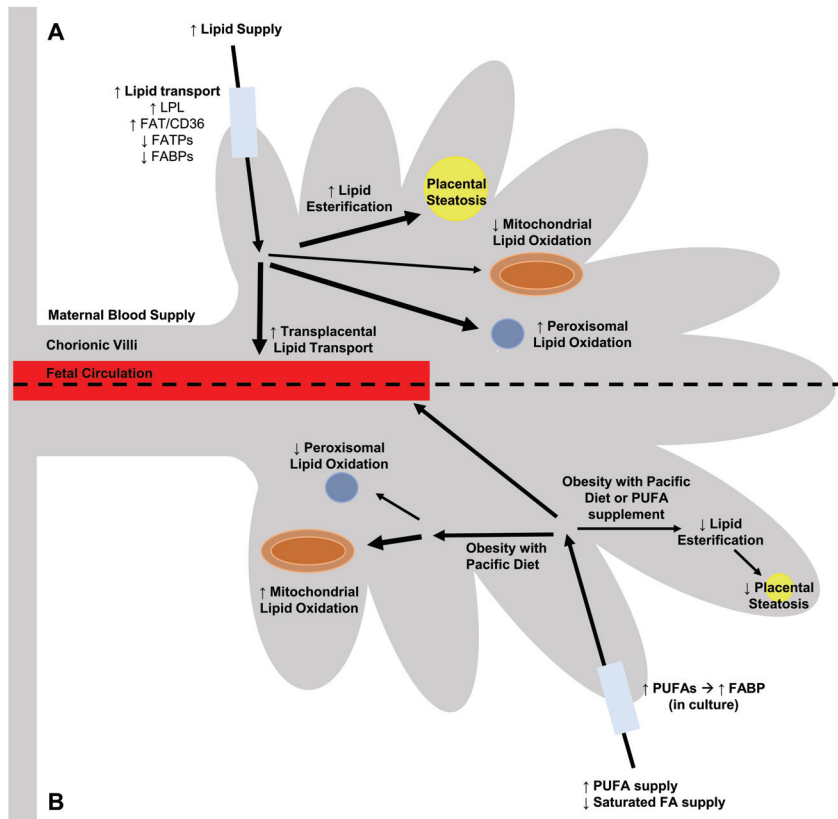
Figure 1. Summary description of alterations to the placental lipid processing functions of fatty acid (FA) transport, esterification and beta-oxidation under conditions of (**A**) maternal obesity and (**B**) with maternal diet improvement. Maternal gestational obesity has been associated with increased (↑) transplacental lipid transport (highlighted by increased expression of lipoprotein lipase (LPL) and fatty acid translocase (FAT/CD36) as well as decreased (↓) expression of fatty acid transport proteins (FATP) and fatty acid binding proteins (FABP)), increased placental lipid esterification and lipid droplet formation as well as decreased placental mitochondrial beta-oxidation with concomitant increased peroxisomal beta-oxidation. These changes are understood to be important in utero insults that program the development of early-life metabolic disease in the offspring from obesity-exposed pregnancies. Improved maternal diet under conditions of obesity, such as with consumption of a 'pacific diet' or use of dietary polyunsaturated FA (PUFA) supplements, have been associated with reduced placental steatosis and improved placental beta-oxidative function (increased mitochondrial beta-oxidation with simultaneous decreased peroxisomal beta-oxidation).

7. Obesity, Diet and Placental Lipid Accumulation

The villous trophoblast cells of the placenta not only have the capability to uptake and transfer NEFAs from maternal circulation to the fetus, but also to store them as lipid droplets for future metabolic needs [80–82]. Analysis of the activity of FA transport proteins on placental membranes has indicated that placental lipid uptake is greater on maternal-facing membranes than on fetal-facing membranes, highlighting that placental lipid storage and/or metabolism is an important aspect of placental lipid processing [82]. More recently, CT cells were demonstrated to be the sole location of lipid esterification in cultured PHTs following treatment with fluorescent-conjugated FA derivatives [83].

This suggests that the CT cells of the villous trophoblast layer may be more important than SCT cells for lipid metabolic function in the placenta and may be a potential target of future pharmacological therapies [83].

Maternal gestational obesity has been well demonstrated to alter placental lipid storage resulting in a pathological accumulation of lipid droplets (steatosis) at term, suggesting that placental lipid droplets may be a mechanism by which the placenta modulates FA transfer to the fetus [82,84–87] (Figure 1). Analysis of the composition of these lipid droplets has demonstrated that saturated FAs and MUFAs are the predominate lipid species that are stored in obese placentae, [88]. The increase in lipid esterification and lipid droplet formation in obese placentae is potentially the result of increased formation of MUFA species via Stearoyl-CoA Desaturase (SCD-1) [85]. SCD-1 is an enzyme that is overexpressed within the obese placenta and converts the saturated FAs palmitate (16:0) and stearate (18:0) into less the lipotoxic MUFAs palmitoleate (16:1n7) and oleate (18:1n9), respectively [89]. The formation of MUFA species via SCD-1 has been previously been identified as a precursor step in the activation of WNT signaling proteins via palmitoylation [90]. More importantly, increased activity of WNT signaling proteins is involved in the pathology of placental steatosis in obesity-prone rats [91].

Maternal dietary supplementation with omega-3 PUFAs alone has been demonstrated to decrease placental lipid accumulation at term in obese pregnancies [86] (Figure 1). In addition, human population data have demonstrated that obese women from pacific regions such as Hawaii who naturally consume greater levels of omega-3-rich fatty foods, such as fish, have less severe placental steatosis than obese women from landlocked areas such Ohio who consume diets less plentiful in omega-3 fats [85,92] (Figure 1). These studies further highlight that maternal diet is an important regulator of placental lipid processing independent from maternal body composition. However, as previously stated, lipid esterification is also an important regulator of transplacental lipid transport. Thus, an improvement in placental steatosis with omega-3 PUFA supplements without correcting an underlying maternal overconsumption of saturated fats may be harmful to the fetus through increased transplacental lipid transport. In fact, there may be an increased risk that offspring are born LGA in pregnancies that are supplemented with omega-3 PUFA, which itself may promote the development of later life metabolic disease [93,94]. Overall, a simple dietary supplementation may not be sufficient to improve adverse fetal outcomes, and a more rigorous dietary intervention may be needed in women who overconsume saturated fats.

8. Diet and Placental Lipid Oxidation and Acylcarnitine Production in the Obese Environment

The dietary FA that are transported into the villous trophoblast cells from maternal circulation can additionally be metabolized via mitochondrial beta-oxidation to produce ATP necessary for the placenta to perform its biological functions. In brief, mitochondrial beta-oxidation occurs through 4 enzymatic steps in which the carbon backbone of the FA species is shortened to produce acetyl-CoA that can enter The Citric Acid Cycle.

Immunohistological staining of isolated placental cells and western blot protein analysis of term and early gestation human placental explants has revealed that villous trophoblast cells express enzyme isoforms for all enzymatic steps in the mitochondrial beta-oxidation pathway. Both SCT and CT cells are found to express the Acyl-CoA dehydrogenase isoforms very-long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), and medium-chain acyl-CoA dehydrogenase (MCAD); enolyl-CoA hydratase; the 3-hydroxyacyl-CoA dehydrogenase enzyme isofroms short-chain L-3 hydroxyacyl-CoA dehydrogenase (SCHAD) and long-chain L-3 hydroxyacyl-CoA dehydrogenase (LCHAD); as well as the 3-ketoacyl-CoA thiolase enzyme isoforms long-chain 3-ketoacyl-CoA thiolase (LKAT) and short-chain 3-ketoacyl-CoA thiolase (SKAT) [95–97]. It is of particular interest to note that the expression levels of these beta-oxidation enzymes within placental explants is similar to that of skeletal muscle—a tissue known to be highly dependent on beta-oxidation for ATP production—highlighting that FA oxidation is critical for placental [95].Additionally, the ability of placental mitochondria to utilize lipid substrates for ATP production has been demonstrated to vary over gestation [97]. Specifically,

mid-gestational placental explants display an elevated expression of mitochondrial beta-oxidation enzymes compared to term samples, indicating that the capacity of the placenta to utilize FA as a metabolic substrate diminishes as pregnancy progresses [97]. These findings suggest that the fetus may be more susceptible to influences from a maternal diet overabundant in saturated FA during late gestation when the placenta limits FA oxidation and increases trans-placental lipid transport to support rapid fetal growth.

Independently, maternal gestational obesity has been shown to impede the ability of term placental mitochondria to oxidize FA species for energy (ATP) production [85,98] (Figure 1). Observed decreases in intra-placental concentrations of acylcarnitine species (a marker of beta-oxidation) combined with an overall reduction in mitochondrial content within term obese placentae suggests that the maternal environment can negatively impact placental beta-oxidation activity [85]. However, while beta-oxidation primarily occurs within the mitochondria, placental peroxisomes have also been found to express enzymes for FA beta-oxidation [65,99,100]. Specifically, the enzymes involved in peroxisomal beta-oxidation are acyl-CoA oxidases (ACOX), D-bifunctional protein (DBP) and 3-ketoacyl-CoA thiolases [99,101]. In brief, peroxisomal beta-oxidation shortens long-chain FA species into acetyl-CoA and short-chain acyl-CoAs such as octanoyl-CoA which can then be exported into the mitochondria for complete oxidation [99,101]. More importantly, environmental cues such as fatty acid overabundance in obesity have been associated with increases in both the size and number of peroxisomes [85,102]. Additionally, maternal obesity has been linked to specific increases in the mRNA expression of peroxisomal beta-oxidation enzymes, suggesting that peroxisomal beta-oxidation is a major component of placental lipid handling in obese pregnancies [85] (Figure 1). Obese placentae were further found to have greater rates of oxidation of radio-labelled palmitate following treatment with etomoxir (a mitochondrial beta-oxidation inhibitor) than non-obese placentae highlighting that increases in peroxisomal beta-oxidation may act to modulate lipid oxidation in obese pregnancies with poor mitochondrial function [85]. Overall, these results suggest that the balance between mitochondrial and peroxisomal beta-oxidation in the placenta is disrupted by obesity.

Maternal diet has been identified to impact placental lipid oxidative function in some obese women. Specifically, obese Hawaiian women, who consume the Pacific diet, have been found to have similar mRNA expression levels of mitochondrial and peroxisomal beta-oxidation enzymes as lean Hawaiian women [92] (Figure 1). This may suggest that the increased PUFA content of the Pacific diet could moderate the balance between mitochondrial and peroxisomal lipid oxidation. In contrast, dietary omega-3 PUFA supplementation in obese pregnancies from landlocked areas (Ohio) was not linked to alterations in mRNA expression of mitochondrial and peroxisomal beta-oxidative enzymes [86]. Additionally, omega-3 PUFA treatments did not alter [^{3}H]palmitate oxidation rates in cultured villous trophoblast cells from otherwise healthy obese Ohioan women [86]. While PUFA supplementation studies have highlighted some favourable outcomes, further studies of the impact upon mitochondrial and peroxisomal beta-oxidation pathways are warranted. Furthermore, placental beta-oxidation biomarker signatures must be identified in order to appropriately monitor the effects of any dietary intervention in real time during gestation, especially in women from landlocked areas.

One potential method to quantify placental beta-oxidative function is to examine the acylcarnitine profiles of maternal blood products. Under normal physiological conditions, complete beta-oxidation occurs whereby all carbon atoms in the FA backbone are converted into acetyl-CoA molecules that are oxidized for ATP production [95,96]. However, under pathological conditions such as lipid overload, mitochondrial beta-oxidation may become incomplete resulting in accumulation of shortened chain acyl-CoA molecules within the mitochondrial matrix that may then be exported into circulation [103,104]. Analysis of differences in acylcarnitine profiles has previously been utilized to predict the presence of aberrant metabolic function in tissues including cardiac and skeletal muscle [105–109]. Thus, analysis of blood acylcarnitine profiles of mothers who consume poor diets throughout the gestational period may allow for the real-time identification of specific placental-derived acylcarnitine species that are predictive of aberrant placental mitochondrial beta-oxidative function.

Acylcarnitine profiles have previously been examined as potential biomarkers for the early detection of other placental diseases such as pre-eclampsia [110,111]. Specifically, potential acylcarnitine biomarkers for the early detection of pre-eclampsia were found in both maternal serum and plasma [110,111]. In addition, acylcarnitines have also been examined as potential non-invasive biomarkers to examine placental metabolic function under conditions of maternal obesity [85,112,113]. As this field of investigation develops, it is important to note that these studies highlight that different maternal blood fractions may have differing capabilities to estimate placental metabolic function. For example, increases in some short chain acylcarnitine species are reported in maternal serum with increasing BMI [112], while no differences are found in acylcarnitine profiles in maternal plasma [113].

Accumulation of shortened acylcarnitine species has also previously been linked to an increased expression of pro-inflammatory molecules [104]. For example, mouse macrophage cells cultured with short-chain acylcarnitine species displayed a marked increase in the phosphorylation of the downstream effector proteins JNK and ERK which are involved in the signaling cascade of many inflammatory peptides [104]. If a maternal diet high in saturated fat can lead to incomplete placental beta-oxidation that promotes an inflammatory response, acylcarnitine analysis may be beneficial in explaining the presence of increased placental inflammation that often accompanies maternal obesity [114].

Overall, acylcarnitine analysis may represent a relatively unexplored field in placenta physiology. Analysis of differences within these profiles of obese and lean women may allow clinicians to diagnose placental mitochondrial dysfunctions in conjunction with inflammatory responses early during the gestation period. In turn, acylcarnitine biomarkers may allow clinicians to monitor the impact of dietary interventions on placental lipid handling during gestational period and modulate the course of treatment to limit the risks of offspring development of later life disease.

9. Conclusions

A maternal consumption of a diet high in saturated FA species and low in PUFA species during the gestational period may promote adverse placental function that underlies the development of placental and fetal metabolic dysfunction, independent to maternal body composition. Understanding the mechanisms that underlie placental metabolic dysfunctions associated with dietary fat in obese pregnancies and the accompanying offspring metabolic disorders will require a robust understanding of placental lipid transport, esterification and oxidation (Figure 1). A greater understanding of these processes will yield information that will provide frameworks from which to develop diagnostic tests to monitor the efficacy of gestational dietary interventions. Proper implementation of gestational diet improvements in obese women has the potential to limit future harm to the placenta and overall reduce risk of early-onset metabolic disease development in obesity-exposed offspring.

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Article

Omega-6:Omega-3 Fatty Acid Ratio and Total Fat Content of the Maternal Diet Alter Offspring Growth and Fat Deposition in the Rat

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Abstract: Omega-3 long-chain polyunsaturated fatty acids (LCPUFA) have been shown to inhibit lipogenesis and adipogenesis in adult rats. Their possible early life effects on offspring fat deposition, however, remain to be established. To investigate this, female Wistar rats ($n = 6$–9 per group) were fed either a 9:1 ratio of linoleic acid (LA) to alpha-linolenic acid (ALA) or a lower 1:1.5 ratio during pregnancy and lactation. Each ratio was fed at two total fat levels (18% vs. 36% fat *w/w*) and offspring were weaned onto standard laboratory chow. Offspring exposed to a 36% fat diet, irrespective of maternal dietary LA:ALA ratio, were lighter (male, 27 g lighter; female 19 g lighter; $p < 0.0001$) than those exposed to an 18% fat diet between 3 and 8 weeks of age. Offspring exposed to a low LA (18% fat) diet had higher proportions of circulating omega-3 LCPUFA and increased gonadal fat mass at 4 weeks of age ($p < 0.05$). Reduced Srebf1 mRNA expression of hepatic ($p < 0.01$), gonadal fat ($p < 0.05$) and retroperitoneal fat ($p < 0.05$) tissue was observed at 4 weeks of age in male and female offspring exposed to a 36% fat diet, and hepatic Srebf1 mRNA was also reduced in male offspring at 8 weeks of age ($p < 0.05$). Thus, while offspring fat deposition appeared to be sensitive to both maternal dietary LA:ALA ratio and total fat content, offspring growth and lipogenic capacity of tissues appeared to be more sensitive to maternal dietary fat content.

Keywords: maternal nutrition; omega-6; omega-3; pregnancy; obesity; fatty acids; lipogenesis

1. Introduction

Risk of obesity may be partially attributed to the nutritional environment encountered during early life [1]. Interventions that target these critical life stages exert a greater preventative effect than those applied later in life [2]. Epidemiological as well as experimental animal studies have shown that exposure to a hypercaloric or high-fat diet during early development is associated with increased adiposity in the offspring in later life [3–5]. Emerging evidence, however, suggests that the type of fat an individual is exposed to during development may also play a key role in determining their future metabolic health. Of increasing interest, due to the significant increase in their consumption over the past 60 years, is the role of dietary omega-6 polyunsaturated fatty acids (PUFA) [6,7].

Knowledge of the biological effects of omega-6 fatty acids (which have pro-adipogenic and pro-inflammatory properties), as well as the evidence suggesting that increased maternal omega-6 PUFA intake is associated with offspring adiposity [8,9], has led to the hypothesis that a diet high in omega-6 PUFA may be contributing to the increased incidence of obesity [10]. Furthermore, due to

the effects of the omega-3 PUFA alpha-linolenic acid (ALA) and its derivatives, which are primarily anti-inflammatory in nature, it has been hypothesised that a diet high in these fatty acids may reduce fat deposition [11,12]. Substantial increases in population level intakes of omega-6 PUFA, in particular linoleic acid (LA; precursor to longer omega-6 derivatives), have not coincided with any increases in omega-3 consumption [7], resulting in a significant increase in the ratio of omega-6 to omega-3 fatty acids in typical Western diets over the last forty years. Formation of longer-chain PUFA, such as arachidonic acid (AA; omega-6), eicosapentaenoic acid (EPA; omega-3) and docosahexaenoic acid (DHA; omega-3), relies on a common set of enzymes utilised by both families of PUFA. As such, competition exists between the two families such that the levels of omega-6 PUFA within the body can directly affect the levels of omega-3 PUFA, therefore, implying that alterations in the ratio of these two families of PUFA, as well as their overall amount, may impact on fat deposition and lipogenesis.

The potential mechanism through which variation in the omega-6:omega-3 ratio in early life may programme long-term metabolic health is unknown. It is possible that early changes in the patterns of expression of key genes involved in lipogenesis within the liver and adipose tissue have a long-term impact on fat deposition and accumulation. These genes include sterol regulatory element-binding protein 1c (*Srebf1*), peroxisome proliferator-activated receptor gamma (*Pparg*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*), and leptin (*Lep*). Previous studies in adult animals have also demonstrated that increased omega-3 PUFA intake can reduce lipid accumulation resulting in an overall reduction in body fat [11–13], and that this is mediated through modulation of the expression of *Srebf1* [14] and *Pparg* [15,16]. There have been few studies, however, investigating whether these anti-lipogenic effects are observed in offspring exposed to a maternal diet that is high in omega-3 fats. Conflicting results have been reported in this regard, with some studies reporting decreased [9,17,18] and others reporting increased [19] offspring adiposity.

The aim of this study was to investigate the effects of feeding a maternal dietary LA:ALA ratio similar to that of the Western diet (9:1) [7], compared to a proposed 'ideal' ratio of ~1:1.5 [20,21] on offspring adiposity and other health indicators in rats. To elucidate any additive effects of altering the maternal dietary LA:ALA ratio, each diet was fed at either 18% fat *w/w* or at a higher fat content of 36% fat *w/w*. This paper focusses specifically on the effects of pre- and early postnatal exposure to altered dietary fat content and fatty acid ratio on offspring that have been weaned onto a standard laboratory diet. As such, offspring are no longer directly exposed to the maternal dietary intervention postweaning. We hypothesised that exposure to a high LA diet during pregnancy and lactation would lead to increased adiposity in the offspring, in conjunction with an increased expression of lipogenic genes, and that this effect may be exacerbated with exposure to a high-fat diet.

2. Materials and Methods

2.1. Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office licence and were approved by the Animal Ethics Committee of the University of Nottingham, UK (Project code 40/3598; approved 02/03/2015). Virgin female Wistar rats ($n = 30$; 75–100 g; Charles River, UK) were maintained as previously described [22]. After acclimatisation, a tail vein blood sample was taken from each animal for the determination of fatty acid status and individuals were then randomly allocated to experimental groups. Animals were maintained on their allocated diet for a four week 'pre-feeding' period, after which they were mated. Conception was confirmed by the presence of a semen plug and this was recorded as day 0 of pregnancy. Animals were housed in individual cages and remained on their respective diets throughout pregnancy and lactation. All maternal data are reported elsewhere [22].

Litters were standardised to 8 pups within 24 h of birth (4 males and 4 females, where possible). At 1 and 2 weeks of age, one male and one female from each litter were euthanised and tissues collected for analyses, the results of which are published elsewhere [22]. At 3 weeks of age, the remaining

offspring were weaned and dams were euthanised by CO_2 asphyxiation and cervical dislocation for collection of maternal blood and tissues. Offspring were weaned onto a standard laboratory chow diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Derby, UK) and pair-housed with the remaining same sex littermate. Offspring bodyweight was measured weekly and all animals had blood pressure measured at 4 weeks of age. At this time, one male and one female were euthanised by CO_2 asphyxiation and cervical dislocation. Blood pressure was measured again at 8 weeks of age in all remaining animals after which the experiment ended and all remaining animals were euthanised by CO_2 asphyxiation and cervical dislocation.

2.2. Diets

Diets were designed to provide either a high (9:1, high LA) or low (1:1.5, low LA) ratio of LA to ALA. For each level of LA, diets containing either 18% or 36% fat (*w/w*) were developed. This resulted in four experimental diets; high LA (18% fat; *n* = 6), high LA (36% fat; *n* = 8), low LA (18% fat; *n* = 7) and low LA (36% fat; *n* = 9). The list of ingredients and final fatty acid composition of the four experimental diets are reported elsewhere [22].

2.3. Tail Cuff Plethysmography

This experiment utilised a non-invasive method for measuring blood pressure validated by Feng, et al. [23]. A volume pressure recording (VPR) sensor was used to measure tail blood volume to assess systolic, diastolic and mean arterial blood pressure as well as heart rate. Prior to blood pressure measurements, animals were placed in a heat box set to 30 °C for 15 min to enhance blood flow to the tail. Animals were then restrained in individual restraint tubes with an adjustable nose cone, fitted with the deflated occlusion and VPR cuff (CODA System, Kent Scientific, Torrington, CT, USA), and left to acclimatise to the restraint tube for 10 min to minimise the impact of stress before measurements began. After acclimatisation, animals underwent 10 cycles of blood pressure measurements; of these 10 cycles, the first three were disregarded as acclimatisation cycles and an average for each measurement was taken from the remaining seven. Animals were restrained for no longer than 30 min and removed if they exhibited any signs of stress.

2.4. Blood Sample and Tissue Collection

Blood samples were collected from the offspring, when culled, at 4 and 8 weeks of age via cardiac puncture and ~30 µL was spotted onto PUFAcoat™ dried blood spot (DBS) collection paper (Waite Lipid Analysis Service, Adelaide, Australia [24]), allowed to dry at room temperature and stored at −20 °C for subsequent fatty acid analysis. The remainder of the blood sample was centrifuged at 13,000 rpm for 10 min at 4 °C. The plasma was isolated from the whole blood sample and stored at −80 °C until further analysis. Offspring body and organ weights were measured and samples of liver, gonadal fat and retroperitoneal fat were collected at each time point. All tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until determination of gene expression by quantitative reverse transcriptase PCR (qRT-PCR).

2.5. Lipid Extraction

Total lipids were extracted from liver samples of 4- and 8-week-old offspring. For each sample, ~300 mg of crushed, frozen liver was homogenised in 1.6 mL of 0.5M Na_2SO_4. The homogenate was decanted into 5.4 mL of hexane-isopropanol (3:2, *v/v*) and 2 mL of 0.5M Na_2SO_4 was added. Samples were vortexed and then centrifuged at 3000 rpm for 15 min. The supernatant was removed into a fresh tube, dried under nitrogen and the resultant lipid content was weighed. Samples were resuspended in 1 mL of hexane and 100 µL of resuspended sample was removed into a fresh tube, re-dried under nitrogen and resuspended in 100 µL of isopropanol for the determination of cholesterol and triglyceride content. The remaining sample was stored at −20 °C for fatty acid analysis.

2.6. Determination of Circulating and Hepatic Lipids

Plasma and liver cholesterol and triacylglycerol (TAG) content was determined by a quantitative enzymatic colorimetric assay as per the manufacturer's protocol (Infinity™ cholesterol and Infinity™ triglyceride reagent; Thermo Scientific, Abingdon, UK).

2.7. Fatty Acid Methylation and Fatty Acid Analysis of Whole Blood and Liver Samples

Fatty acid composition in maternal and foetal whole blood, and in lipids extracted from liver samples from offspring at 4 weeks of age, was determined by Gas Chromatography (GC) on a Hewlett-Packard 6890 gas chromatograph using methods that have previously been described in detail [22,24]. Individual fatty acid content was calculated based on peak area and response factors normalised to total fatty acid content and expressed as a percentage of total fatty acids.

2.8. Isolation of RNA and cDNA Synthesis and Reverse Transcription Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated from crushed snap-frozen samples of ~25 mg of liver using the Roche High Pure Tissue kit (Roche Diagnostics Ltd., Burgess Hill, UK). Adipose RNA was extracted, after homogenisation of ~100 mg of tissue with MagNA Lyser green green beads and instrument (Roche Diagnostics Ltd., Burgess Hill, UK), using the RNeasy Mini Kit (QIAGEN Ltd., Manchester, UK). RNA concentration was determined using a Nanodrop 2000 (Thermo Scientific, Abingdon, UK) and RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesised using a RevertAid™ reverse transcriptase kit (Thermo Fisher Scientific, Abingdon, UK) with random hexamer primers.

Lipogenic pathway and adipokine target genes included peroxisome proliferator-activated receptor gamma (*Pparg*), sterol regulatory element-binding protein (variant 1c; *Srebf1*), fatty acid synthase (*Fasn*), lipoprotein lipase (*Lpl*) and leptin (Lep). Primer sequences for these gene targets have previously been published elsewhere [22]. Hepatic expression of delta-5 (*Fads1*; Rn_Fads1_1_SG QuantiTect Primer Assay, Qiagen) and delta-6 (*Fads2*; Rn_Fads2_1_SG QuantiTect Primer Assay, Qiagen) desaturase enzymes were also determined. Cyclophilin A (*Ppia*) and β-actin (*Actb*) were used as housekeeper genes. Adipocyte and hepatic gene expression was quantified using SYBR Green (Roche Diagnostics) in a Light-Cycler 480 (Roche Diagnostics). Samples were analysed against a standard curve of a serially diluted cDNA pool to produce quantitative data and expression was normalised to the housekeeping gene using LightCycler® 480 software (version 1.5.1) as previously described [25]. The expression of the housekeeper genes were not different between treatment groups.

2.9. Statistical Analysis

Data are presented as the mean ± SEM. Data were analysed using the Statistical Package for Social Sciences (Version 24, SPSS Inc., IBM, Chicago, IL, USA). The effect of maternal dietary fatty acid ratio, maternal dietary fat content and sex on dependent variables was assessed using a three-way ANOVA. Where sex had a main effect on variables but no interaction with maternal dietary factors, data were split for male and female offspring and a two-way ANOVA was then used to assess the effect of maternal dietary fat content and fatty acid ratio on male and female offspring separately. Where longitudinal data were analysed, as with bodyweight, the impact of maternal dietary LA:ALA ratio and maternal dietary fat content was analysed using a two-way repeated-measures ANOVA. A value of $p < 0.05$ was considered to be statistically significant and dams were used as the unit of analysis.

3. Results

3.1. Offspring Bodyweight, Body Composition and Blood Pressure

Figure 1 shows bodyweights of offspring from 3 to 8 weeks of age. Offspring birthweight and bodyweight prior to this are reported elsewhere [22]. Offspring of dams consuming a 36% fat diet,

irrespective of maternal dietary LA:ALA ratio, were lighter than offspring of dams fed on an 18% fat diet from 3 to 8 weeks of age in both male (on average 27 g lighter) and female (on average 19 g lighter) offspring ($p < 0.0001$).
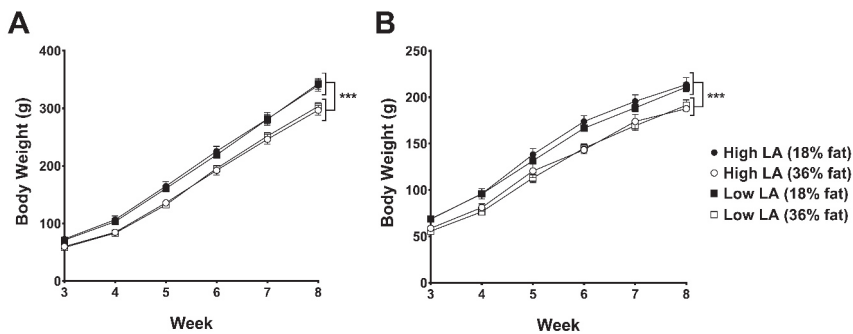

Figure 1. Body weights of (**A**) male and (**B**) female offspring postweaning up to 8 weeks of age exposed to either a high LA (18% fat) diet (closed circles), high LA (36% fat) diet (open circles), low LA (18% fat) diet (closed squares) or a low LA (36% fat) diet (open squares) during gestation and lactation. Offspring were weaned onto a chow diet. Values are means ± SEM and *n* = 6–9 per group. The effects of dietary fatty acid ratio and dietary fat content were determined using a two-way repeated measures ANOVA. *** indicates a significant effect of maternal dietary fat content ($p < 0.0001$) on body weight.

Table 1 shows the organ and fat depot weights of male and female offspring normalised to bodyweight at 4 and 8 weeks of age (absolute organ weights can be found in Supplementary Table S1). At 4 weeks of age, relative heart weight was 5% higher and relative liver weight was 4% lower in female offspring of dams exposed to a 36% fat diet compared to those exposed to an 18% fat diet, irrespective of maternal dietary LA:ALA ratio ($p < 0.05$). Relative liver weight at 4 weeks also tended ($p = 0.075$) to be lower in male offspring of dams consuming the 36% vs. 18% fat diet. A significant ($p < 0.05$) interaction between maternal dietary fatty acid ratio and maternal dietary fat content on relative gonadal fat weight was observed for both male and female offspring at 4 weeks of age. This manifested as ~30% lower weight of the gonadal fat depots in the low LA group, but only if exposed to a 36% fat diet in early life. There were no differences in the relative weight of lungs, kidneys or retroperitoneal fat pads between experimental groups at 4 weeks of age. Differences in relative organ and fat weights measured in offspring at 4 weeks of age appeared to be transient, as no differences were observed at 8 weeks of age for any of these organs or fat depots.

Blood pressure at 4 weeks of age was not influenced by maternal diet. At 8 weeks of age, female offspring exposed to a 36% diet during gestation and lactation had significantly lower systolic (16 mmHg; $p = 0.024$) and tended to have lower diastolic (11 mmHg; $p = 0.068$) blood pressure than offspring exposed to an 18% fat diet (Table 1). Blood pressure in males was not influenced by either LA:ALA ratio or fat content of the maternal diet.

Table 1. Offspring body composition and blood pressure at 4 and 8 weeks of age.

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
4 Week Offspring								
Heart (% BW)	0.49 ± 0.02	0.53 ± 0.02	0.50 ± 0.02	0.51 ± 0.02	0.52 ± 0.003 [a]	0.55 ± 0.002 [b]	0.51 ± 0.02 [a]	0.53 ± 0.01 [b]
Lungs (% BW)	1.05 ± 0.07	1.07 ± 0.10	1.02 ± 0.07	1.29 ± 0.06	1.39 ± 0.09	1.28 ± 0.12	1.20 ± 0.11	1.19 ± 0.08
Kidney (%BW)	1.08 ± 0.03	1.12 ± 0.03	1.10 ± 0.02	1.06 ± 0.01	1.02 ± 0.10	1.11 ± 0.02	1.13 ± 0.04	1.05± 0.02
Liver (% BW)	5.05 ± 0.15	4.92 ± 0.13	5.05 ± 0.12	4.71 ± 0.10	4.98 ± 0.07 [a]	4.73 ± 0.08 [b]	4.76 ± 0.16 [a]	4.60 ± 0.11 [b]
Gonadal fat (%BW) *	0.49 ± 0.03	0.52 ± 0.04	0.56 ± 0.03	0.39 ± 0.03	0.64 ± 0.09	0.66 ± 0.05	0.79 ± 0.05	0.54 ± 0.04
Retroperitoneal fat (%BW)	0.57 ± 0.06	0.52 ± 0.04	0.52 ± 0.07	0.47 ± 0.05	0.42 ± 0.06	0.37 ± 0.03	0.42 ± 0.03	0.34 ± 0.03
Systolic BP (mmHg)	86.2 ± 4.0	92.6 ± 4.6	89.0 ± 5.9	91.9 ± 3.8	85.8 ± 7.2	87.4 ± 3.3	91.8 ± 4.1	91.3 ± 5.2
Diastolic BP (mmHg)	65.5 ± 3.6	68.2 ± 3.7	65.4 ± 4.6	68.3 ± 3.3	61.9 ± 7.1	61.1 ± 2.1	68.9 ± 4.1	64.5 ± 2.2
8 Week Offspring								
Brain (%BW)	0.56 ± 0.02	0.57 ± 0.02	0.55 ± 0.02	0.57 ± 0.01	0.80 ± 0.01	0.84 ± 0.02	0.79 ± 0.01	0.79 ± 0.03
Heart (% BW)	0.34 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.39 ± 0.02	0.40 ± 0.01
Lungs (% BW)	0.61 ± 0.06	0.62 ± 0.05	0.61 ± 0.05	0.63 ± 0.04	0.66 ± 0.04	0.68 ± 0.04	0.60 ± 0.02	0.68 ± 0.05
Kidney (%BW)	0.83 ± 0.04	0.83 ± 0.02	0.84 ± 0.02	0.88 ± 0.03	0.88 ± 0.03	0.87 ± 0.03	0.85 ± 0.03	0.81 ± 0.02
Liver (% BW)	4.80 ± 0.08	5.00 ± 0.14	4.96 ± 0.09	5.00 ± 0.08	4.49 ± 0.16	4.63 ± 0.08	4.43 ± 0.10	4.56 ± 0.08
Gonadal fat (%BW)	1.30 ± 0.10	1.25 ± 0.08	1.34 ± 0.08	1.19 ± 0.09	1.38 ± 0.15	1.56 ± 0.07	1.53 ± 0.19	1.57 ± 0.23
Retroperitoneal fat (%BW)	1.22 ± 0.09	1.29 ± 0.11	1.32 ± 0.11	1.16 ± 0.10	0.88 ± 0.10	0.94 ± 0.11	0.94 ± 0.09	0.86 ± 0.08
Systolic BP (mmHg)	116.0 ± 5.0	109.1 ± 5.2	113.4 ± 3.4	105.8 ± 6.6	122.7 ± 10.1 [a]	103.2 ± 3.0 [b]	120.4 ± 8.2 [a]	107.4 ± 5.9 [b]
Diastolic BP (mmHg)	82.9 ± 0.2	73.5 ± 5.3	80.3 ± 3.3	74.4 ± 5.7	82.3 ± 5.1	71.8 ± 2.3	86.0 ± 7.1	74.5 ± 6.3

All values are mean ± SEM and organ weights are expressed as a percentage of bodyweight (%BW). A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors, all comparisons are made within sex groups. Different superscripts (a, b) denote values which are significantly different ($p < 0.05$). * indicates a significant interaction effect of maternal dietary fatty acid ratio and maternal fat content on gonadal fat weight in male ($p < 0.01$) and female ($p < 0.05$) offspring. $n = 6$–9 per dietary group.

3.2. Offspring Whole Blood and Hepatic Fatty Acid Profile

A significant effect of sex was observed for some of the fatty acids measured in whole blood and liver at 4 and 8 weeks of age. However, no interactions were observed between sex and maternal dietary treatment, so male and female data were split for further analysis. Figure 2 shows the fatty acid profile of whole blood in offspring at 4 weeks of age. In male offspring, exposure to a 36% fat diet was associated with increased proportions of saturated fatty acids (SFA; $p < 0.05$) and monounsaturated fatty acid (MUFA; $p < 0.05$) as well as decreased proportions of LA ($p < 0.05$) and AA ($p < 0.05$), resulting in lower overall total omega-6 in response to a maternal 36% fat diet. Proportions of MUFA and AA were also influenced by maternal dietary fatty acid ratio such that a low LA diet was associated with increased MUFA ($p < 0.01$) and decreased AA ($p < 0.01$) levels. A similar pattern was observed for the proportions of SFA, MUFA and omega-6 fatty acids in whole blood of female offspring. In females, a significant interaction was observed for the proportions of long-chain omega-3 fatty acids (EPA, $p < 0.05$; DPA, $p < 0.01$ and DHA; $p < 0.05$). Interestingly, female offspring exposed to a low LA (18% fat) diet had higher proportions of these fatty acids and as a result, higher total omega-3 proportions. Similar patterns were observed in male offspring. However, only a significant main effect of maternal dietary LA:ALA ratio was observed.

Figure 2. Whole blood fatty acid profile in (**A**) male and (**B**) female offspring at 4 weeks of age. Values are means ± SEM and *n* = 6–9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA; all comparisons were made within sex group. * Indicates significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). † indicates a significant interaction effect ($p < 0.05$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

The elevated omega-3 proportions in offspring of dams exposed to a low LA (18% fat) diet at 4 weeks of age, prompted investigation into the liver fatty acid profile at this time point (Figure 3). Interestingly, the composition of fatty acids in the liver did not completely reflect that of the whole blood and were only influenced by maternal dietary fatty acid ratio. In male offspring, exposure to a

low LA diet during pregnancy and lactation was associated with lower proportions of total omega-6, LA and AA and higher proportions of total omega-3, ALA, EPA, DHA and total SFA in the liver (Figure 3A). Similar observations were made for the fatty acid composition of the liver in female offspring at this time point. A key difference, however, was that maternal diet appeared to have no effect on total SFA in the female offspring (Figure 3B).
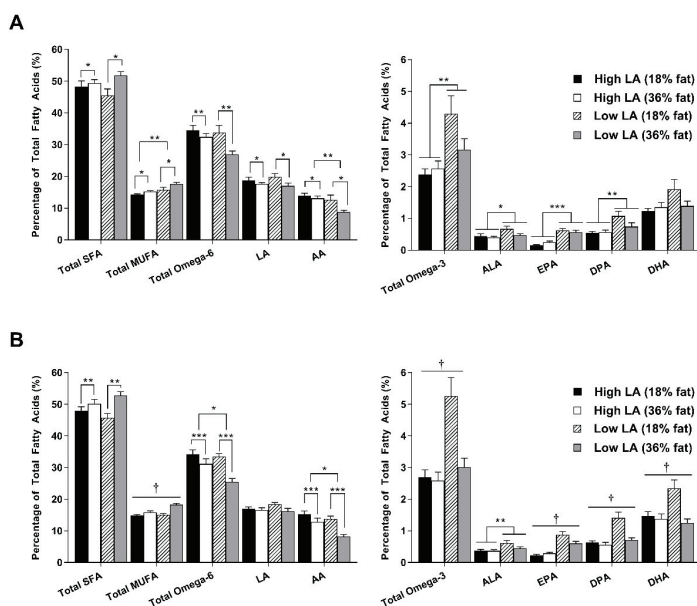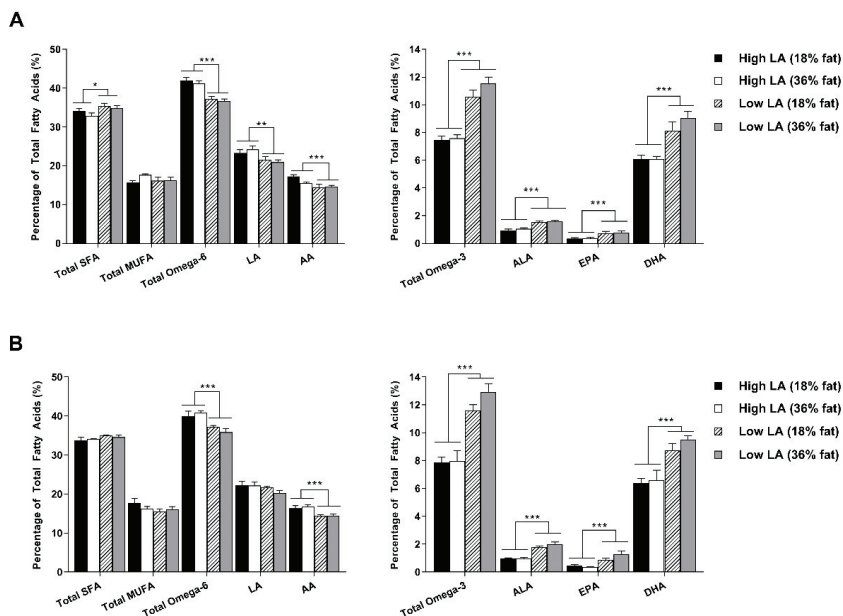

Figure 3. Liver fatty acid profile in (**A**) male and (**B**) female offspring at 4 weeks of age. Values are means ± SEM and *n* = 6–9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA; all comparisons were made within sex group. * Indicates significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

At 8 weeks of age, whole blood fatty acid profile was reassessed (Figure 4). In male offspring, there were no longer any differences in proportions of SFA, MUFA, total omega-6, LA, AA or ALA between experimental groups. Total omega-3 ($p < 0.001$), EPA ($p < 0.01$), DPA ($p < 0.05$) and DHA ($p = 0.052$) proportions all remained elevated in male offspring of dams exposed to a low LA diet during pregnancy and lactation. Similar observations were made for the fatty acid composition of female whole blood at 8 weeks of age. Proportions of AA, and consequently levels of total omega-6, were, however, higher in female offspring exposed to a high LA diet, irrespective of maternal dietary fat content. In both male and female whole blood, DPA proportions appeared to be influenced by maternal dietary fat content such that a 36% fat diet was associated with lower proportions of this fatty acid. This was significant in female offspring ($p < 0.05$) and tended towards significance in male offspring ($p = 0.057$). Unlike in male offspring, DHA proportions in female offspring whole blood at 8 weeks of age were not associated with maternal dietary intake.
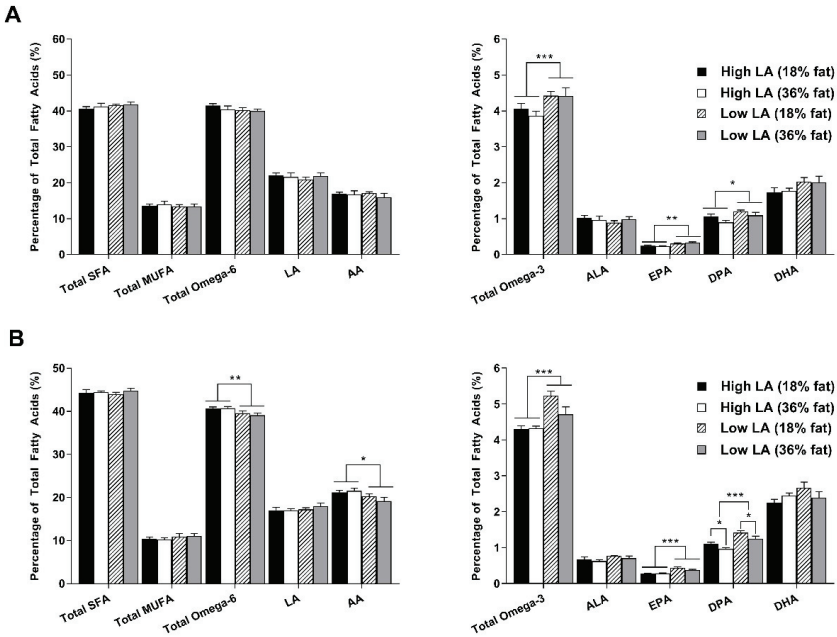
Figure 4. Offspring whole blood fatty acid profile in (**A**) male and (**B**) female offspring at 8 weeks of age. Values are means ± SEM and *n* = 6–9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were determined using a two-way ANOVA. All comparisons were made within sex group. * Indicates significant difference (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

3.3. Circulating and Hepatic Lipid Profile

At 4 weeks of age, male offspring exposed to a 36% fat diet had lower circulating plasma TAG concentrations (*p* = 0.01) and reduced liver cholesterol concentrations (*p* < 0.05) when compared to offspring exposed to an 18% fat diet (Table 2). In the female liver, however, TAG concentrations were affected by maternal dietary ratio such that female offspring exposed to a low LA diet had lower concentrations of liver TAG (*p* < 0.05), irrespective of maternal dietary fat level. There was no effect of maternal diet on plasma cholesterol or total liver lipid at 4 weeks of age. By 8 weeks of age, there were no significant differences in any of the variables measured in female offspring. In males, however, there was a significant interaction of maternal dietary fat content and fatty acid ratio such that exposure to a high LA (36% fat) diet resulted in increased circulating cholesterol (*p* < 0.05) but reduced liver TAG concentrations in 8-week-old male offspring (Table 2).

Table 2. Offspring lipid and liver characteristics at 4 and 8 weeks of age.

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
4 Week Offspring								
Plasma cholesterol (mmol/L)	2.93 ± 0.13	3.08 ± 0.10	3.08 ± 0.13	2.90 ± 0.15	2.83 ± 0.09	2.90 ± 0.18	2.80 ± 0.14	2.55 ± 0.10
Plasma TAG (mmol/L)	0.89 ± 0.07 [a]	0.74 ± 0.03 [b]	1.03 ± 0.12 [a]	0.75 ± 0.06 [b]	0.72 ± 0.06	0.76 ± 0.05	0.83 ± 0.09	0.80 ± 0.09
Liver lipid (mg/g tissue)	30.59 ± 4.37	33.60 ± 3.51	28.55 ± 6.07	33.53 ± 1.28	34.33 ± 7.35	36.61 ± 3.25	26.03 ± 3.01	28.57 ± 4.00
Liver cholesterol (mg/g tissue)	1.93 ± 0.20 [a]	1.46 ± 0.12 [b]	1.78 ± 0.15 [a]	1.63 ± 0.11 [b]	1.63 ± 0.18	1.58 ± 0.12	1.56 ± 0.15	1.55 ± 0.09
Liver TAG (mg/g tissue)	18.01 ± 1.71	16.40 ± 1.54	16.01 ± 2.64	13.23 ± 1.90	17.05 ± 2.42 [a]	15.75 ± 1.37 [a]	13.99 ± 1.03 [b]	11.56 ± 0.85 [b]
Liver Fads1 #	0.99 ± 0.15	1.31 ± 0.21	0.88 ± 0.07	0.97 ± 0.20	1.09 ± 0.06	1.48 ± 0.21	1.02 ± 0.10	0.82 ± 0.08
Liver Fads2 #	1.08 ± 0.13	1.42 ± 0.18	1.27 ± 0.19	1.16 ± 0.16	1.19 ± 0.06	1.54 ± 0.16	1.33 ± 0.19	0.95 ± 0.06
8 Week Offspring								
Plasma cholesterol (mmol/L) *	2.39 ± 0.14	3.47 ± 0.33	2.87 ± 0.14	2.97 ± 0.15	2.30 ± 0.30	2.72 ± 0.21	2.72 ± 0.17	2.64 ± 0.15
Plasma TAG (mmol/L)	1.57 ± 0.18	1.48 ± 0.17	1.42 ± 0.09	1.51 ± 0.11	1.10 ± 0.16	0.92 ± 0.06	1.11 ± 0.13	1.03 ± 0.11
Liver lipid (mg/g tissue)	37.56 ± 3.69	36.18 ± 6.82	28.22 ± 10.63	26.27 ± 3.47	26.81 ± 2.27	32.73 ± 6.80	30.99 ± 5.70	37.27 ± 7.01
Liver cholesterol (mg/g tissue)	1.20 ± 0.06	1.06 ± 0.12	1.11 ± 0.12	0.97 ± 0.10	0.87 ± 0.13	1.16 ± 1.38	0.89 ± 0.17	0.94 ± 0.12
Liver TAG (mg/g tissue) *	19.87 ± 1.80	9.81 ± 1.13	13.68 ± 1.99	12.50 ± 1.87	14.53 ± 1.29	12.15 ± 2.53	11.72 ± 0.89	8.91 ± 1.24

All values are mean ± SEM. A two-way ANOVA was used to analyse results with maternal dietary fatty acid ratio and maternal dietary fat content as factors; all comparisons are made within sex group. Different superscripts (a, b) denote values which are significantly different ($p < 0.05$). A significant interaction of maternal dietary fat content and maternal dietary fatty acid is denoted for * males ($p < 0.05$) and # females ($p < 0.05$). $n = 6$–9 per dietary group.

3.4. Gene Expression

An interaction between maternal dietary fatty acid ratio and fat content on hepatic expression of *Fads1* and *Fads2* was observed in female offspring (Table 2). This resulted in increased expression of both genes in female offspring of dams exposed to a high LA (36% fat) diet suggesting an increased capacity for synthesis of long-chain fatty acids in this group. There were no differences in the expression of these genes in male offspring. Expression of key lipogenic genes (*Fasn*, *Lpl*, *Pparg*, *Srebf1* and *Lep*) was measured in the liver as well as gonadal and retroperitoneal fat depots (*Lep* was only measured in the fat depots due to limited hepatic expression). At 4 weeks of age, a consistent effect of maternal dietary fat content on expression of *Srebf1* mRNA was observed (Table 3). Offspring of dams exposed to a 36% fat diet had lower expression of hepatic ($p < 0.01$), gonadal fat ($p < 0.05$; significant in female offspring only) and retroperitoneal fat ($p < 0.05$) *Srebf1* mRNA compared to offspring of dams consuming an 18% fat diet. A similar pattern was observed for other genes in the retroperitoneal fat depot, such that offspring of dams exposed to a 36% fat diet exhibited lower mRNA expression of *Fasn* ($p < 0.01$; male offspring only), *Lpl* ($p < 0.05$; male and female offspring) and *Lep* ($p < 0.05$; male offspring only). At 4 weeks of age, hepatic *Lpl* expression was higher in offspring of dams consuming a low LA 36% fat diet compared to other groups in both male and female offspring ($p < 0.05$). In female offspring at 4 weeks of age, a significant interaction between maternal dietary fatty acid ratio and maternal dietary fat content was observed in gonadal fat expression of *Fasn* ($p < 0.05$) and *Lep* ($p < 0.05$) as well as retroperitoneal fat expression of *Pparg* ($p < 0.05$) and *Lep* ($p < 0.01$). This interaction manifested as increased expression of these genes in offspring exposed to 36% fat with a high LA:ALA ratio, but decreased expression when the diet consisted of a low LA:ALA ratio. As such, offspring exposed to a low LA (18% fat) diet consistently exhibited the highest expression of these genes.

Table 4 summarises the mRNA expression at 8 weeks of age. Male offspring exposed to a 36% fat diet during gestation and lactation showed lower hepatic *Srebf1* and gonadal fat *Lep* mRNA expression ($p < 0.05$) when compared to an 18% fat diet, irrespective of maternal dietary fatty acid ratio. In females, and other tissues measured in male offspring, the differences in *Srebf1* expression observed at 4 weeks of age appeared to be transient, as no differences were observed between groups at 8 weeks of age. *Fasn* mRNA at 8 weeks of age was significantly higher in female offspring of dams consuming a 36% fat diet in both the gonadal ($p < 0.05$) and retroperitoneal ($p < 0.01$) fat depots, irrespective of maternal dietary ratio.

Table 3. Offspring lipogenic gene expression in male and female offspring at 4 weeks of age.

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
4 Week Offspring								
Liver								
Fasn	0.91 ± 0.12	0.93 ± 0.15	1.49 ± 0.42	0.66 ± 0.08	0.97 ± 0.14	1.08 ± 0.10	1.12 ± 0.19	0.90 ± 0.08
Lpl [†,#]	0.12 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	0.18 ± 0.02	0.15 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.18 ± 0.03
Pparg	0.95 ± 0.18	0.91 ± 0.11	0.94 ± 0.17	0.91 ± 0.17	0.96 ± 0.22	0.78 ± 0.12	0.70 ± 0.10	0.75 ± 0.13
Srebf1	0.82 ± 0.13 [a]	0.64 ± 0.02 [b]	0.89 ± 0.11 [a]	0.52 ± 0.07 [b]	0.86 ± 0.10 [a]	0.65 ± 0.07 [b]	0.79 ± 0.10 [a]	0.40 ± 0.05 [b]
Gonadal Fat								
Fasn [#]	3.23 ± 1.17	2.22 ± 0.52	2.36 ± 0.37	1.17 ± 0.06	0.85 ± 0.16	2.42 ± 0.46	3.08 ± 0.47	2.29 ± 0.59
Lpl	0.90 ± 0.05	0.94 ± 0.13	1.30 ± 0.33	0.80 ± 0.12	1.04 ± 0.30	0.88 ± 0.04	1.47 ± 0.27	1.07 ± 0.17
Pparg	0.84 ± 0.04	0.87 ± 0.08	0.95 ± 0.12	0.86 ± 0.03	0.68 ± 0.14	0.80 ± 0.12	0.81 ± 0.06	0.56 ± 0.09
Srebf1	3.12 ± 0.65	3.05 ± 0.50	3.29 ± 0.51	2.21 ± 0.27	3.15 ± 0.64 [a]	2.23 ± 0.18 [b]	3.68 ± 0.63 [a]	2.57 ± 0.45 [b]
Lep [#]	0.90 ± 0.22	0.83 ± 0.08	1.01 ± 0.12	0.62 ± 0.12	0.38 ± 0.05	0.72 ± 0.12	1.32 ± 0.25	0.70 ± 0.14
Retroperitoneal Fat								
Fasn [#]	5.19 ± 1.09 [a]	2.35 ± 0.37 [b]	3.01 ± 0.37 [a]	2.19 ± 0.45 [b]	1.86 ± 0.42	2.11 ± 0.47	2.13 ± 0.16	1.77 ± 0.57
Lpl	1.67 ± 0.16 [a]	1.13 ± 0.17 [b]	1.46 ± 0.11 [a]	1.19 ± 0.18 [b]	1.21 ± 0.14 [a]	0.99 ± 0.11 [b]	1.41 ± 0.13 [a]	0.86 ± 0.04 [b]
Pparg [#]	0.72 ± 0.03	0.95 ± 0.09	1.03 ± 0.20	1.15 ± 0.25	0.70 ± 0.07	1.01 ± 0.11	1.12 ± 0.22	0.71 ± 0.05
Srebf1	2.71 ± 0.19 [a]	1.89 ± 0.10 [b]	2.47 ± 0.33 [a]	1.74 ± 0.15 [b]	2.93 ± 0.53 [a]	2.27 ± 0.17 [b]	2.61 ± 0.40 [a]	1.77 ± 0.20 [b]
Lep [#]	2.23 ± 0.39 [a]	1.31 ± 0.10 [b]	1.84 ± 0.15 [a]	1.43 ± 0.20 [b]	1.00 ± 0.08	1.03 ± 0.11	1.77 ± 0.13	0.95 ± 0.17

Values are means ± SEM and n = 6–9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were analysed using a two-way ANOVA; all comparisons are made within sex group. Different superscripts (a, b) denote statistical different ($p < 0.05$). A significant interaction of maternal dietary fat content and maternal dietary fatty acid is denoted for [†] males ($p < 0.05$) and [#] females ($p < 0.05$). *Fasn*, fatty acid synthase; *Lpl*, lipoprotein lipase; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf1*, sterol regulatory element-binding protein (variant 1c); *Lep*, leptin.

Table 4. Offspring lipogenic gene expression in male and female offspring at 8 weeks of age.

Experimental Group	Male				Female			
	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)	High LA (18% Fat)	High LA (36% Fat)	Low LA (18% Fat)	Low LA (36% Fat)
Liver								
Fasn [†]	1.40 ± 0.14	2.23 ± 0.5	2.20 ± 0.34	1.58 ± 0.13	1.75 ± 0.16	2.45 ± 0.45	1.59 ± 0.29	2.43 ± 0.40
Lpl	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.13 ± 0.01
Pparg	2.57 ± 0.53	2.25 ± 0.30	2.03 ± 0.22	1.71 ± 0.07	1.43 ± 0.43	1.16 ± 0.29	0.85 ± 0.15	0.94 ± 0.15
Srebf1	1.80 ± 0.21 [a]	1.63 ± 0.12 [b]	2.12 ± 0.25 [a]	1.56 ± 0.07 [b]	1.39 ± 0.21	1.27 ± 0.14	1.10 ± 0.18	1.20 ± 0.17
Gonadal Fat								
Fasn	2.24 ± 0.64	2.70 ± 0.53	2.83 ± 0.51	4.03 ± 0.88	1.22 ± 0.35 [a]	2.54 ± 0.23 [b]	2.06 ± 0.59 [a]	4.36 ± 1.10 [b]
Lpl	2.91 ± 0.31	3.55 ± 0.52	3.50 ± 0.63	3.12 ± 0.36	2.00 ± 0.38	3.65 ± 0.78	3.06 ± 0.43	3.27 ± 0.53
Pparg	1.02 ± 0.08	0.90 ± 0.11	0.82 ± 0.06	1.00 ± 0.09	0.92 ± 0.07	0.94 ± 0.12	0.89 ± 0.14	1.02 ± 0.11
Srebf1	3.18 ± 0.46	2.98 ± 0.17	3.65 ± 0.47	3.33 ± 0.56	2.48 ± 0.27	2.97 ± 0.33	3.14 ± 0.55	3.81 ± 0.69
Lep	4.79 ± 0.76 [a]	4.12 ± 0.52 [b]	5.82 ± 0.83 [a]	3.58 ± 0.44 [b]	2.34 ± 0.53	2.99 ± 0.47	4.44 ± 0.87	3.31 ± 0.58
Retroperitoneal Fat								
Fasn	1.58 ± 0.34	1.85 ± 0.31	1.75 ± 0.17	2.25 ± 0.37	0.64 ± 0.11 [a]	2.24 ± 0.43 [b]	1.11 ± 0.36 [a]	1.53 ± 0.26 [b]
Lpl	2.05 ± 0.45	2.01 ± 0.32	2.01 ± 0.19	2.41 ± 0.42	1.30 ± 0.21	2.08 ± 0.28	1.68 ± 0.21	1.54 ± 0.18
Pparg	0.94 ± 0.17	0.92 ± 0.04	0.88 ± 0.05	1.00 ± 0.06	0.92 ± 0.14	1.07 ± 0.09	1.06 ± 0.13	1.01 ± 0.04
Srebf1	1.58 ± 0.30	1.48 ± 0.23	1.64 ± 0.10	1.79 ± 0.24	1.06 ± 0.07	1.81 ± 0.24	1.30 ± 0.12	1.29 ± 0.21
Lep	2.98 ± 0.68	2.62 ± 0.49	2.85 ± 0.33	2.47 ± 0.47	1.49 ± 0.33	1.95 ± 0.21	1.72 ± 0.24	1.49 ± 0.17

Values are means ± SEM and n = 6–9 per group. The effects of maternal dietary fatty acid ratio and maternal dietary fat content were analysed using a two-way ANOVA; all comparisons are made within sex group. Different superscripts (a, b) denote statistical different (p < 0.05). [†] Indicates a significant interaction of maternal dietary fat content and maternal dietary fatty acid in male offspring (p < 0.05). *Fasn*, fatty acid synthase; *Lpl*, lipoprotein lipase; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf1*, sterol regulatory element-binding protein (variant 1c); *Lep*, leptin.

4. Discussion

This study aimed to investigate the effect of an altered maternal dietary LA:ALA ratio, as well as total dietary fat content, on offspring growth, adiposity, lipid profiles and expression of key genes associated with lipogenesis. We have shown that the maternal dietary LA:ALA ratio is a key driver of the fatty acid profile in whole blood and liver of adult offspring. Additionally, we found that exposure to a high-fat diet, irrespective of dietary LA:ALA ratio, was associated with a reduction in offspring bodyweight that persisted after the offspring were weaned onto a standard, nutritionally balanced rodent diet. Differences in adipose tissue weight were determined by maternal dietary LA:ALA ratio as well as total fat content, whilst the expression of key lipogenic genes was predominantly affected by the latter. These data suggest that a maternal diet high in fat can have detrimental effects on offspring growth whilst an interaction between total fat intake and maternal dietary PUFA ratio appears to affect offspring adiposity via alterations in the expression of lipogenic genes.

We have previously shown that exposure to a varying LA:ALA ratio and fat content in the diet influences the circulating fatty acid profile of dams [22] as well as offspring directly exposed to the maternal diet [26]. In the present study, we have demonstrated that the circulating and hepatic fatty acid profiles of offspring at 4 weeks of age, as well as the circulating fatty acid profile at 8 weeks of age, are still influenced by maternal dietary factors despite the offspring no longer being directly exposed to dietary interventions. Of particular interest is the elevated proportions of long-chain omega-3 PUFA in whole blood samples of offspring exposed to a low LA (18% fat) diet but not in those exposed to a low LA (36% fat) diet. The experimental diet, as well as the chow diet that offspring were weaned onto, only contained the omega-6 and omega-3 precursors, LA and ALA. This implies, therefore, that the increased levels of long-chain omega-3 PUFA (LCPUFA) are due to an increased capacity within these offspring to convert ALA to its longer-chain derivatives through elongation and desaturation and/or remnants of preferential transfer of these fatty acids from the mother during pregnancy and/or lactation. We are inclined to believe this is a result of the latter as our previous study indicated a similar fatty acid profile in the dams during the lactation period [26]. Further to this, studies in other species have provided no evidence of increased desaturation and elongation capacity of offspring exposed to higher omega-3 levels [27]. However, a combination of these factors, as well as the possible influence of fatty acid release from adipose tissue, is conceivable and should not be completely ruled out.

This interesting finding in the offspring whole blood fatty acid profile at 4 weeks of age encouraged investigation into the hepatic fatty acid profile and capacity for long-chain PUFA synthesis. Interestingly, and despite evidence of strong correlations between circulating and hepatic liver fatty acid profiles [28], we found that the increased omega-3 LCPUFA observed in whole blood of offspring exposed to a low LA (18% fat) diet was not apparent in the liver. Investigation into the desaturation capacity of the liver in these animals revealed some sex-specific interactions of maternal diet and key genes associated with this pathway. The observation that female offspring exposed to a high LA (36% fat) diet exhibited increased levels of *Fads1* and *Fads2*, does in fact suggest that these individuals may have an increased capacity for LCPUFA synthesis. This did not, however, appear to translate into any physiological differences in the composition of fatty acids in the liver between experimental groups and the mRNA levels measured in this study may not be reflective of protein levels and/or activity of these enzymes. In addition, assessments of enzyme activity and mRNA expression of elongase enzymes would provide further insights into the capacity for LCPUFA synthesis in the liver. These findings do, however, highlight the potential for prolonged biological effects of fatty acids incorporated into phospholipid membranes and/or stored in tissues during gestation and lactation. Further experiments investigating the longevity of changes in offspring fatty acid profiles would confirm if there is a programmed effect of increased capacity for LCPUFA synthesis or if this is an artefact of direct exposure to the maternal dietary intervention. Even if transient, the effects of altered fatty acid composition of tissues, restriction of growth and greater adiposity that we have observed are likely to potentiate long-term metabolic consequences.

Offspring of dams exposed to a 36% fat diet exhibited consistently lower bodyweights than offspring exposed to an 18% fat diet during gestation and lactation. Importantly, this effect was apparent from birth [26] and persisted after the offspring had been weaned onto a standard laboratory diet, suggesting a long-term effect of exposure to a maternal high-fat diet that is persistent beyond direct dietary exposure. This is consistent with many studies reporting decreased foetal [29,30], birth [31] and weaning weight [32] in offspring of dams exposed to a high-fat diet during gestation and lactation periods. Early life growth restriction is often proceeded by a period of "catch-up" growth in which offspring gain weight rapidly and is often associated with increased adiposity [33] and increased risk of metabolic disease and hypertension in the offspring [34]. One possibility is that the decreased bodyweight observed in this group was due to reduced feed intake, although we were not able to assess this in the current study as animals were group housed. Previous studies have, however, reported alterations in the energy intake and neuroendocrine control of bodyweight in offspring of dams exposed to a high-fat diet [35,36] adding feasibility to this hypothesis, and it would be interesting to assess these parameters in future studies using similar diets to the current study. Surprisingly, further to a reduced bodyweight in response to a maternal high-fat diet, female offspring at 8 weeks of age also had reduced blood pressure. This apparent increased sensitivity of female rather than male offspring to maternal dietary treatments has been noted previously [37], although, as in a number of other studies [38,39], maternal high-fat diets resulted in increased as opposed to decreased blood pressure. It is important to note, however, that many of these studies utilised a maternal diet high in saturated fat and studies using diets high in polyunsaturated fats, as with this study, have shown reductions in blood pressure when compared to a maternal diet high in saturated fats [40]. In support of this, we have previously noted that offspring hypertension associated with low protein feeding during rat pregnancy is modified by other components of the experimental diet, including the source of fat [41,42].

An unexpected finding of this study was that offspring exposed to a low LA (18% fat) diet had the highest relative gonadal fat mass at 4 weeks of age, in conjunction with higher *Lep* mRNA expression in the gonadal fat adipose tissue. This was consistent across sexes and conflicted with our hypothesis as well as the evidence linking increased omega-3 intake with reduced fat deposition and accumulation in in vitro and rodent models [9,18]. This finding was, however, in line with other rodent studies in which the higher omega-3 exposure was restricted to the gestation and lactation periods [19] and adds to the disparity observed in reports of human [43] as well as animal studies [44] investigating the role of increased maternal dietary omega-3 on offspring body composition. The increased gonadal fat weight of the low LA (18% fat) group at 4 weeks of age coincided with increased *Fasn* expression in this tissue, suggesting that the higher gonadal fat deposition may have been driven by an increased capacity for de novo lipogenesis in this group. Interestingly, however, this increased *Fasn* expression was only observed in females, raising the possibility of different underlying mechanisms for increased gonadal fat accumulation between males and females which has also been suggested by previous work [45].

Interestingly, and despite no effects on relative fat mass, expression of lipogenic genes in the retroperitoneal fat depot appeared to be more susceptible to maternal dietary effects compared to the gonadal fat depot. In both male and female offspring at 4 weeks of age, there appeared to be a decrease in lipogenic capacity in offspring of dams fed high-fat diets. This apparent reduction in lipogenic capacity may be a compensatory response to mitigate the effects of a maternal high-fat diet and limit excessive fat accumulation in these groups which has been demonstrated in rodent models directly consuming a high-fat diet [46–49]. Alternatively, dietary PUFA can act as potent inhibitors of lipogenesis [50]. It may be that the high amount of PUFA that dams are consuming as part of the high-fat diets within this study was sufficient to reduce expression of lipogenic genes in the offspring. To our knowledge, few studies have investigated the effect of maternal dietary PUFA on offspring lipogenic capacity, and one such study found no effect of maternal high omega-3 diet on the expression of key lipogenic genes in the offspring [19]. Therefore, further studies are required to more fully understand the impact of maternal fat intake on lipogenesis in the offspring. Indications of reduced

lipogenic capacity were also apparent in the liver in offspring of dams exposed to a high-fat diet. A significant reduction in hepatic *Srebf1* expression was observed in the offspring of dams receiving a 36% fat diet; this was accompanied by proportionally lower liver weights in these groups at 4 weeks of age. In other models of maternal dietary insult, such as the low protein model, studies have shown that early reductions in the lipogenic capacity of tissues, through reduced gene expression, are often followed by an upregulation in lipogenesis between 9 and 18 months of age [51], but can occur much earlier if the individual encounters further dietary challenge [52]. Whilst this study only followed offspring until 8 weeks of age, some indications of this shift in lipogenic capacity were apparent in female offspring at this time point. Female offspring of dams exposed to a 36% fat diet exhibited increased *Fasn* levels suggesting increased de novo lipogenesis in these tissues. It is important to note, however, that although the genes investigated within this study are key regulators of lipogenesis, the list is not exhaustive, and inclusion of additional lipogenic genes, as well as genes involved in inflammatory pathways, would provide additional insights. Further to this, it will be of interest in future studies to determine whether the observed changes in phenotype and/or gene expression persist or change as the offspring age.

In conclusion, we have shown that, despite significant alteration in the ratio of omega-3 and omega-6 fatty acids in offspring of dams fed either a high or low LA diet, offspring growth and lipogenic capacity of adipose tissue are more susceptible to changes in the total fat content of the maternal diet rather than changes in the types of fats consumed. Whilst there appears to be more robust data supporting the beneficial effects of omega-3 fatty acids on mature adipocytes [11–13], their biological effects on developing adipose tissue are far less clear. Evidence suggesting beneficial or detrimental effects of the two families of PUFA in the maternal diet on offspring growth and adiposity, have largely been based on in vitro studies or animal experiments and recent data have suggested limited reproduction of these results in human trials [53]. Further studies are required to investigate the effects of maternal dietary PUFA on developing tissues but caution should be exercised in the meantime not to extrapolate from data on mature tissues and to highlight the detrimental effects of a maternal high-fat intake regardless of the types of fats consumed.

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Article

Fatty Acid Reference Intervals in Red Blood Cells among Pregnant Women in Norway–Cross Sectional Data from the 'Little in Norway' Cohort

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Abstract: There is a growing interest in determining fatty acid reference intervals from pregnancy cohort, especially considering the lack of reference values for pregnant women in the literature and the generalized misconception of equating reference intervals for nonpregnant women as equivalent to pregnant women. Seafood and supplements are important dietary sources for the omega-3 long-chain polyunsaturated fatty acids (ω-3 LCPUFA), such as eicosapentaenoic acid (EPA, 20:5ω-3), docosapentaenoic acid (DPA, 22:55ω-3), and docosahexaenoic acid (DHA, 22:6ω-3). Sufficient intake of EPA and DHA is vital during pregnancy for the development of the fetus, as well as for maintaining adequate levels for the mother. This study describes the fatty acid status and suggests reference values and cut-offs for fatty acids in red blood cells (RBC) from pregnant women (*n* = 247). An electronic food frequency questionnaire (e-FFQ) mapped the dietary habits of the participants, and gas chromatography was used to determine the fatty acid levels in RBC. The association between e-FFQ variables and fatty acid concentrations was established using a principal component analysis (PCA). Twenty-nine-point-one percent (29.1%) of the participants reported eating seafood as dinner according to the Norwegian recommendations, and they added in their diet as well a high percentage (76.9%) intake of ω-3 supplements. The concentration levels of fatty acids in RBC were in agreement with those reported in similar populations from different countries. The reference interval 2.5/97.5 percentiles for EPA, DPA, DHA were 0.23/2.12, 0.56/2.80, 3.76/10.12 in relative concentration units (%), and 5.99/51.25, 11.08/61.97, 64.25/218.08 in absolute concentration units (μg/g), respectively. The number of participants and their selection from all over Norway vouch for the representativeness of the study and the validity of the proposed reference values, and therefore, the study may be a useful tool when studying associations between fatty acid status and health outcome in future studies. To the best of our knowledge, this is the first PCA study reporting a direct association between ω-3 LCPUFA and intake of seafood and ω-3 supplements in a pregnancy cohort.

Keywords: fatty acid status; pregnancy; nutrition; biomarker; seafood intake; ω-3 supplement

1. Introduction

Nutrient deficiencies may lead to undesirable health outcomes. Pregnant women are considered vulnerable, as the mother is the sole provider of nutrients for the fetus [1–3]. During pregnancy and lactation, the maternal fatty acid status declines [4,5], which may lead to a suboptimal supply for the fetus, principally in cases where the dietary intake of these fatty acids is low or absent. In addition,

fatty acids are released from maternal adipose tissue stores to the fetus, especially docosahexaenoic acid (DHA, 22:6ω-3), and marginally change blood levels [3,6]. The rapid growth of the fetal brain during pregnancy and the first two years of childhood demand adequate levels of nutrients, such as the omega-3 long-chain polyunsaturated fatty acids (ω-3 LCPUFA), eicosapentaenoic acid (EPA, 20:5ω-3), and DHA. Experimental evidence suggests that DHA is the major structural and functional fatty acid in the central nervous system [5,7]. Consequently, the maintenance of maternal fatty acid supply is crucial.

Norway recommends a daily intake of 200 mg DHA for pregnant women [8]. Aquatic foods and ω-3 supplements are the main dietary sources of EPA and DHA [9]. Pregnant women are advised to follow the general dietary recommendations, which is to consume 300–450 g of fish per week, corresponding to fish or fish products for dinner 2–3 times per week, of which a minimum of 200 g should be fatty fish. There is inconsistency regarding the effects of DHA supplementation during pregnancy and in the early phase of infant cognitive development. Some research suggests a beneficial effect of DHA supplementation during pregnancy and/or lactation on mental development and on long-term cognition [10]. However, the evidence on cognitive development is inconclusive [11–15]. Recent studies have also concluded that low levels of ω-3 LCPUFA in the blood are a risk factor for early preterm birth and that an increased intake of ω-3 LCPUFA (via fish or supplements) is advisable [6,16]. Some studies suggested that pregnant and lactating women should consume 225–350 g (8–12 oz.) per week (~250–375 mg/day of EPA and DHA) of a variety of seafood [17]. However, a study on DHA and the increased risk for early preterm birth recommends a range of 600–800 mg/day of DHA for women with levels of DHA in red blood cells (RBC) lower than 5% [6]. Some authors who support the supplementation of ω-3 LCPUFA as an effective strategy for reducing preterm birth advise that a follow-up of completed trials is needed to assess long-term outcomes [18]. Lands and collaborators emphasize that careful handling of data on fatty acid composition is needed when interpreting evidence of dietary fatty acids on health outcomes [19].

Determination of fatty acid levels in RBC is a well-known approach for assessing fatty acid status as it reflects the last 30–60 days of intake [20]. EPA and DHA, accompanied by some other fatty acids, for example, short-chain fatty acids present in milk, are indirect biomarkers of specific foods as these foods are the primary dietary source of the respective fatty acids [21].

Reference intervals provide information on specific biomarkers in population-based cohort studies and offer a clear understanding of the initial status, as well as provide the basis for comparison over time. Most laboratories and scientific reference tables offer information derived from healthy nonpregnant women, but lack reference intervals for pregnant women. During pregnancy, there are changes in many biological markers, and therefore, reliable reference values derived from a healthy pregnant population are of importance for correct clinical decisions. Without adequate reference intervals, there is an increased risk of missing important changes, due to pathological conditions and to erroneously interpretation of normal changes as pathological events [22]. Hence, reference intervals are the most widely used tool for medical decision-making, therapeutic management decisions, and other physiological assessments [23,24]. The present study aims at suggesting reference intervals and cut-offs for fatty acids in maternal RBC on a sufficiently large healthy population that can be used in future studies to identify women who are at risk of adverse health outcomes as a result of under or overexposure to fatty acids. In addition, the relationship between the intake of seafood and ω-3 LCPUFA, generally characterized as poor in many pregnancy cohort studies [25,26], is thoroughly investigated using a principal component analysis.

2. Materials and Methods

2.1. Study Design

The present research is based on data from the national Little in Norway (LiN) cohort project (ISRCTN registry number 66710572) that is a cross-disciplinary prospective longitudinal study starting in

pregnancy. The overall study design for the LiN-cohort has been described in more detail elsewhere [27]. The LiN-cohort included nine health care centers from northern, mid, western, and eastern Norway (Table 1).

Table 1. Background characteristics of the population.

Maternal age (years)	30.1 ± 4.6 *
Gestation (weeks)	16–32
Median (weeks)	28
Range (weeks)	17–40
	%
Body mass index (BMI **) in kg/m^2	
<18.5	3.5
18.5–24.9	68.8
≥25	27.7
Educational level	
<4 years of higher education [†]	60.7
≥4 years of higher education	39.3
Marital status	
Living with partner/married	96.8
Not living with partner/other	3.2
Use of smoke/snuff tobacco during pregnancy	
Yes	6.5
No	93.5
Percentage of population per region	
Northern Norway	13.8
Mid Norway	31.6
Western Norway	30.4
Eastern Norway	24.3

* mean ± standard deviation; ** BMI estimated for *n* = 202; [†] University or University College.

The study was conducted from September 2011 to October 2012 according to the guidelines laid down in the Declaration of Helsinki. The procedures involving human subjects were approved by the Regional Committees for Medical and Health Research Ethics in Norway (REK 2011/560). Informed written consent was obtained from all subjects participating in the study. The flow of participants and data relevant for this research is outlined in Figure 1.
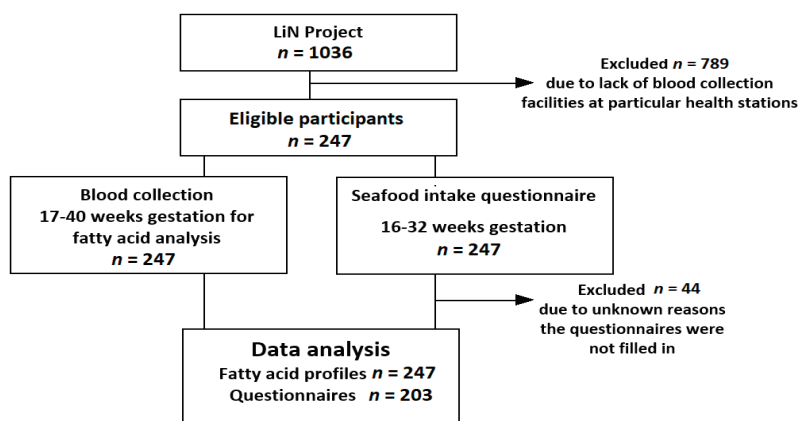

Figure 1. Flow chart of the study population, including reasons behind patient exclusion and refusals.

2.2. Population

Pregnant women (*n* = 247), at different gestational periods and from different geographical regions in Norway (Figure 2), were recruited and their blood collected at the first prenatal appointment in the health centers. The characteristics of the population, including age, gestational weeks, and demographic information is presented in Table 1.
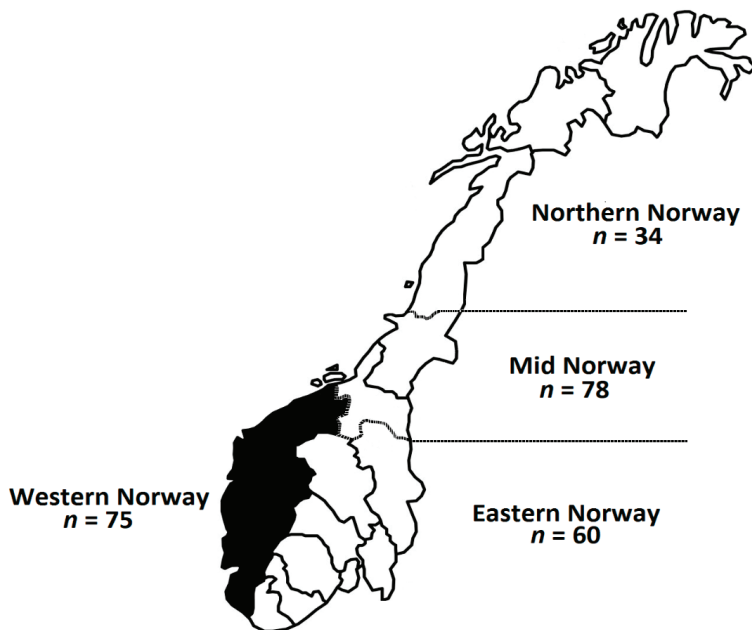

Figure 2. Norwegian map showing the geographical location and distribution of the participants (*n* = 247).

2.3. Dietary Assessment

The validated electronic semi-quantitative food frequency questionnaire (e-FFQ) [28] was implemented on 203 out of the 247 participants to determine the dietary intake of seafood between gestational weeks 16 and 32. The e-FFQ considers questions, such as "How often have you consumed fish, fish products or other seafood as lunch, spread or snack meal during the last three months?" and also a question regarding intake of ω-3 supplements, with the alternatives "yes" and "no". The e-FFQ was designed to capture the whole seafood diet, including seafood from all meals during the day [28]. Educational level, demographic information, and tobacco use questions are also included in the e-FFQ. The participants were anonymized by giving a unique ID number and corresponding password for entering the electronic questionnaire. It is important to mention that before starting the LiN project, all the available brands of omega-3 supplements in Norway were analyzed, and the results were published elsewhere [29]. The fatty acid composition in mg/capsule of the different brands of ω-3 supplements that were consumed by the participants is reported in Table S1. The declared content of a capsule was always 1 g of oil. The minimum/maximum levels of EPA, DPA, DHA, and EPA+DHA in mg/capsule were 174.55/282.65, 25.05/41.45 167.60/190.75, and 349.65/457.00, respectively (Table S1).

2.4. RBC Collection

The sample collection procedure has been described elsewhere [4]. Briefly, non-fasting venous blood samples from the participants were collected by venepuncture in 4 mL BD Vacutainer K_2EDTA (7.2 mg) vials (Becton, Dickinson and Company, Franklin Lakes, USA) at the first prenatal appointment.

The vials were centrifuged (1000–1300× *g*, 20 °C, 10 min) within 30 min. The RBC were adequately separated from plasma and buffy coat to ensure a clean RBC fraction. The samples were stored at the sites of the collection at −18 °C for up to a maximum of four weeks, and thereafter shipped to the Institute of Marine Research (IMR) in Bergen, Western Norway, for further storage at −80 °C prior to analysis. Regarding the stability of the fatty acids in the RBC samples, some studies recommend temperatures between 1 °C and 6 °C to preserve RBC quality for up to 42 days [30]. In addition, a recent pilot biobank study, at IMR, demonstrated that fatty acid profiles from RBC, with or without antioxidants, remain stable for up to 13 weeks at −20 °C and −80 °C [31].

2.5. Fatty Acids

The preparation of the fatty acid methyl ester (FAME) is an accredited method granted by the Norwegian Accreditation Authority and published elsewhere [32]. Briefly, 50 μL of the RBC sample was mixed with 2 mL BF_3 in methanol, and 5 μg of 19:0 internal standard. The mixture was heated at 100 °C for 1 h and cooled until it reached room temperature. Aliquots of 1 mL of hexane and 2 mL of H_2O were added, vortex-mixed for 15 s, placed in a centrifuge at 1620× *g* for 2 min, and the hexane phase (containing the FAME) was collected, evaporated under nitrogen, dissolved in hexane, and submitted to gas chromatography analysis at IMR on a Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer, Norwalk, CT, USA) equipped with a liquid autosampler and a flame ionization detector. The FAME samples were analyzed on a CP-Sil 88 capillary column (50 m × 0.32 mm I.D. 0.2 μm film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer TotalChrom Data System software version 6.3 (Perkin-Elmer, Somerset, MA, USA). The temperature program was as follows: The oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C/min, held at 160 °C for 28 min, ramped to 190 °C at 25 °C/min, held at 190 °C for 17 min, ramped to 220 °C at 25 °C/min and finally held at 220 °C for 10 min. The direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C, and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 82 Kpa. The analysis time was 60 min. This time interval was sufficient to detect FAME with chains from 10 to 24 carbons in length. The FAME peaks were identified by comparing their retention times with the retention times of highly purified FAME standards. The fatty acid results were expressed as relative (%) and absolute (mg/g RBC wet weight) units. The omega-3-index was calculated as the sum of EPA and DHA in relative units (Σ(%EPA + %DHA) [33].

2.6. Statistics

An Excel-based platform (Table S2) was developed for the automatic analysis of the chromatographic data. The Excel-based platform consists of three workbooks: (1) Data entry, where a maximum of five fatty acid concentration profiles can be entered; (2) FA distribution per station, where the distributions of the different fatty acids at the different health care stations are displayed automatically; (3) total FA distribution, to visualize automatically whether the total number of measured concentrations (*n* = 247) for specific fatty acids are normally distributed. The percentiles of the fatty acids were derived from the normal distribution. After transforming the e-FFQ nominal variables into numerical values, they were submitted to principal component analysis (PCA) along with the chromatographic data to detect meaningful relationships between the different fatty acids and the intake of seafood and ω-3 supplements. Statgraphics Centurion XVI (Version 16.1.11, StatPoint Technologies, Inc., Warrenton, VA, USA) was used for data analysis.

3. Results

3.1. Characterization of Study Population

Demographic information of the population, such as age, gestation period, body mass index (BMI), education, marital status, smoking habits (Table 1), and geographical region (Figure 2), are

described. The different characteristics were estimated from the total number of participants (*n* = 247), except the BMI values that were estimated from 202 participants and categorized as underweight (3.5%), normal weight (68.8%), and overweight (27.7%). All the participants attended university or university college, and the majority of them (86.2%) were located in geographical regions under Northern Norway (13.8%) (Table 1, Figure 2).

3.2. Seafood Intake

The results of the e-FFQ (Table 2) revealed that 76.4% (47.3 + 29.1) of the pregnant women consumed seafood as dinner with a frequency of 1–3 times/week. However, only 29.1% of the participants were following the Norwegian recommendations of seafood intake as dinner 2–3 times/week (Table 2). A percentage of 4.4% of the population reported a frequency intake of seafood as dinner lower than once a month, and from this group of participants, only 2.5% reported consuming ω-3 supplements. For the intake of seafood as spread or snack, similar frequencies were reported for 1–3 times per month (27.6%) and 1–2 times per week (29.1%), and they were ascribed to a relatively high intake of bread and spread in Norway. These particular frequency groups, reported the highest intake of ω-3 supplements, 20.2% (1–3 times per month) and 24.6% (1–2 times per week).

Table 2. Intake frequencies for seafood as dinner, seafood as spread/snack and omega-3 (ω-3) supplements among Norwegian pregnant women (*n* = 203). Unbracketed and bracketed figures represent the actual number of participants and the corresponding percentage (%).

	Assigned Score for PCA *		ω-3-Supplement Intake Distribution	
			Yes	No
Seafood as dinner				
<1time/month	1	9 (4.4)	5 (2.5)	4 (2.0)
1–3 times/month	2	35 (17.2)	27 (13.3)	8 (3.9)
1 time/week	3	96 (47.3)	74 (36.5)	22 (10.8)
2–3 times/week	4	59 (29.1)	46 (22.7)	13 (6.4)
≥4 times/week	5	4 (2)	4 (2.9)	
Seafood as spread or snack				
Never	1	24 (11.8)	15 (7.4)	9 (4.4)
Rare	2	45 (22,2)	35 (17.2)	10 (4.9)
1–3 times/month	3	56 (27.6)	41 (20.2)	15 (7.4)
1–2 times/week	4	59 (29.1)	50 (24.6)	9 (4.4)
3–5 times/week	5	17 (8.4)	15 (7.4)	2 (1.0)
≥5 times/week	6	2 (1)		2 (1.0)
Total ω-3-supplement intake	0 or 1		156 (76.9)	47 (23.2)

* PCA: principal component analysis.

3.3. Fatty Acid Status

A total of 247 fatty acid concentration profiles were estimated from seven health stations (two out of the total nine health stations lacked facilities for blood collection and sample preservation) and reported in both relative (%) and absolute (mg/g) units (Table S3). The relative concentrations of the fatty acids (14:0, 16:0, 18:0, 22:0, 16:1, 18:1, 24.1ω-9, 18:2ω-6, 20:3ω-6, 20:4ω-6, 22:4ω-6, 18:3ω-3, 20:5ω-3, 22:5ω-3 and 22:6ω-3) at the different health stations were normally distributed. After demonstrating data normality at the different stations, the concentrations of the different fatty acids (unsaturated, monounsaturated, and polyunsaturated) were added together, and graphs of the probability density function against the concentration of fatty acid in the relative unit (%) were plotted (Figure 3) and used for computing the corresponding percentiles (Table 3). Although the distributions of the fatty acids in mg/g units are not shown, they were also normally distributed. The reader can automatically generate the normal distributions (for % or mg/g) by copy-pasting the experimental results in Table S3 into the provided calculation platform in Table S2.
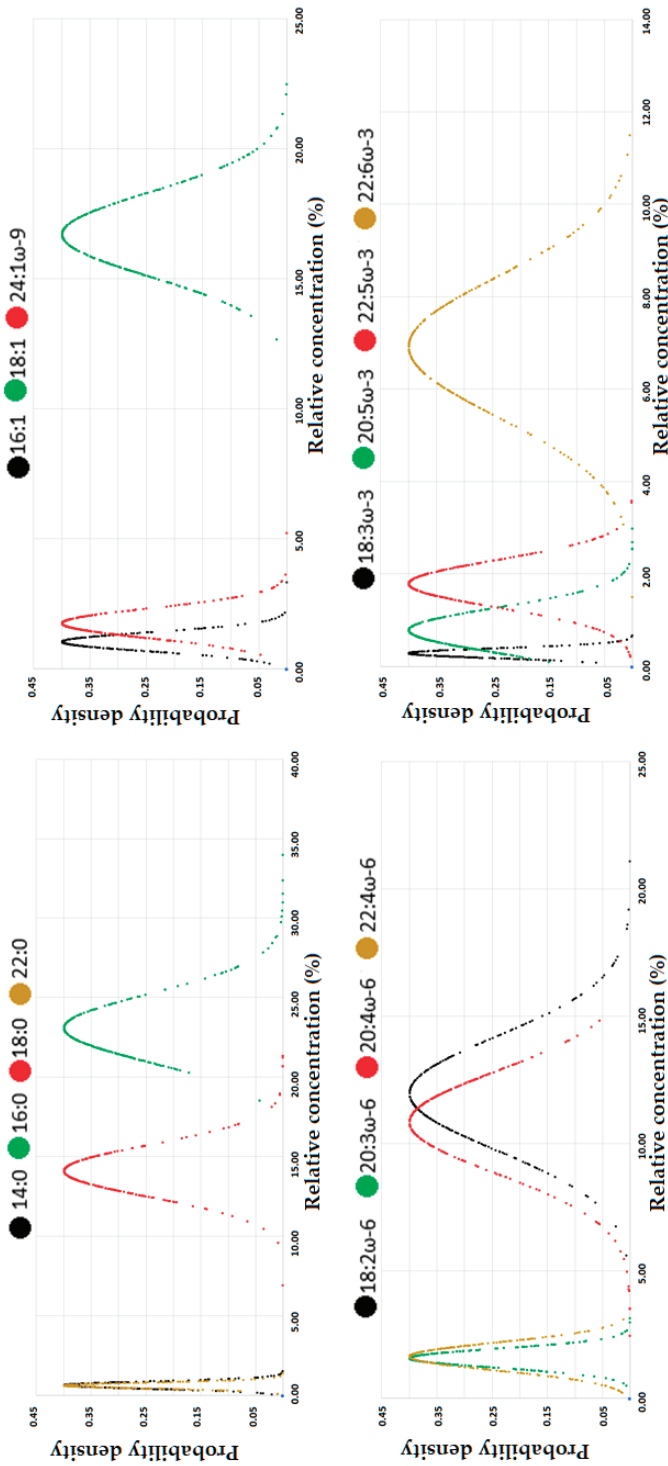
Figure 3. Normal distribution of the different groups of fatty acids (unsaturated, monounsaturated, and polyunsaturated). The graphs can be generated automatically (for % or mg/g) by copy-pasting the experimental results in Table S3 into the provided Excel-based platform in Table S2.

Table 3. Percentiles for selected fatty acids in red blood cells from pregnant women (n = 247) were recruited all over Norway.

		Percentiles (%)									Mean	Percentiles (µg/g)								
	Mean	2.5	5	10	25	50	75	90	95	97.5		2.5	5	10	25	50	75	90	95	97.5
%																				
14:0	0.65	0.27	0.35	0.39	0.49	0.62	0.77	0.96	1.11	1.22	14.75	7.14	7.48	8.68	10.38	13.48	17.33	22.24	26.97	30.66
16:0	23.10	20.60	20.83	21.19	21.72	22.70	23.68	25.51	26.96	29.62	509.41	400.25	413.49	430.96	458.06	500.95	548.11	599.90	623.18	652.36
18:0	14.12	11.72	12.22	12.56	13.16	14.04	14.82	15.55	16.85	18.82	308.39	266.68	277.86	282.71	290.24	307.58	324.14	339.64	348.88	359.40
22:0	0.63	0.31	0.34	0.40	0.50	0.61	0.74	0.86	0.94	1.00	13.79	7.26	8.24	9.34	11.18	13.73	16.30	18.46	19.91	21.21
16:1	1.04	0.36	0.51	0.65	0.84	0.98	1.20	1.50	1.67	1.90	23.58	7.54	10.33	13.27	17.08	21.07	27.79	35.97	43.36	47.43
18:1	16.71	14.00	14.45	14.80	15.45	16.58	17.63	18.88	19.68	20.18	371.49	264.10	280.12	298.38	319.83	358.57	409.14	476.29	510.59	536.99
24:1ω-9	1.75	0.88	1.07	1.17	1.34	1.64	2.01	2.50	2.76	3.13	38.07	20.36	22.90	25.49	30.42	36.73	44.04	51.83	57.73	64.76
18:2ω-6	11.98	8.34	9.01	9.52	10.55	11.52	13.13	14.74	16.10	17.08	268.62	160.28	173.28	191.40	220.38	255.68	301.01	359.55	402.95	453.61
20:3ω-6	1.57	0.93	1.06	1.14	1.35	1.56	1.76	2.05	2.19	2.41	35.03	17.29	20.70	24.04	29.43	34.13	40.77	46.03	49.96	54.82
20:4ω-6	10.84	5.05	6.87	8.56	9.95	11.28	12.11	12.77	13.14	13.52	240.27	92.32	135.45	182.19	223.29	247.75	273.12	289.50	297.74	313.66
22:4ω-6	1.64	0.41	0.69	0.91	1.31	1.67	2.03	2.31	2.44	2.62	36.31	10.00	13.49	20.00	28.77	37.33	45.15	50.55	55.20	57.91
18:3ω-3	0.29	0.13	0.15	0.17	0.21	0.27	0.35	0.44	0.51	0.54	8.70	5.14	5.32	5.51	6.50	9.49	10.00	10.63	12.27	13.52
20:5ω-3	0.79	0.23	0.27	0.32	0.45	0.64	1.01	1.33	1.82	2.12	17.86	5.99	6.61	7.83	10.00	15.06	22.17	29.85	40.73	51.25
22:5ω-3	1.79	0.56	0.83	1.26	1.53	1.82	2.09	2.36	2.65	2.80	39.71	11.08	17.34	28.62	34.32	40.41	45.75	52.72	55.66	61.97
22:6ω-3	6.92	3.76	4.18	5.14	5.98	6.94	7.86	8.63	9.38	10.12	152.74	64.25	82.84	114.74	135.66	153.41	172.38	194.38	209.45	218.08
Omega-3 Index *	7.71	4.14	4.66	5.53	6.60	7.70	8.86	9.87	10.67	11.90										
ω6/ω3	2.71	1.63	1.85	1.97	2.29	2.65	3.03	3.54	3.76	3.93										
Total ω-6	26.65	17.83	21.01	23.44	25.62	27.16	28.52	29.74	30.34	30.85										
Total ω-3	10.24	5.68	6.28	7.85	8.89	10.32	11.67	12.66	13.33	15.14										

* Omega-3 Index is defined as the summation of 20:5ω-3 and 22:6ω-3.

The most concentrated saturated, monounsaturated, ω-6 polyunsaturated fatty acids (PUFA) and ω-3 PUFA in Table 3 were 16:0, 18:1, 18:2ω-6, and 22:6ω-3, respectively. The computed median/average ratios for these major fatty acids were 0.98 (22.7/23.1), 0.99 (16.6/16.7), 0.96 (11.5/12.0), and 1.00 (6.9/6.9) in % units and 0.98 (500.9/509.4), 0.97 (358.6/371.5), 0.95 (255.7/268.6), and 1.00 (153.4/152.7) in µg/g units. Similarly, the rest of the fatty acids exhibited mean/average ratios close to 1.00, indicating that the graph's probability density versus concentration (Figure 3) provides a good approximation of the sampling distribution of the fatty acid of interest. A comparison of the results in Table 3 with those reported in similar studies was performed, and presented in Table 4.

The PCA of the e-FFQ and fatty acid data was performed after transforming the e-FFQ nominal data into numerical variables. The transformation consisted of assigning scores of 1 (lowest frequency), 5 or 6 (highest frequency) to the seafood frequency, and scores of 0 (negative answer) or 1 (affirmative answer) to the intake of ω-3 supplements (Table 2). The PCA revealed a positive correlation between EPA (20:5ω-3), docosapentaenoic acid (DPA, 22:5ω-3), DHA (22:6ω-3), the intake of seafood (designated as WI and WII variables in Figure 4) and the intake of ω-3 supplements (designated as WIII variable in Figure 4). These variables (EPA, DPA, DHA, WI, WII, WIII) are close to each other (framed in black in Figure 4) and display negative PC3 values. In contrast, the ω-6 PUFA, more specifically 20:3ω-6, 20:4ω-6, and 22:6ω-3 (framed in green in Figure 4) do not correlate with the e-FFQ variables and display positive PC3 values, which in turn discriminates the ω-3 PUFA. Linoleic acid (LA, 18:2ω-6) and alpha linolenic acid (ALA, 18:3ω-3) emerge as a cluster (framed in blue in Figure 4) and do not exhibit any association with the ω-6 and ω-3 PUFA or any of the studied e-FFQ variables. The remaining fatty acids were independent of the intake of seafood or ω-3 supplements, as observed in Figure 4.
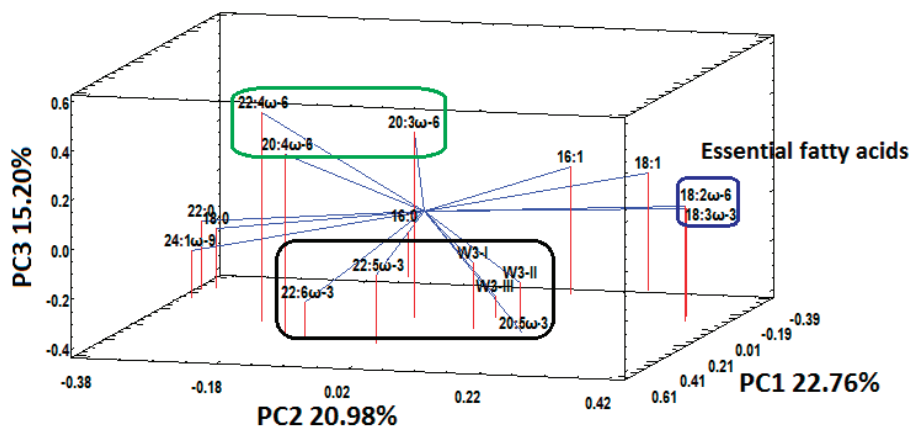

Figure 4. Principal components 1, 2 and 3 (PC1, PC2 and PC3, respectively) to study the correlation between selected fatty acids in maternal red blood cells and electronic food frequency questionnaire (e-FFQ) variables (WI = seafood as dinner, WII = seafood as spread or snack, WIII = ω-3 supplements) as. There is an association between 20:5ω-3 (EPA), 22:5ω-3 (DPA), 22:6ω-3 (DHA) and WI, WII, WIII (black frame), while their ω-6 counterparts (green frame) and essential fatty acids (blue frame) do not correlate with the e-FFQ variables.

4. Discussion

The applied e-FFQ was not focused on ω-3 fatty acids originating from plants, but from the habitual intake of seafood (fish and shellfish) and the use of dietary supplements, because the endogenous metabolization of ALA (18:3 n-3) from plants to ω-3 PUFA (e.g., EPA, DPA, and DHA) is minimal. Furthermore, the e-FFQ considered different forms of seafood individually. For example, the indexes for dinners were grouped into five categories comprising dinner items of oily fish, lean fish, shellfish,

processed fish, and freshwater fish. Additionally, freshwater fish consumption was divided into two separate questions, frequency of perch/pike (lean fish) and frequency of char/whitefish (oily fish) [28].

The e-FFQ indicated that 29.1% of the participants reported an intake of fish for dinner that was in accordance with dietary guidelines from the Norwegian Directorate of Health (Table 2). However, a high percentage of participants from all the assessed groups (under and over seafood as dinner 2–3 times/week) reported the intake of ω-3 supplements. In addition, it was remarkable that the intake of ω-3 supplements was almost identical (around 77%) for all the observed groups, 1–3 times per month ($27/35 \times 100 = 77.14\%$), one time per week ($74/96 \times 100 = 77.08\%$) and 2–3 times per week ($46/59 \times 100 = 77.97\%$), as shown in Table 2. The high intake of omega-3 supplements in this particular cohort of Norway is in accordance with global awareness towards the beneficial effects of these dietary products as they improve the levels of omega-3 PUFA by covering dietary seafood shortfalls, particularly for those who dislike the taste or smell of fish.

The observed frequencies for gestational weeks 16 and 32 of 68.97, 29.06 and 76.85% for the categories seafood intake under dietary guidelines ($n = 140$), 2–3 times/week ($n = 59$) and intake of ω-3 supplements ($n = 156$), respectively (Table 2) are consistent with those reported for gestational week 22 and 32 by The Norwegian Mother and Child Cohort Study ($n = 67007$) of 60.06, 23.47 and 63.95 for the categories seafood intake under 2–3 servings/week ($n = 40244$), seafood intake of 2–3 servings/week ($n = 15724$) and intake of ω-3 supplements ($n = 428852$), respectively [34]. In addition, the observed 29.06% frequency (for those Norwegian pregnant women (30.1 ± 4.6 years) in accord with the national dietary guidelines), is in close agreement with the latest national dietary survey conducted among adults in Norway (2010–2011) where women in the age group 30–39 reported a frequency of 21% for the intake of seafood for dinner three times per week or more [35]. The agreement with previous studies confirms the robustness of the semi-quantitative e-FFQ to assess the dietary intake of seafood and ω-3 supplements.

The PCA plot (Figure 4) detected a correlation between the ω-3 PUFA and the e-FFQ variables, and it discriminated the ω-6 and ω-3 PUFA into three clusters that can be intuitively explained, as follow: The concentration levels of 20:3ω-6, 20:4ω-6, and 22:4ω-6 (inside the green frame in Figure 4) and 20:5ω-3, 22:5ω-3 and 22:6ω-3 (inside the black frame in Figure 4) reflect both endogenous (de novo lipogenesis) and exogenous (dietary intake) sources; whereas, the concentration levels of essential fatty acids, such as 18:2ω-6 and 18:3ω-3 (inside the blue frame in Figure 4), exclusively reflect the dietary intake of the participants. In addition, Figure 4 reveals that neither 18:2ω-6 nor 18:3ω-3 are correlated with any of the e-FFQ variables.

The associations between qualitative variables (e.g., frequency of consumption of fish, BMI, ethnicity, etc.) and fatty acids in plasma from pregnant adolescents (14–18 years old) by using PCA has been published elsewhere [36]. Although this particular study did not discuss in detail the PCA results, an analysis of its reported PC1 and PC2 loadings revealed that the association 20:4ω-6/fish was stronger than the association ω-3 PUFA/fish (e.g., 18:3ω-3, EPA, DPA); and also the lack of correlation between essential fatty acids (e.g., 18:3ω-3, 18:2ω-6) which should exclusively reflect the dietary intake. In general, studies on the association between food intake variables from FFQ and fatty acids from pregnant women, by using techniques different to PCA, have consistently demonstrated poor correlations between dietary fatty acid intake and blood levels [25,26]. The present pregnant cohort study is the first to report a clear association between e-FFQ variables and fatty acids in RBC from the pregnant cohort by using PCA.

Except for 18:2ω-6, the sequence of most concentrated fatty acids reported in the present study (16:0, 18:1, 18:2ω-6, 22:6ω-3) has been also observed in studies with pregnant women from Belgium [37], Netherlands [38], Germany [39], and Japan [40,41]. In these countries, the major ω-6 PUFA was 20:4ω-6, and its level was consistently higher than 18:2ω-6 by 69.6, 2.8, 1.0, and 28.2% (average from References [40,41]), respectively; whereas, in the present study 20:4ω-6 was lower than 18:2ω-6 by 9.5%. Possible explanations behind the observed reduction in the present study might be the high intake of ω-3 supplements (76.9%) compared to the studies from Belgium (24.6%), Netherlands (14.3%),

Germany (20%), and Japan (2.2% in Reference [41]). In addition, an analysis of the estimated global seafood consumption per country [42] by the time these specific studies were performed indicated that Norway had the highest seafood consumption per capita (52.9 Kg in 2012) compared to Belgium (23.8 Kg in 2016), Netherlands (22.11 Kg in 2000), Germany (14.3 Kg in 2011) and Japan (48.6 Kg in 2013).

In the present study, the ω-3 PUFA sequence ranked from lowest to highest concentration was 18:3ω-3, 20:5ω-3, 22:5ω-3, and 22:6ω-3. This specific sequence is in agreement with similar studies from the Netherlands [38], Germany [39], and Japan [41]. Other studies from Japan [40], Belgium [37], and Iceland [43] have not reported the concentration levels of 18:3ω-3 or 22:5ω-3. However, in these studies, the declared ω-3 PUFA followed the aforementioned order.

In general, the range of concentrations for selected fatty acids in RBC from pregnant women in Table 3 is in agreement with reported median or average values in similar studies from different countries, as indicated in Table 4 in green color. However, in some countries, the levels of particular fatty acids were distinct from the 2.5 or 97.5 percentiles of the present study, as indicated in Table 4 in yellow and red colors, respectively. The reasons behind the observed discrepancies are beyond the scope of the present article.

Table 4. A comparison of reported mean [37–39,43] or median [40,41] concentrations (%) of fatty acids in different countries with those selected in the present study. The symbol × indicates that the fatty acid is not reported in the particular reference number. The green, yellow and red colors denote: Between 2.5 and 97.5 percentiles, under 2.5 percentile and over 97.5 percentile of the present study, respectively.

	Belgium [37]	Iceland [43]	Germany [39]	Japan [41]	Netherland [38]	Japan [40]
14:0	green	×	green	green	×	×
16:0	green	×	red	green	×	green
18:0	green	×	green	green	×	green
22:0	×	×	×	red	×	red
16:1	yellow	×	×	yellow	×	×
18:1	green	×	green	yellow	×	green
24:1ω-9	×	×	×	red	×	red
18:2ω-6	green	green	green	green	green	green
20:3ω-6	green	green	×	green	green	green
20:4ω-6	red	green	green	green	green	green
22:4ω-6	×	green	yellow	green	red	green
18:3ω-3	green	×	green	green	green	×
20:5ω-3	green	green	green	green	green	green
22:5ω-3	×	green	green	green	green	green
22:6ω-3	green	green	yellow	green	yellow	green

Some studies have indicated that values ≥8% or <5% are associated with the lowest risk for cardiovascular events [44] or the highest risk of depressive episodes [45], respectively. Despite these observations, an optimal range of omega-3 index for pregnant women has not been defined yet. A recent study has indicated that no human being has an omega-3 index <2% [44]. Contrary to this observation, in the present study that involved only healthy pregnant women, a participant (hereinafter referred to as p#159) with an omega-3 index of 1.93% was recorded. The relative concentrations of EPA (0.43%) and DHA (1.50%) for p#159 were allocated inside the range and under the lowest percentiles for these fatty acids (Table 3). In addition, p#159 exhibited the largest DPA concentration level (3.59%). A close inspection of the same fatty acids in μg/g units for p#159 revealed that EPA, DHA, and DPA were allocated in the 55, 35, and 55 percentiles, respectively, and consequently, the values in μg/g units are inside the range of the studied population. It is equally important to mention that the ω-6/ω-3 index is another key player in epidemiological studies that are generally associated with depression [45] and cardiovascular events [46]. Some studies have indicated that ω-6/ω-3 >9 is associated with postpartum depression [47]; whereas, an ω-6/ω-3 around 4 exerts cardioprotective effects [46]. Experimental evidence suggests that the optimum ω-6/ω-3 ratio must be kept around 4 and 5 and should not exceed 10 [48]. The computed ω-6/ω-3 ratio for p#159 was 3.84 (95 percentile in Table 3), and it can be regarded as optimum. The previous observations about the different indexes and measurement units, do not try

to draw general conclusions based on the results of just one participant, but to highlight the importance of a comprehensive evaluation of the implications in human health of the different indexes and their corresponding threshold not only from the perspective of relative units (%), but also absolute units (mg/g). In addition, it is important to highlight that published randomized trials have not provided conclusive evidence yet about the effect of ω-3/ω-6 PUFA on postpartum depression.

In the present research, 42% of the pregnant women had an omega-3 index above 8%. It was mentioned that this index plays a pathophysiologic role in depressive symptoms [45,49,50]. The International Society for Nutritional Psychiatry Research Practice Guidelines for ω-3 fatty acids has recently recommended therapeutic dosages of pure EPA or a combination of EPA and DHA (with net EPA starting from at least 1 up to 2 g/day) for at least eight weeks as a potential treatment for major depressive disorders [51]. We have previously shown that low omega-3 index in pregnancy is a possible risk factor for postpartum depression [52], with a cut-off at 4%. This cut-off is similar to the 2.5 percentile in Table 3 and in accordance with the cut-off for those at high risk of developing coronary heart disease [53]. Thus, the suggested reference values and omega-3 index cut-off could help to identify women who might benefit from increasing the dietary intake of EPA and DHA, like seafood and supplements that are important dietary sources of these long-chain PUFA, and hence, will influence their nutritional status. It must be mentioned that there are no specific recommendations on the intake of EPA or DHA for the general population, including prenatal women, in Norway [54].

Cohort studies for establishing national reference intervals for fatty acids in RBC of pregnant women are largely dependent, among other things, on the number of participants, the number of health stations, the geographical distribution of the health stations along with their inherent infrastructure for collecting and preserving samples long-term at appropriate temperatures. For instance, fatty acids in RBC are susceptible to degradation and remain stable for 42 or 91 days at 1 °C or −20°C, respectively [30,31]. Failure to comply with these requirements might be regarded as a drawback. Some of the apparent limitations of the present study are the lack of blood collection/preservation facilities (namely seven well-equipped facilities). However, most of the studies in Table 4 were performed in one specific geographical region by using just one blood collection facility. In some cases, the selected geographical regions represented a very low percentage of the total female population of the country in question. For example, the studies from Belgium [37], the Netherlands [38], and Germany [39] represented ~1.71, ~0.71%, and ~0.13% of the total female population, respectively. Moreover, the studies from Iceland [43] and Japan [40,41] constituted approximately 33.73 and 18.23% of the total female population, respectively, they were carried out in specific regions (Reykjavik and the Miyagi Prefecture), and they do not contain all the important characteristics of the country population from which they were drawn. The present study collected samples from the main geographical regions of Norway, which account for a ~91.5% of the targeted population. In addition, the present study with seven collection facilities has a higher level of enrolment per thousand pregnant women than Japan with 15 collection facilities, namely, 4.16‰ and 1.63‰ by the time these specific studies were performed, respectively. The expression $n = N/[1 + N(e/100)^2]$ (aka Slovin formula) [55], that is generally considered to estimate the sample size (n) given the population size (N) and a percentage of margin error (e) was used to judge whether $n = 247$ was an appropriate sample size. By the time the samples were collected (2011–2012), the parameter N was estimated using the Statistics Bureau of Norway's records of the average number of births (59410 ± 10) between 2011–2012 [56], while the parameter e was set at 7.5% (half the maximum margin of error of 15% proposed by IUPAC for monitoring fatty acid concentrations by gas chromatography [57]). A minimum value of $n = 177$ was calculated by introducing the aforementioned parameters in the Slovin expression, which in turn concluded that the sample size of the present research ($n = 247$) was sufficient to determine reliable reference intervals for fatty acids in maternal RBC. An important feature of a selected sample size should be its ability to make projections or generalizations regarding an entire population. The information in Table 1 and Figure 2 indicates that pregnant women were recruited from all over the Norwegian territory, which emphasizes the strength and representativeness of the sample size, and consequently, the validity of the proposed reference values in the present

study. The previous observations indicate that there is not any suspicion of misrepresentation of the population of interest in the present study.

5. Conclusions

Reference intervals and cut-offs for fatty acids in RBC from a pregnancy cohort from all over Norway and in agreement with those reported in other countries were established. A direct association between ω-3 LCPUFA (EPA, DPA, DHA, but not ALA) in maternal RBC and the intake of seafood and ω-3 supplements was found. The findings from the e-FFQ were in accordance with national surveys and highlighted the awareness of the participants about the importance of dietary ω-3 in maternal health. Given the importance of seafood and ω-3 supplements during pregnancy, further studies are warranted to investigate comprehensively the impact on the health of the various indexes (e.g., omega-3 index, ω6/ω3) associated with fatty acid status and by using relative and absolute units. The proposed reference intervals in RBC may be a useful tool when studying associations between fatty acids and health outcomes.

Abbreviations

ALA	=	Alpha Linolenic Acid
DHA	=	DocosaHexaenoic Acid
e-FFQ	=	electronic-Food Frequency Questionnaire
EPA	=	EicosaPentaenoic Acid
FAME	=	Fatty Acid Methyl Esters
HUFA	=	Highly Unsaturated Fatty acids
LCPUFA	=	Long-Chain Polyunsaturated Fatty Acids
LiN	=	Little in Norway
PCA	=	Principal Component Analysis
PC1	=	Principal Component 1
PC2	=	Principal Component 2
PC3	=	Principal Component 3
PUFA	=	Polyunsaturated Fatty Acids
ω-3	=	Omega-3
ω-6	=	Omega-6
ω-9	=	Omega-9
RBC	=	Red Blood Cells

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Multiple Micronutrients and Docosahexaenoic Acid Supplementation during Pregnancy: A Randomized Controlled Study

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Abstract: Maternal dietary intake during pregnancy needs to meet increased nutritional demands to maintain metabolism and to support fetal development. Docosahexaenoic acid (DHA) is essential for fetal neuro-/visual development and in immunomodulation, accumulating rapidly within the developing brain and central nervous system. Levels available to the fetus are governed by the maternal diet. In this multicenter, parallel, randomized controlled trial, we evaluated once-daily supplementation with multiple micronutrients and DHA (i.e., multiple micronutrient supplementation, MMS) on maternal biomarkers and infant anthropometric parameters during the second and third trimesters of pregnancy compared with no supplementation. Primary efficacy endpoint: change in maternal red blood cell (RBC) DHA (*wt%* total fatty acids) during the study. Secondary variables: other biomarkers of fatty acid and oxidative status, vitamin D, and infant anthropometric parameters at delivery. Supplementation significantly increased RBC DHA levels, the omega-3 index, and vitamin D levels. Subscapular skinfold thickness was significantly greater with MMS in infants. Safety outcomes were comparable between groups. This first randomized controlled trial of supplementation with multiple micronutrients and DHA in pregnant women indicated that MMS significantly improved maternal DHA and vitamin D status in an industrialized setting—an important finding considering the essential roles of DHA and vitamin D.

Keywords: docosahexaenoic acid; long-chain polyunsaturated fatty acids; maternal biomarkers; micronutrients; neurodevelopment; pregnant women; supplementation; vitamin D

1. Introduction

During pregnancy, an adequate maternal dietary intake is essential to meet the increased nutritional demands required to maintain metabolism and support fetal development [1]. Micronutrients such as folic acid and other B vitamins, vitamin D, vitamin C, calcium, copper, magnesium, iodine, selenium, zinc, and iron all have vital roles throughout all stages of pregnancy [2–4]. Poor dietary intake or deficiencies in both micro- and macronutrients can have adverse effects on pregnancy outcomes and neonatal health [5], including an increased risk of neural tube defects, preeclampsia,

miscarriage, and low birth weight [6,7]. Many women are at risk of insufficient nutrient intake in industrialized as well as developing countries [8–10]. Therefore, micronutrient supplementation is frequently recommended during pregnancy to help improve pregnancy outcomes in the mother and child [11,12]. International guidelines (i.e., from the World Health Organization) currently recommend supplementation of iron and folic acid (0.4 mg/day) during the whole pregnancy for the purpose of improving pregnancy outcomes and for reducing maternal anemia in pregnancy [13]. Recently, there have been extensive scientific and medical discussions around the need to include vitamin D as a standard nutrient to be supplemented during pregnancy, due to low intake. Vitamin D regulates calcium and phosphate body stores and is therefore critical for bone health [14]. Furthermore, low concentrations of blood vitamin D in pregnant women have been associated with pregnancy complications [15,16].

In addition to micronutrients, a balanced macronutrient intake is recommended. In particular, the long-chain polyunsaturated fatty acids (LCPUFAs) found at high concentrations within the brain and central nervous system are essential for the development of the fetal brain [17]. Docosahexaenoic acid (DHA)—representing the largest proportion of LCPUFAs in the brain and retina—plays a key role during the pre- and early postnatal period [17–20]. After the first trimester, when the neural tube has closed and grey matter begins to form [21], DHA begins to rapidly accumulate in the brain [18,22]; accumulation continues for up to two years [23,24].

However, the human body is not efficient at producing essential LCPUFAs [22], and maternal concentrations decrease over the course of gestation [25]. Of note, the levels of DHA available to the fetus during pregnancy are governed by the diet of the mother [17,26–28]. Studies suggest that consumption of a diet rich in omega-3 LCPUFAs including DHA may have a reduced risk of common pregnancy complications such as intrauterine growth restriction, preeclampsia, and preterm deliveries [29–31]. Supplementation with DHA can also increase the expression of fatty acid transport proteins, thus increasing transport through the placenta and improving the fatty acid status of both the mother and child [32,33].

Meta-analyses have demonstrated that there are clinical benefits associated with prenatal multiple micronutrient [34] and LCPUFA supplementation [35] during pregnancy. However, there is limited data on the effects of prenatal supplementation in industrialized countries, particularly when used in combination. Clinical guidelines for pregnant women tend to focus on single nutrients for supplementation [36,37]. Given the interest in the potential beneficial effects of supplementation with micronutrients and DHA during pregnancy, we carried out a randomized trial to evaluate the effects of multiple micronutrients plus DHA supplementation during the second and third trimesters of pregnancy on maternal biomarkers compared with no supplementation in the control group in an industrialized country. The primary variable, i.e., the concentration of DHA (weight percent of total fatty acids (*wt*% TFA)) in maternal red blood cells (RBC), was considered indicative of LCPUFA status. Secondary explorative variables were other biomarkers of fatty acid and oxidative status, vitamin D, and anthropometric parameters of infants at delivery. We included vitamin D status as a secondary endpoint to investigate whether vitamin D supplementation is needed to maintain adequate status, and whether the levels of vitamin D in the supplement would be sufficient to maintain an adequate status. We hypothesized that supplementation might help to improve maternal DHA and vitamin D status in a healthy population of pregnant women, whereas dietary intake would be insufficient to meet the increased needs during pregnancy.

2. Materials and Methods

2.1. Trial Design

This was a multicenter, parallel, randomized controlled trial conducted at two centers in Italy to compare the effects of once daily supplementation with multiple micronutrients plus DHA (hereafter referred to as multiple micronutrient supplementation, or MMS) versus no supplementation during

pregnancy on maternal biomarkers and infant anthropometric parameters. Supplementation began at gestational week 13–15 until delivery. Six visits were conducted during the trial, from screening to final follow-up, as outlined in Figure 1 and Supplementary Table S1. At baseline (Visit 2; gestational week 13–15), women who fulfilled the eligibility criteria were randomized to the supplementation or control group in a 1:1 ratio. The sequential randomization list (generated through a validated SAS program by an independent statistician) was generated according to permutated block codes. A randomization number was assigned to each woman at each site by means of randomization cards. The study was not blinded. All blood parameters were measured at Visits 1, 3, and 4 in all women, while dietary intake was recorded at Visits 2, 3, and 4.
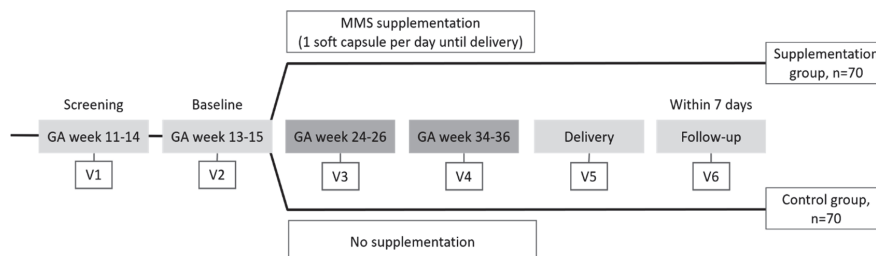

Figure 1. Study design. Visit 1 (V1, screening): pregnant women were screened for study eligibility and blood collection was performed. Visit 2 (V2, baseline): eligible women meeting the inclusion and exclusion criteria were randomized equally to one of the two study groups; nutritional status was assessed using a semi-quantitative FFQ. Visits 3 and 4 (V3 &V4, MMS supplementation or no supplementation): FFQ was administered and blood sampling took place—the red blood cell DHA level measured at Visit 4 was compared with the value measured at Visit 1 to assess the primary endpoint. Visit 5 (V5, delivery): obstetric evaluations were performed in all women and infant anthropometric parameters were measured. Concomitant medications and adverse events were assessed at all Visits. GA, gestational age; DHA, docosahexaenoic acid; FFQ, food frequency questionnaire; MMS, multiple micronutrients and DHA supplementation.

The study was approved by an independent ethics committee (Comitato Etico Milano, Milan, Italy). The Institutional Review board Project no. of the study: 2016/ST/024. The study was approved on 30 March 2016. The study was conducted in accordance with the Declaration of Helsinki and in compliance with all current Good Clinical Practice guidelines, local laws, regulations, and organizations. The trial was registered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT04438928). The trial protocol can be obtained from the corresponding author, upon reasonable request.

2.2. Study Population

Healthy, pregnant Caucasian women aged 18–42 years were screened during their first trimester prenatal visit (gestational age (GA), week 11–14) at Hospital Sacco and Hospital Buzzi in Milan, Italy. The study was proposed to all pregnant women with a singleton pregnancy within the gestational age indicated. Women were included in the study if they were having a singleton pregnancy, hemoglobin level >105 g/L, normal ultrasound examination, and inconspicuous fetal anomaly screening, taking at least 400 µg folate per day, and provided written, signed informed consent for participation in the study. Women were excluded if they had experienced previous adverse pregnancy outcomes, followed a specific diet, or were already taking DHA/multivitamin supplements (except folate or iron). Full inclusion and exclusion criteria are listed in Supplementary Table S2.

2.3. Study Product

The study product was an oral MMS soft gel capsule (Elevit, Bayer) that contained 12 vitamins, six minerals, and DHA (200 mg) to meet the requirements of women during pregnancy, especially during

the second and third trimester [38,39] (Supplementary Table S3). One capsule was taken per day with a sufficient amount of liquid, from GA week 13–15 (Visit 2, baseline) until delivery (Visit 5; approximately 27 weeks of supplementation). The control group did not receive a placebo during this time.

2.4. Parameters Assessed

Analyses were performed at the "Luigi Sacco" Department of Biomedical and Clinical Sciences (Università degli Studi di Milano) and ASST Fatebenefratelli Sacco, Milan, Italy. In total, approximately 56 mL of blood was taken in the fasted state from each subject for the efficacy and safety assessments during the whole study. Blood samples were centrifuged for 10 min at 1000 g at 4 °C; plasma for 8-isoprostane and dROMs analysis was separated from the erythrocyte pellet, and the buffy coat was discarded. Erythrocytes for fatty acid and glutathione analyses were washed once with a 0.2 M EDTA + 150 nM NaCl solution through gentle inversion, and then 15 min centrifugation at 2000 g at 4 °C.

The efficacy parameters assessed are outlined in Supplementary Table S4. The change in RBC DHA (*wt*% TFA) from Visit 1 to Visit 4 was the primary maternal variable to assess the beneficial effects of supplementation with micronutrients and DHA during the second and third trimesters of pregnancy. Secondary maternal variables included other RBC fatty acid parameters (TFA, eicosapentaenoic acid (EPA), *wt*% TFA, DHA/TFA ratio, and omega-3 index), calcidiol (25-hydroxyvitamin D), and oxidative stress markers in blood including reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio, plasma reactive oxygen metabolites (ROMs, which are hydroperoxides), and plasma 8-isoprostane. The erythrocyte membrane fatty acid composition was determined by gas chromatography of fatty acid methyl esters [40–42]. The amount of each considered fatty acid was calculated as μg/mL of RBCs and expressed as a percentage of the total fatty acid concentration. The omega-3 index was calculated by summing the percentage of EPA and DHA [43]. Calcidiol levels were measured using radioimmunoassay [44], the GSH/GSSG ratio using fluorimetric assay [1], ROMs using photometric assay [45,46], and 8-isoprostane using competitive enzyme immunoassay with an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions [47]. Briefly, 500 uL of heparinated plasma were stored at –80 °C with a preservative ethanol solution containing butylated hydroxytoluene (BHT) until analysis. Alkaline hydrolysis was performed to allow total 8-isoprostane (both free and esterified fractions) quantification; after neutralization, proteins were removed by ethanol precipitation and samples were purified by solid phase extraction (SPE) using octadecyl (C-18) silica affinity cartridges. Total 8-isoprostane levels in purified plasma samples were then analyzed by ELISA. Dietary intake was evaluated using a semi-quantitative Food Frequency Questionnaire of five food categories to assess the usual daily intake of foods and nutrients (adapted from Vioque et al. [48], which was validated in pregnant women) at Visits 2, 3, and 4. Dietary intake data and results of a small subgroup analysis in women who underwent a cesarean section (cord blood and placenta samples) will be presented elsewhere.

Safety and tolerability were assessed by evaluating the incidence and severity of adverse events (AEs) and their relationship to trial treatment. Laboratory parameters, physical examination, and vital signs were also recorded.

2.5. Statistical Analysis

Assuming a treatment difference of 1.6 (standard deviation (SD) 3.4), as observed by Bergmann et al. 2008 [49], 70 subjects per arm were required to achieve 80% power with 0.05 of alpha to detect the treatment difference between the supplementation and control groups. To account for a drop-out rate of 15%, approximately 164 subjects (82 per treatment group) were to be randomized to get 140 evaluable subjects.

The primary efficacy analysis was performed on the per protocol (PP) population (all subjects with efficacy data for the primary efficacy endpoint at Visit 4 who did not have protocol violations). Results were corroborated using data from the intent-to-treat (ITT) population (i.e., all subjects in

the safety population who had at least one post-baseline measurement of efficacy data). The safety population comprised all subjects who were randomized into the study, and took at least one dose of the supplement for those randomized to the treatment group.

The primary efficacy endpoint was defined as the change in maternal RBC DHA (*wt*% total fatty acids) from Visit 1 to Visit 4, analyzed using the analysis of covariance (ANCOVA) with treatment as a fixed effect and the Visit 1 value as covariate. Secondary maternal efficacy endpoints were changes from Visit 1 to Visit 4 in blood fatty acid parameters (RBC EPA (*wt*% total fatty acids), DHA/EPA ratio, RBC omega-3 index), 25-hydroxyvitamin D, and antioxidant status (GSH/GSSG ratio, plasma ROMs, 8-isoprostane). All secondary endpoints were analyzed similarly to the primary endpoint. Secondary infant efficacy endpoints (gestational age, head circumference, weight and length measurements, ponderal index, infant skinfold thickness, Apgar score, bone density) were collected at delivery (Visit 5) or within 10 days after delivery for bone density and analyzed using ANCOVA with treatment as fixed effect.

Safety and tolerability variables were assessed by evaluating incidence and severity of AEs, their relationship to trial treatment, and the incidence of abnormal findings in measurement of objective tolerability through vital signs, physical examination, and clinical laboratory findings. Only treatment-emergent AEs (TEAEs) were analyzed, i.e., AEs that began or worsened after randomization.

Two-sided p-values < 0.05 were considered statistically significant. Results are presented as mean ± standard deviation (range), n (%), or LSMEANS (least squares means) of change from Visit 1 (95% confidence interval, CI), as appropriate. All statistical tables, listings, and analyses were produced using SAS® release 9.4 or later (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Subject Characteristics

The study took place between September 2016 to December 2019. After screening, 176 subjects were randomized to the MMS ($n = 87$) or control ($n = 89$) groups (Figure 2). All subjects were included in the safety population. Forty-six subjects discontinued the study, mainly because of adverse events (32 (69.6%) subjects). The PP population comprised 141 subjects (MMS, $n = 65$; control, $n = 76$). The mean study duration was 24.5 ± 6.49 (1.0–30.9) weeks, and was comparable in both groups. Overall compliance was ≥80% in 63 (72.4%) of MMS subjects, ≤80% in four (4.6%), and unknown in 20 (23%).

Subject baseline demographics, clinical characteristics, and delivery information are shown in Table 1. The mean age was 31.9 ± 4.64 (18–41) years and all subjects were Caucasian. All demographics were similar between groups, with no significant differences. No abnormalities in physical or gynecological examinations were reported at Visit 1 or Visit 2. Although not statistically significant, a higher proportion of subjects in the control group compared with the MMS group experienced delivery complications (16 (23.2%) vs. eight (12.9%) subjects, respectively) or had an induced labor (13 (18.8%) vs. nine (14.5%) subjects). The groups were well balanced regarding infant sex (male 58.1% in the MMS group, 56.5% in the control group).
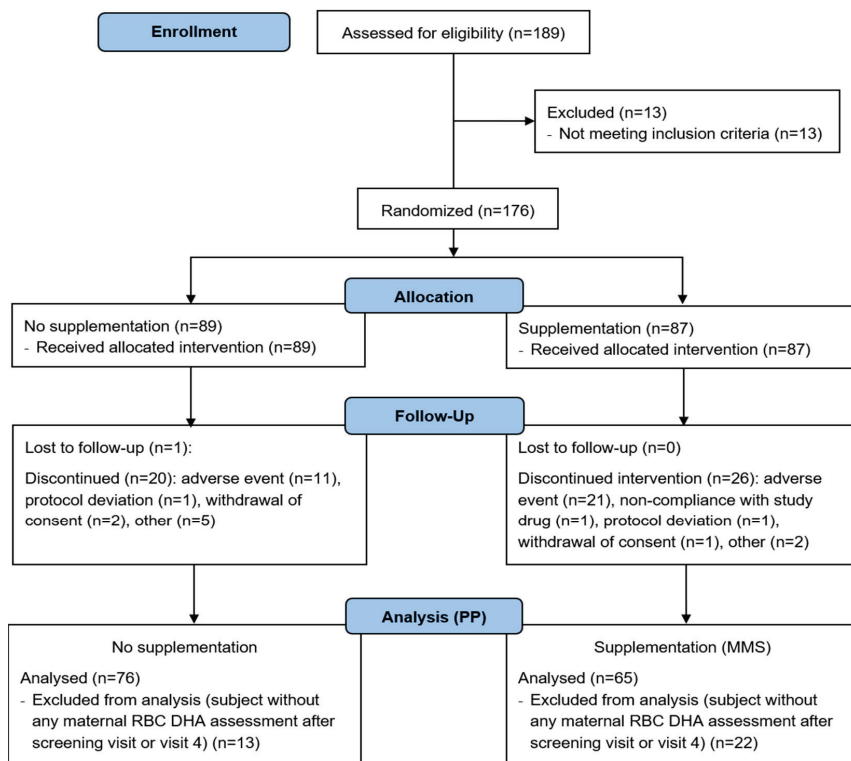
Figure 2. Flow diagram for study participants. DHA, docosahexaenoic acid; MMS, multiple micronutrients and DHA supplementation; RBC, red blood cells; PP, per protocol.

Table 1. Subject characteristics at baseline (values expressed as *n*, mean ± standard deviation, and median (range), unless otherwise stated) and delivery information (values expressed as *n* (%), unless otherwise stated) (per protocol population).

Characteristics	No Supplementation (*n* = 76)	MMS (*n* = 65)
Age (years)	76	65
	32.3 ± 4.72	31.4 ± 4.52
	33.0 (18–41)	32.0 (20–40)
Weight (kg)	76	65
	61.5 ± 9.96	63.2 ± 9.48
	59.0 (45–87)	47.0 (47–95)
Height (cm)	76	65
	164.1 ± 7.08	165.9 ± 5.60
	165.0 (147–184)	165.0 (150–178)
Body mass index (kg/m^2)	76	65
	22.8 ± 3.24	22.9 ± 3.10
	21.7 (18.0–29.7)	22.0 (18.1–29.9)
Previous pregnancy, *n* (%)		
No	30 (39.5)	30 (46.2)
Yes	46 (60.5)	35 (53.9)
Smoking status, *n* (%)		
Never	49 (64.5)	49 (75.4)
Former [a]	27 (35.5)	16 (24.6)

Table 1. *Cont.*

Characteristics	No Supplementation (*n* = 76)	MMS (*n* = 65)
Delivery information		
Subjects performing delivery visit	69	62
Type of delivery, *n* (%)		
Vaginal	55 (79.7)	49 (79.0)
Caesarean	14 (20.3)	13 (21.0)
Delivery complications, *n* (%)		
No	53 (76.8)	54 (87.1)
Yes	16 (23.2)	8 (12.9)
Induced labor, *n* (%)		
No	56 (81.2)	53 (85.5)
Yes	13 (18.8)	9 (14.5)
Infant sex, *n* (%)		
Male	39 (56.5)	36 (58.1)
Female	30 (43.5)	26 (41.9)

[a] Stopped smoking prior to pregnancy/when becoming aware of pregnancy consent signature plus one day. MMS, multiple micronutrients and docosahexaenoic acid supplementation.

3.2. Efficacy Endpoints

Primary. Maternal RBC DHA (*wt*% TFA) increased every visit in both groups (Figure 3 and Table 2), but the mean change from Visit 1 to Visit 4 was significantly greater in the MMS group compared with the control group, with an estimated treatment difference of 0.96 (95% CI 0.61, 1.31) ($p < 0.0001$) (Table 2). Furthermore, RBC DHA levels in women at the lower ranges increased by a greater extent in the MMS group (1.1% at Visit 3 and 1.6% at Visit 4 vs. Visit 1) compared to those in the control group (increase of 0.2% at Visit 3 and 0.5% at Visit 4 vs. Visit 1), and reached threshold levels (5% [50]) by Visit 4 (Table 2).

Secondary maternal endpoints. Significant differences were observed in favor of MMS for maternal RBC DHA/TFA ratio (estimated difference 0.01 (95% CI 0.006, 0.013); $p < 0.0001$), omega-3 index (estimated difference 1.00 (95% CI 0.64, 1.37); $p < 0.0001$), and calcidiol (estimated difference 3.96 (95% CI 0.88, 7.04) μg/L; $p = 0.0122$) (Figure 3 and Table 2).

The remaining secondary efficacy endpoints (maternal RBC TFA, RBC EPA (*wt*% TFA, GSH/GSSG ratio, ROMs, 8-isoprostane)) were comparable between groups, albeit slightly higher in the MMS group, with no significant differences (Supplementary Table S5).

Secondary infant endpoints. As outlined in Supplementary Table S6, infant variables were comparable between groups, with no statistically significant differences apart from subscapular skinfold thickness (thicker in the MMS group, $p = 0.0292$) and bone density in m^2 (borderline significantly greater in the control group, $p = 0.0486$).

Dietary intake. Assessment of dietary intake showed that consumption of the macro- and micronutrients measured was comparable between groups at each visit (Supplementary Table S7).
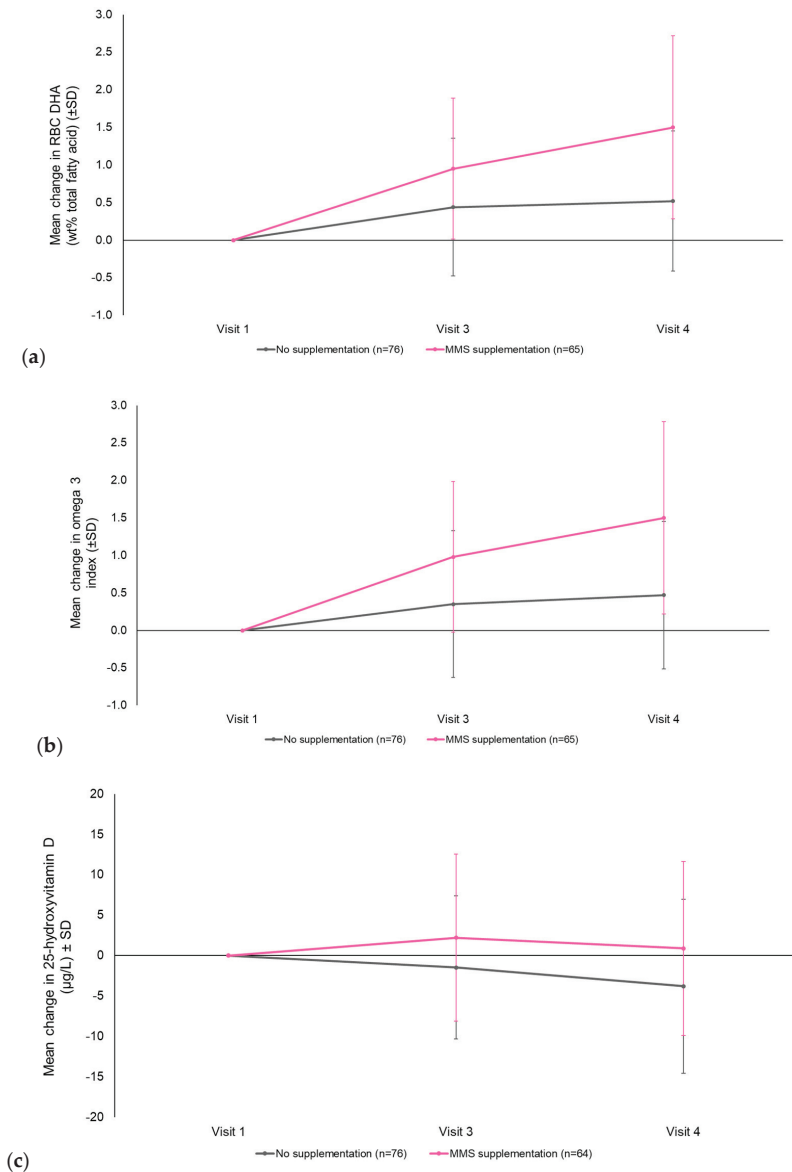
Figure 3. Mean change (± standard deviation) from Visit 1 to Visit 4 in maternal (**a**) RBC DHA (*wt*% TFA) ($p < 0.0001$ in favor of MMS), (**b**) omega 3 index ($p < 0.0001$ in favor of MMS), and (**c**) calcidiol (25-hydroxyvitamin D) ($p = 0.0122$ in favor of MMS) (per protocol population; LOCF approach). Visit 1: Screening (GA Week 11/14); Visit 3: GA Week 24/26; Visit 4: GA Week 34/36. DHA, docosahexaenoic acid; GA, gestational age; LOCF, last observation carried forward; MMS, multiple micronutrients and DHA supplementation; RBC, red blood cells; SD, standard deviation; TFA, total fatty acids; *wt*, weight.

Table 2. Primary and secondary outcomes at each visit (LOCF approach; values presented as mean ± standard deviation (range)) and differences between groups from Visit 1 to Visit 4 (presented as LSMEANS (95% confidence interval), ANCOVA model) (per protocol population).

	No Supplementation (n = 76)			MMS (n = 65)		
	Visit 1	Visit 3	Visit 4	Visit 1	Visit 3	Visit 4
RBC DHA (wt% TFA)	6.1 ± 1.23 (3.8–9.3)	6.6 ± 1.30 (4.0–10.4)	6.7 ± 1.34 (4.3–9.6)	6.1 ± 1.26 (3.4–10.2)	7.0 ± 1.30 (4.5–10.5)	7.5 ± 1.48 (5.0–13.0)
LSMEANS difference/*p* value						0.96 (0.61, 1.31)/ < 0.0001 *
RBC DHA/TFA ratio	0.06 ± 0.01 (0.04–0.09)	0.07 ± 0.01 (0.04–0.10)	0.07 ± 0.01 (0.04–0.10)	0.06 ± 0.01 (0.03–0.10)	0.07 ± 0.01 (0.04–0.11)	0.08 ± 0.01 (0.05–0.13)
LSMEANS difference/*p* value	—	—	—	—	—	0.010 (0.006, 0.013)/ <0.0001 *
Omega 3 index (%)	6.7 ± 1.38 (4.2–10.1)	7.0 ± 1.43 (4.2–10.7)	7.1 ± 1.45 (4.5–10.0)	6.5 ± 1.40 (3.7–10.9)	7.5 ± 1.43 (4.7–11.1)	8.0 ± 1.59 (5.3–13.6)
LSMEANS difference/*p* value	—	—	—	—	—	1.00 (0.64, 1.37)/ <0.0001 *
Calcidiol (ug/L)	21.6 ± 8.94 (5.5–48.8)	19.9 ± 9.87 (4.6–64.1)	17.8 ± 9.72 (4.0–45.0)	20.5 ± 7.54 (4.4–36.5)	22.8 ± 8.94 (4.0–48.6)	21.4 ± 9.07 (5.5–42.7)
LSMEANS difference/*p* value	—	—	—	—	—	3.96 (0.88, 7.04)/ 0.0122 *

* Two-sided *p* value <0.05 considered statistically significant. Visit 1: Screening (GA Week 11/14); Visit 3: GA Week 24/26; Visit 4: GA Week 34/36. DHA, docosahexaenoic acid; GA, gestational age; LOCF, last observation carried forward; LSMEANS, least squares means (difference = supplementation—no supplementation); MMS, multiple micronutrients and DHA supplementation; RBC, red blood cells; TFA, total fatty acids; wt, weight.

3.3. Safety Analysis

As outlined in Table 3, 125 (71.0%) subjects reported at least one TEAE pertinent to the mother (232 TEAEs overall) and 23 (13.1%) subjects reported them as serious, with a comparable number in each group. In the MMS group, 19 (21.8%) had one TEAE that led to permanent treatment discontinuation. Only three (3.5%) subjects in the MMS group had at least one suspected related TEAE (vomiting, with mild severity). At least one TEAE pertinent to the fetus/child were reported in ten (5.7%) subjects (13 TEAEs overall), and five (2.8%) reported them as serious. A higher proportion of subjects reported a TEAE in the MMS group, but none were considered to be treatment related. One (1.6%) subject had one TEAE pertinent to the fetus/child that led to permanent discontinuation. There was one fatality in the MMS group unrelated to study treatment. No relevant changes in clinical laboratory parameters (i.e., hematology, kidney function, liver function, blood coagulation, CRP) were observed, although there was a decrease in mean ferritin levels in both groups over the course of the study. Physical and gynecological examinations were normal throughout.

Table 3. Summary of participants with treatment-emergent adverse event (safety population; values expressed as *n* (%) subjects).

Parameters	No Supplementation (*n* = 89)	MMS (*n* = 87)	Total (*n* = 176)
Number of TEAEs pertinent to the mother	114	118	232
Any TEAEs pertinent to the mother	64 (71.9)	61 (70.1)	125 (71.0)
At least one suspected related [a]	NA	3 (3.5)	3 (1.7)
At least one serious TEAE	11 (12.4)	12 (13.8)	23 (13.1)
At least one leading to temporary treatment interruption [b]	NA	1 (1.2)	1 (0.6)
At least one leading to permanent treatment discontinuation [c]	NA	19 (21.8)	19 (10.8)
Fatal outcome	0	0	0
Number of TEAEs pertinent to fetus/child	4	9	13
Any TEAEs pertinent to fetus/child	3 (3.4)	7 (8.1)	10 (5.7)
At least one suspected related [a]	NA	0	0
At least one serious TEAE [b]	2 (2.3)	3 (3.5)	5 (2.8)
At least one leading to temporary treatment interruption [c]	NA	1 (1.2)	1 (0.6)
At least one leading to permanent treatment discontinuation [d]	NA	1 (1.2)	1 (0.6)
Fatal outcome	0	1 (1.2)	1 (0.6)

[a] Suspected related adverse events were those events with causal relationship equal to related; [b] No Supplementation group, the TEAEs pertinent to the fetus/child classified as severe were: fetal distress syndrome 1 (1.12%), fetal growth restriction 1 (1.12%); MMS group, the TEAEs pertinent to the fetus/child classified as severe were "Duodenal atresia" (1, 1.15%), "Fetal compartment fluid collection "(1, 1.15%), "Fetal growth restriction" (1, 1.15%) and "Polyhydramnios" (1, 1.15%). No TEAE pertinent to the fetus/child was suspected of being related to the study product; [c] adverse events leading to temporary treatment interruption were those events with action taken equal to drug interrupted; [d] adverse events leading to permanent treatment discontinuation were those events with action taken equal to drugs withdrawn. MMS, multiple micronutrients and docosahexaenoic acid supplementation; NA, not applicable; TEAEs, treatment-emergent adverse events.

4. Discussion

Supplementation with MMS plus DHA throughout the second and third trimester of pregnancy led to a significant increase in RBC levels of DHA, as well as the proportion of DHA compared with EPA and TFA. There was also a significant increase in the omega-3 index, while vitamin D levels increased during the course of the study compared to a decrease in women who did not receive supplementation. In the infant, a significantly greater subscapular skinfold thickness was observed in the MMS group. Safety outcomes were comparable between groups and MMS was well tolerated.

Our findings demonstrate that RBC DHA levels were significantly higher in the MMS group than in the control group. In pregnant women, the target RBC DHA level is 5% [50] (with <4.3% considered very low [51]). In our study, although average RBC DHA levels were above 6% at each visit (with higher levels in the MMS group), the lower ranges indicated that some women in both groups fell below this value. Nevertheless, RBC DHA levels in women at the lower ranges increased by a

greater extent in the MMS group compared to those in the control group over the course of the study, and reached the threshold by the third trimester (Table 2).

The omega-3 index was also significantly higher after supplementation. As RBC EPA values were comparable between groups, the increase in omega-3 index must be the result of an increase in DHA. In cardiovascular disease, the target range for the omega-3 index is 8–11%; it has been suggested that this range might also be suitable during pregnancy and lactation [52]. Reference values of 7.5–10.0% have also been recommended in pregnant women [53]. In our study, while the omega-3 index increased from 6.7% to 7.1% in the control group, the increase was greater (6.5% to 8.0%) in the MMS group. Therefore, supplementation with DHA helped women to reach target levels during pregnancy.

Current nutritional recommendations indicate that pregnant and lactating women should aim to achieve an average dietary intake of at least 200 mg DHA/day [54]. However, consumption of omega-3 fatty acids remains low particularly in pregnant and lactating women [55]. This is of relevance considering the vital roles of DHA in neurodevelopment, visual development, and neuroinflammation [56]. Moreover, pregnancy syndromes such as gestational diabetes and preeclampsia have also been associated with altered maternal omega-3 status and placental omega-3 metabolism [57–59].

The finding that there was a significant increase in calcidiol levels in supplemented women, but not in the non-supplemented control group, is also of interest. Vitamin D is essential for the health of both the developing fetus and the mother [60], and insufficient levels may have an adverse effect on skeletal homeostasis in the infant [61] and increase the maternal risk of preeclampsia [5].

In our study, no significant differences were observed between supplemented and control women regarding the markers of oxidative status. Oxidative stress has been implicated in many pathological processes during pregnancy [5]. However, this particular population of pregnant women was selectively chosen as a low-risk population, likely not at risk for decreased antioxidant status. Moreover, the sample size of the study was calculated based on the primary outcome; therefore, these results must be considered exploratory.

To our knowledge, this is the first randomized controlled trial evaluating the combination of MMS plus DHA in pregnant women. Our results indicate that in a high-income country setting, supplementation with micronutrients in combination with DHA can optimize maternal DHA status [49,62,63], despite the women in our supplemented group having a slightly lower intake of DHA from food. The timing of supplementation is important, and should occur in line with the development and growth of the embryonic brain, particularly during the later stages of pregnancy [17,21] when DHA rapidly begins to accumulate [18,22]. Furthermore, supplementation with MMS during pregnancy, as in our study, can improve maternal and infant outcomes, leading to reductions in the incidence of pre-eclampsia [64], neural-tube defects [64,65], low birthweight and small-for-gestational age babies [3], limb reduction defects, and congenital urinary tract abnormalities [64]. There may also be long-term benefits in children [4] (e.g., cognitive development [66,67]). Although many of these results have been reported from low- to middle-income countries, micronutrient levels in pregnant women are often insufficient even in industrialized countries, where dietary resources are more readily available [12]. However, the routine use of multivitamins during pregnancy has not yet been recommended in high-income countries, despite the benefits on clinical outcomes [68]. Currently, only folic acid and iron are recommended as standard interventions in pregnancy in industrialized countries [37].

Further research is necessary to better understand whether the improvements in maternal DHA status, as well as other improvements in omega-3 index and calcidiol levels, have a positive impact on maternal and infant clinical outcomes. Large, long-term randomized controlled trials on MMS supplementation including DHA are essential.

Our study has some limitations, including the lack of a placebo control group and the consequent unblinded nature of the study (which could have led to expectation bias [69]), the small sample size, and the fact that only Caucasian women were included (which limits the generalizability of the results). Adequately powered studies with a varied study population are necessary to better establish the impact of different baseline characteristics in pregnant women and to evaluate clinical outcomes.

5. Conclusions

Supplementation with MMS plus DHA in pregnant women can complement dietary intake and significantly improve maternal DHA and vitamin D status. This finding is important in light of the essential roles of DHA in the developing brain of the fetus, in visual development, and in immunomodulation.

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Serum 25 Hydroxyvitamin D Levels During Pregnancy in Women with Asthma: Associations with Maternal Characteristics and Adverse Maternal and Neonatal Outcomes

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Abstract: Low 25-hydroxyvitamin D (25(OH)D) levels are common in pregnancy and associated with adverse maternal/neonatal outcomes. In pregnant women with asthma, this study examined the association of lifestyle- and asthma-related factors on 25(OH)D levels and maternal/neonatal outcomes by vitamin D status. Serum 25(OH)D was measured at 16 and 35 weeks gestation in women with asthma (n = 103). Body mass index (BMI), gestational weight gain (GWG), smoking status, inhaled corticosteroid (ICS) use, asthma control, airway inflammation, and exacerbations, and maternal/neonatal outcomes were collected. Baseline and change (Δ) in 25(OH)D were modelled separately using backward stepwise regression, adjusted for season and ethnicity. Maternal/neonatal outcomes were compared between low (25(OH)D < 75 nmol/L at both time points) and high (≥75 nmol/L at one or both time points) vitamin D status. Fifty-six percent of women had low vitamin D status. Obesity was significantly associated with lower baseline 25(OH)D (Adj-R^2 = 0.126, p = 0.008); ICS and airway inflammation were not. Excess GWG and season of baseline sample collection were significantly associated with Δ25(OH)D (Adj-R^2 = 0.405, p < 0.0001); asthma-related variables were excluded (p > 0.2). Preeclampsia was more common in the low (8.6%) vs. high (0%) vitamin D group (p < 0.05). Obesity and excess GWG may be associated with gestational 25(OH)D levels, highlighting the importance of antenatal weight management.

Keywords: maternal nutrition physiology; vitamin D; pregnancy; asthma; maternal obesity; gestational weight gain; infant; newborn

1. Introduction

Maternal nutritional status is a major modifiable determinant of neonatal nutritional status and both maternal and offspring health outcomes. A low circulating level of 25 hydroxyvitamin D (25(OH)D) is a preventable, but common problem during pregnancy [1,2]. The role of vitamin D in health and disease prevention involves effects on hormonal pathways, immune system development and infection, as well as cell proliferation and differentiation [3,4]. Moreover, vitamin D is important for the growth

and development of the skeletal system via calcium metabolism [3,5]. Low maternal 25(OH)D levels have also been associated with an increased risk of poor maternal and neonatal outcomes, including gestational diabetes, preeclampsia, preterm birth, and low-birth-weight infants [6–8], with vitamin D implicated in placental and immune function, neurodevelopment and lung development [9]. Adequate 25(OH)D levels during pregnancy are therefore necessary for optimal maternal, fetal and infant health. Neonatal vitamin D levels are largely determined by maternal vitamin D levels during pregnancy [10]. Therefore, low 25(OH)D levels during pregnancy directly affect neonatal 25(OH)D levels [11,12] and the associated health consequences.

Blood 25(OH)D levels have been shown to be low in women with asthma during pregnancy [13]. Asthma affects approximately 12% of women during pregnancy [14] and has been associated with an increased risk of adverse maternal and neonatal outcomes including preeclampsia [15], preterm birth, small-for-gestational age (SGA) infants, and neonatal hospitalization and mortality, highlighting this as a high risk group [16]. In a cohort of pregnant women with and without asthma, higher 25(OH)D levels (≥75 nmol/L) have been associated with a lower risk of preeclampsia, as well as better asthma control during pregnancy [15,17]. Furthermore, in women with asthma, low 25(OH)D levels during pregnancy have been associated with a higher prevalence of infant wheeze, acute-care presentations and oral corticosteroid (OCS) use [13], suggesting that maternal 25(OH)D levels may affect both maternal and infant health outcomes. A recent meta-analysis has also linked maternal vitamin D sufficiency during pregnancy to a decreased risk of asthma or recurrent wheeze in children whose mothers have asthma [18]. Factors influencing 25(OH)D levels in women with asthma during pregnancy require further investigation.

Several studies have examined factors associated with 25(OH)D levels in pregnancy in the general population [19–32]. Certain environmental factors have been associated with 25(OH)D levels during pregnancy including sun exposure [19], with dermal synthesis of cholecalciferol due to ultraviolet (UV) B radiation one of the main contributors to circulating 25(OH)D levels, and season of blood draw, with higher serum 25(OH)D concentrations expected in summer months [20,21,23,24,26,33]. In addition, low 25(OH)D levels are more common in non-white populations and those with higher melanin pigmentation [22,27–29,32], with race demonstrated to be the most important risk factor for vitamin D deficiency or insufficiency in a previous study [2]. Other factors that have been linked to low 25(OH)D levels in pregnancy include maternal smoking and alcohol use [20,23,30], lower education level [28], and low total dietary vitamin D intake and supplement use [22,27,28,31].

Body mass index (BMI) has previously been linked to lower 25(OH)D levels in the non-pregnant population [34,35], with percentage fat mass inversely related to 25(OH)D levels [36]; however, results in pregnant cohorts are less clear, with three studies showing a negative association between BMI and pregnancy 25(OH)D levels [20,37,38], one study showing a positive association [19], and four studies reporting no association [21,23,24,33]. The impact of gestational weight gain (GWG) on 25(OH)D levels is also unclear, with one study finding no association between GWG and 25(OH)D levels during pregnancy [39], a second reporting a negative association [37], and a third reporting a negative association, but only among women with pregestational overweight [25]. It is also not clear how weight status or weight gain interacts with 25(OH)D levels during pregnancy in women with asthma. We have previously demonstrated a high prevalence of overweight and obesity, and excessive GWG, in pregnant women with asthma [13,14]; therefore, examining whether weight status affects maternal 25(OH)D levels during pregnancy is particularly relevant to this group. In addition, whether asthma-related factors, namely airway inflammation, asthma control, inhaled corticosteroid (ICS) use, and exacerbations of asthma, are associated with 25(OH)D levels during pregnancy has not been examined.

Therefore, in pregnant women with asthma, the aims of this study were to: (1) explore the association between lifestyle (weight status, GWG, smoking) and asthma-related factors (airway inflammation, asthma control, ICS use, exacerbations) and 25(OH)D levels during pregnancy; and (2) compare the incidence of adverse maternal and neonatal outcomes by low vs. high vitamin D status during pregnancy.

2. Materials and Methods

This is a secondary analysis of data collected during the period 2007–2009 from a cohort of 168 pregnant women with asthma, aged ≥18 years, recruited between 12 and 20 weeks gestation via the John Hunter Hospital Antenatal Clinic (Newcastle, Australia [latitude 32.93 °S]) into a study of respiratory viral infection in pregnancy [40]. Concurrently, the majority ($n = 157$, 93%) of women also participated in the Managing Asthma in Pregnancy (MAP, 2007–2010) study, a RCT of monthly fractional exhaled nitric oxide (FENO)-guided asthma management versus symptoms-guided management during pregnancy (Hunter New England Human Research Ethics Committee approval # 07/02/21/3.06, Australian and New Zealand Clinical Trials Registry # 12607000561482) [41]. Written informed consent was obtained prior to enrolment in this study. Asthma was determined by self-reported physician diagnosis and recent asthma symptoms or medication use, with confirmation by a respiratory physician at enrolment. Women were excluded if they had used more than three OCS courses in the past 12 months or had another chronic lung disease. All women were followed monthly until birth.

2.1. Measurements

Ethnicity was self-reported at baseline. Tobacco smoke exposure was self-reported and determined objectively by measurement of exhaled carbon monoxide (≥10 ppm, piCO Smokerlyzer Breath CO Monitor, Bedfont, UK) and urinary cotinine (≥level 5 or 2840 nmol/L, Nicalert, NYMOX, Saint-Laurent, Quebec, Canada). Maternal height and weight were measured at each study visit and baseline BMI (kg/m^2) and GWG calculated. Baseline BMI was categorized as non-overweight (<25 kg/m^2), overweight (≥25–<30 kg/m^2) or obese (≥30 kg/m^2). GWG was classified as within or exceeding recommended guidelines [42]. Airway inflammation was measured via FENO (ECOMEDICS online chemiluminescence analyzer, Duernten Switzerland; 50 mL/s flow rate). Lung function was assessed by spirometry (EasyOne Spirometer, Niche Medical, North Sydney Australia), with forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) reported as a percentage of their predicted values (NHANES III) [43], and the ratio documented (FEV_1/FVC). Asthma control was assessed using the validated Asthma Control Questionnaire (ACQ) [44]. Asthma medications and exacerbations requiring medical intervention (unscheduled physician appointment, emergency department presentation, hospitalization, or OCS) were recorded prospectively by participant report. Maternal and neonatal outcomes were documented from the medical records including gestational hypertension (GH, defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, which was not preexisting and developed de novo >20 weeks gestation, without organ disorders), preeclampsia (PE, defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, which developed de novo >20 weeks gestation and the presence of proteinuria >20 weeks gestation), gestational diabetes mellitus (defined as a fasting blood glucose level ≥5.5 mmol/L or a 2-h blood glucose level ≥8.0 mmol/L following a 75 g glucose load, according to guidelines at the time of this study) [45], labor type, mode of birth, infant anthropometry, infant Apgar score at one and five minutes, neonatal respiratory distress and neonatal intensive care unit (NICU) admission. Gestational age was calculated from the estimated date of confinement (based on either the last menstrual period or early ultrasound) and the date of birth, with preterm birth defined as <37 weeks gestation.

A non-fasting blood sample was collected via venepuncture from a subset of participants at baseline and late pregnancy (approximately 16 and 35 weeks gestation, respectively) and stored in serum aliquots at −80 °C. Batch analysis of total 25(OH)D (comprised of 25(OH)D_2 and 25(OH)D_3) using enzyme-linked immunosorbent assay (Abbott Architect assay, Abbott Park, IL; intra- and interassay coefficients of variation <10%) took place at Massachusetts General Hospital (Boston, MA, USA). Season was documented by date of collection (Summer, Winter, Spring, and Autumn). The Massachusetts General Hospital core laboratory is a clinical laboratory improvement amendments-certified facility, which uses rigorous methods and continuously updated reference standards for biomarker assessment. Intra- and interassay coefficients of variation (CV) were both less than 8% for 25(OH)D. Although other metabolites may be included in the future, it is currently accepted that singular measurement of serum 25(OH)D is the biologic marker of vitamin D status clinically [34]. 25(OH)D level was dichotomized at 75 nmol/L, according to Endocrine Society guidelines for vitamin D status [34] and as used in previous studies [13]. Women were grouped by 25(OH)D level during pregnancy: (i) 25(OH)D <75 nmol/L at both time points (low) versus (ii) 25(OH)D ≥75 nmol/L at one or both pregnancy time points (high).

2.2. Analysis

Statistical analyses were conducted using Stata Version 11.1 (StataCorp LP, College Station, TX, USA). Continuous variables were presented as the mean ± standard deviation(SD) or median [interquartile range, IQR] and analyzed using student *t* test or Wilcoxon rank-sum test, with proportions (%) analyzed using X^2 test (demographics, maternal and neonatal outcomes). Statistical significance was set at a two-sided *p* < 0.05. Change (Δ) in 25(OH)D levels were calculated as the difference between the late and early pregnancy measure. Multiple stepwise linear regressions were performed for baseline and Δ25(OH)D as dependent variables and adjusted for ethnicity and season given the known associations between skin color and UVB exposure with 25(OH)D levels. The Δ25(OH)D was also adjusted for baseline 25(OH)D levels. Asthma-related outcomes (FENO, ACQ and ICS use at enrolment, and exacerbations during pregnancy), smoking status and BMI category at enrolment, and GWG above recommendations, were included as independent variables, with backward elimination for parameters with a *p*-value >0.2.

3. Results

Baseline and late serum 25(OH)D measurements, and maternal and neonatal outcomes, were available for 103 women with asthma; all were singleton pregnancies. The majority of women were white (81.6%), with 16.5% current smokers. At enrolment, 32.0% and 40.8% of women were overweight and obese, respectively. The average absolute weight gain during pregnancy (16–35 weeks) was 7.7 ± 4.5 kg (*n* = 88), with GWG exceeding recommendations in 70.5% of women. The mean 25(OH)D level at 16 and 35 weeks was 64.77 ± 20.6 (median [IQR] 60.9 [49.2, 78.10]; range 26.20 to 113.3) nmol/L and 65.59 ± 23.46 (median [IQR] 63.90 [48.90, 81.40]; range 17.50 to 131.0) nmol/L, respectively. The change in 25(OH)D levels from 16 to 35 weeks ranged from 46.20 to 55.40 nmol/L, with an average difference of 1.33 ± 19.79 (median 5.00 [−14.20, 13.20]) nmol/L (Figure 1).

Fifty-six percent of women (*n* = 58) had 25(OH)D <75 nmol/L at both 16 and 35 weeks gestation, and 44% (*n* = 45) had 25(OH)D ≥75 nmol/L at one or both time points. Demographics are presented by vitamin D group in Table 1. There were no significant group differences by vitamin D status, with the exception of BMI, which was higher in the low (vs. high) vitamin D group. Baseline asthma control was similar between the low and high vitamin D group (ACQ: 1.2 (1.0) vs. 1.1 (0.8), *p* = 0.61), as was the proportion prescribed ICS medication (25.9% [*n* = 15] vs. 31.1% [*n* = 14], *p* = 0.56; ICS dose 800 [500, 800] vs. 800 [400, 800] mcg, *p* = 0.52). The proportion of women experiencing an asthma exacerbation during pregnancy was not significantly different between the low and high vitamin D group (41.4% [*n* = 24] vs. 35.6% [*n* = 16], *p* = 0.55).
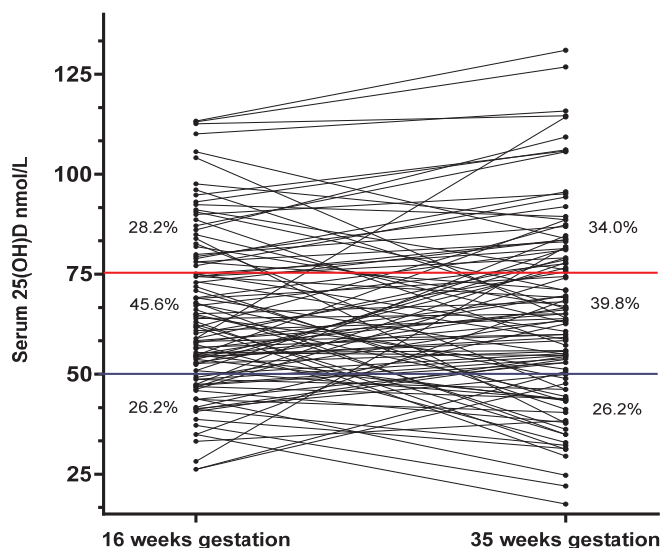
Figure 1. Serum 25(OH)D levels at 16 and 35 weeks gestation in pregnant women with asthma. Serum 25(OH)D at 16 and 35 weeks gestation in 103 pregnant women with asthma. Dots represent individual 25(OH)D values, with connecting lines demonstrating the within-person trajectory from 16 to 35 weeks gestation. Major horizontal lines at 75 and 50 nmol/L represent the cut-points for vitamin D 'sufficiency' and 'deficiency'. The proportion of women who fell into the categories of 'sufficient', 'insufficient' and 'deficient' are presented on the graph at both 16 and 35 weeks gestation. 25(OH)D: 25-hydroxyvitamin D.

Table 1. Demographics by maternal vitamin D status during pregnancy for women with asthma.

Variable	25(OH)D <75 nmol/L (*n* = 58)	25(OH)D ≥75 nmol/L (*n* = 45)	*p*-Value
Age, years	28.4 (5.5)	28.7 (5.9)	0.77
Parity, *n*	1 [0, 1]	1 [0, 1]	0.83
Ethnicity: European, *n* (%)	48 (82.8%)	36 (80.0%)	0.72
Smoking during pregnancy, *n* (%)	9 (15.5%)	8 (17.8%)	0.76
Preexisting diabetes, *n* (%)	3 (5.3%)	1 (2.2%)	0.28
Body mass index, kg/m^2	30.6 (7.8)	27.2 (5.3)	**0.01**
Overweight and obese, %	46 (79.3%)	29 (65.9%)	0.13
Gestational weight gain (16–35 weeks gestation), kg	8.0 (4.6)	7.3 (4.2)	0.45
Weight gain per week above recommendations, *n* (%)	36 (72%)	26 (68.4%)	0.72
FENO, ppb	14.9 [6.7, 29.8]	14.4 [5.9, 31.5]	0.99
FEV$_1$, %predicted	93.7 (15.6)	93.1 (15.9)	0.86
FVC, %predicted	105.0 (2.3)	101.3 (17.7)	0.30
FEV$_1$/FVC, %	77.8 (7.8)	80.3 (7.2)	0.14

FENO, fractional exhaled nitric oxide. FEV$_1$, forced expiratory volume in 1 s. FVC, forced vital capacity. Bolded *p*-values are <0.05.

3.1. Asthma- and Lifestyle-Related Variables Associated with 25(OH)D Levels in Pregnancy

Obesity was associated with a significantly lower 25(OH)D level at 16 weeks gestation, compared to a BMI <25 kg/m^2, after controlling for season of blood collection and ethnicity (Table 2); however,

airway inflammation (measured by FENO) and ICS use were not associated with baseline 25(OH)D levels. Smoking and ACQ score were not retained in the model of baseline 25(OH)D ($p > 0.2$).

Table 2. Asthma- and lifestyle-related variables associated with 25(OH)D levels in pregnant women with asthma.

Final Model	Baseline 25(OH)D $n = 100$, Adj-R^2 = 0.126, p-Value = 0.008		Δ 25(OH)D $n = 86$, Adj-R^2 = 0.405 p-Value <0.0001	
Variable	Coefficient (95% CI)	p-Value	Coefficient (95% CI)	p-Value
Ethnicity: European *	1.18 (−9.42, 11.77)	0.83	2.15 (−7.28, 11.58)	0.65
Season: baseline sample collection *				
Autumn	−2.77 (−15.14, 9.59)	0.66	−11.89 (−22.62, −1.16)	**0.03**
Winter	−9.74 (−21.24, 1.75)	0.10	14.28 (4.64, 23.92)	**0.004**
Spring	−5.56 (−17.88, 6.75)	0.37	16.79 (5.97, 27.60)	**0.003**
BMI category: baseline				
Overweight	−6.81 (−16.91, 3.29)	0.18	5.17 (−3.83, 14.16)	0.26
Obese	−13.70 (−23.48, −3.91)	**0.007**	8.64 (−0.06, 17.35)	0.051
Baseline FENO, ppb	0.10 (−0.03, 0.23)	0.13	-	
ICS use	7.42 (−1.56, 16.41)	0.1	-	
Excessive gestational weight gain	NA		−7.77 (−15.48, −0.05)	**0.048**

25(OH)D, 25-hydroxy vitamin D; Δ25(OH)D, change in 25(OH)D from 16 to 35 weeks gestation; BMI, body mass index; FENO, fractional exhaled nitric oxide; ICS, inhaled corticosteroids; NA, variable non-applicable to model therefore not included. '-' variable excluded from regression model in backward elimination ($p > 0.2$). * variables ethnicity and season forced into both models. Bolded p-values are <0.05.

Only baseline weight status and GWG were retained in the model of Δ25(OH)D, controlling for ethnicity and season; asthma-related variables (FENO, ACQ, ICS use, exacerbations during pregnancy), smoking status and baseline 25(OH)D ≥75 nmol/L were not retained in the model due to a p-value > 0.2. Baseline sample collection in Autumn was associated with a decrease, while sample collection in Winter or Spring were associated with an increase, in 25(OH)D levels from 16 weeks to 35 weeks gestation (Table 2). Excessive GWG was associated with a statistically significant decline in 25(OH)D levels from 16 to 35 weeks gestation.

3.2. Maternal Vitamin D Status and Maternal and Neonatal Outcomes

The incidence of preeclampsia was significantly higher in those with 25(OH)D levels <75 nmol/L during pregnancy, compared with 25(OH)D levels ≥75 nmol/L (Table 3). There were no eclamptic cases in either group. There were no miscarriages; however, one woman in the low vitamin D group, delivered a stillborn infant. There was a clinically important difference in NICU admissions ($p = 0.26$) and neonate respiratory distress ($p = 0.10$) between the low and high vitamin D groups, but this was not statistically significant. There were no statistically significant differences for other maternal or neonatal outcomes by vitamin D status.

Table 3. Maternal and neonatal outcomes by maternal vitamin D status during pregnancy for women with asthma.

Variable	25(OH)D < 75 nmol/L (*n* = 58)	25(OH)D ≥ 75 nmol/L (*n* = 45)	*p*-Value
Gestational hypertension	4 (6.9%)	4 (8.9%)	0.71
Preeclampsia, *n* (%)	5 (8.6%)	0 (0%)	**0.04**
Gestational diabetes, *n* (%)	1 (1.8%)	1 (2.2%)	0.88
Labor type			
Spontaneous	34 (58.6%)	27 (60.0%)	0.89
Induced	16 (27.6%)	11 (24.4%)	0.72
Spontaneous and augmented	1 (1.7%)	0 (0)	0.38
Vaginal birth, *n* (%)	44 (75.9%)	35 (77.8%)	0.82
Gender: male, *n* (%)	28 (48.3%)	21 (46.7%)	0.87
Gestational age at birth, weeks	39.7 (1.3)	39.5 (1.6)	0.27
Preterm birth, *n* (%)	3 (5.2%)	3 (6.7%)	0.75
Birth weight, grams	3498 (591)	3370 (578)	0.14
Birth length, cm	51.3 (2.9)	51.2 (2.7)	0.43
Birth head circumference, cm	34.3 (1.9)	34.0 (1.8)	0.23
Apgar 1, score	9 [7, 9]	9 [7.5, 9]	0.84
Apgar 5, score	9 [9, 9]	9 [9, 9]	0.87
NICU admission, *n* (%)	6 (10.5%)	2 (4.4%)	0.26
Respiratory distress, *n* (%)	6 (10.5%)	1 (2.2%)	0.10

NICU, neonatal intensive care unit. Bolded *p*-values are <0.05.

4. Discussion

This was the first study to report on factors associated with 25(OH)D levels during pregnancy in women with asthma in the Australian context. In predominantly white women of European descent with mild asthma, low vitamin D status was common during pregnancy. Both maternal obesity and GWG above guideline recommendations, regardless of BMI category at enrolment, were significant modifiable factors associated with 25(OH)D levels during pregnancy; however, asthma-related variables were not associated with 25(OH)D levels in this group of women. These results provide evidence to support the importance of early nutrition intervention in pregnant women with asthma.

Our results highlight the importance of achieving a healthy BMI prior to pregnancy and maintaining GWG within recommendations, regardless of BMI category. Obesity, but not overweight, was significantly associated with lower early–mid pregnancy 25(OH)D levels in this group of women with asthma. This is in agreement with a 2011 study conducted in Australia and New Zealand that found BMI had a significant negative association with serum 25(OH)D levels in two general pregnancy cohorts; those with a BMI ≥30 kg/m^2 were three times more likely to have suboptimal 25(OH)D levels, compared to those who had a BMI <30 kg/m^2 (adjusted odds ratio [aOR] 3.0, 95%CI 1.4, 4.3) [27]. BMI was also found to be negatively associated with 25(OH)D levels at 15 weeks gestation in an Irish population (adjusted mean difference −0.3, 95%CI −0.5, −0.04) [38]. This is supported by a Swedish study reporting a weak, but significant, association between both higher pregestational BMI (OR 1.10, 95%CI 1.01, 1.20) and BMI during pregnancy (OR 1.10, 95%CI 1.02, 1.21) and vitamin D deficiency in the first trimester (<50 nmol/L) [20].

In our group of pregnant women with asthma, GWG above recommendations was also negatively associated with the change in 25(OH)D level from 16 to 35 weeks gestation, which has not been previously examined in women with asthma. This is in agreement with a previous study by Moon et al. in 1753 women demonstrating a significant decrease in 25(OH)D levels over pregnancy with greater weight gain [37]. However, these results contrast a second study which found no association, albeit in

a much smaller sample size (*n* = 237) [39]. A third study from Figueiredo et al. found an association between maternal weight gain and 25(OH)D levels but this was limited to women with pregestational overweight (*n* = 163) [25]. The association between excess weight and lower 25(OH)D levels may be attributable to adipose tissue sequestration, or volumetric dilution, of endogenous and exogenous vitamin D [46]. Low vitamin D intake from poor diet and inadequate sun exposure associated with a sedentary lifestyle, notably minimal outdoor activity, is another possible explanation [46]. Our results and the previous literature highlight the role of both prenatal weight status and GWG in influencing 25(OH)D levels during pregnancy, an important factor in both maternal and neonatal health outcomes. Given the high prevalence of obesity and excessive GWG in women, including women with asthma, this area warrants further research into the impacts on maternal and neonatal nutritional health.

Smoking status was also examined in our cohort, with current smoking status not associated with either baseline, or change in, 25(OH)D levels in our group of women with asthma. With the exception of one study in a pregnant Spanish cohort [47], our results contrast the majority of previous studies demonstrating that maternal smoking is associated with low 25(OH)D levels during pregnancy [28,29,38,48]. The prevalence of smoking was similar across studies, so this may be due to our smaller sample size.

There are several other potentially important lifestyle variables that have been associated with pregnancy 25(OH)D levels, which were not available in the present study, nor did we have data on preexisting hypertension in this cohort; these are limitations of this study. Most notably, dietary and supplemental intake were not collected in our cohort, nor was a measure of sun exposure or socio-economic status, and therefore we were unable to account for these variables in our analysis. High vitamin D dietary intake [22,31] and supplementation during pregnancy [22,27], as well as outdoor recreational walking ≥4 times per week (proxy for sun exposure) [38], have been associated with higher 25(OH)D levels during pregnancy. Similarly, low sun exposure time is also a documented risk factor for vitamin D deficiency during pregnancy in a predominantly veiled and dark skinned population in Australia [49]. Therefore, future research in this area would benefit from comprehensively documenting dietary, supplemental and environmental sources of vitamin D.

Previous studies have looked at the association of various factors on 25(OH)D levels in the general population, but not specifically the association of asthma-related variables. Airway inflammation, asthma control, ICS use, and exacerbations were not associated with 25(OH)D levels at 16 weeks gestation, or the change in 25(OH)D levels from 16 to 35 weeks gestation, in this group of women. Indeed, we did not detect a difference in airway inflammation, asthma control or ICS use at enrolment (approximately 16 weeks gestation), or exacerbations requiring medical intervention during pregnancy, by vitamin D status. However, we may have lacked the power to detect a statistically significant difference in such outcomes. Furthermore, the fact that our population had relatively mild asthma may explain why there was no association between asthma-related variables and 25(OH)D levels in this group of pregnant women. Therefore, future work exploring the relationship between asthma and 25(OH)D levels in a larger sample of pregnant women with varying degrees of asthma severity is warranted.

A statistically significant higher incidence of preeclampsia was detected in women who had low (vs. high) 25(OH)D levels during pregnancy; in fact, no women in the high vitamin D group developed preeclampsia. This is in agreement with a secondary analysis of a RCT of vitamin D supplementation during pregnancy, which reported lower preeclampsia rates in women (40% with a self-reported history of physician-diagnosed asthma) with 25(OH)D levels ≥75 nmol/L in early and late pregnancy (10–18 and 32–38 weeks), compared with those who were insufficient at both time points (2.3 vs. 11.9%) [17]. Although previous meta-analyses have found an association between preterm birth and low birth weight in women with vitamin D deficiency (<50 nmol/L) during pregnancy [50,51], we did not detect a difference in these outcomes in our study. Of interest, we did observe a clinically important difference in NICU admissions and neonatal respiratory distress between the low and high vitamin

D groups; however, this was not of statistical significance. This has not been examined in previous studies specifically including pregnant women with asthma, and warrants further investigation.

This study was a secondary analysis of a prospective trial, and thus not primarily designed for the outcomes of interest (i.e., factors associated with gestational 25(OH)D levels and the incidence of adverse maternal and neonatal outcomes by vitamin D status). Nevertheless, this is the first study to examine the effect of lifestyle- and asthma-related factors on 25(OH)D levels during pregnancy, in an Australian context, in a well-defined sample of women with mild asthma. Both obesity and maternal weight gain are modifiable factors that may be associated with 25(OH)D levels in pregnancy. Considering the high prevalence of obesity and GWG in women with asthma [14], this further highlights the importance of dietary intervention in this group of women in the antenatal period. A larger sample size would provide increased power to further examine the association of lifestyle, and disease-related, factors on maternal 25(OH)D levels. Moreover, given that women with indications of more severe asthma, e.g., recent OCS use, were excluded from the trial, it is unclear whether the associations observed would be different if studied in women with more moderate to severe disease. Furthermore, our results suggest that further investigation of the effect of maternal vitamin D status on maternal and neonatal outcomes in women with asthma is warranted. Nutritional therapy to improve vitamin D status may be an acceptable adjunct therapy to the clinical management of pregnant women with asthma and requires further investigation.

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Article

The Impact of Sample Type on Vitamin D Quantification and Clinical Classification during Pregnancy

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Abstract: Measurement of vitamin D status has significant use in clinical and research settings, including during pregnancy. We aimed to assess the agreement of total 25-hydroxyvitamin D (25(OH)D) concentration, and its three analytes (25-hydroxyvitamin D_3 (25(OH)D_3), 25-hydroxyvitamin D_2 (25(OH)D_2) and Epi-25-hydroxyvitamin D_3 (Epi-25(OH)D_3)), in plasma and serum samples collected during pregnancy, and to examine the proportion of women who change vitamin D status category based on sample type. Matching samples were collected from $n = 114$ non-fasting women between 12–25 weeks gestation in a clinical trial in Newcastle, Australia. Samples were analysed by liquid chromatography-tandem mass-spectrometry (LC-MS/MS) to quantify total 25(OH)D and its analytes and examined using Bland-Altman plots, Pearson correlation (r), intraclass correlation coefficient and Cohen's Kappa test. Serum total 25(OH)D ranged from 33.8–169.8 nmol/L and plasma ranged from 28.6–211.2 nmol/L. There was a significant difference for total 25(OH)D based on sample type (measurement bias 7.63 nmol/L for serum vs plasma (95% Confidence Interval (CI) 5.36, 9.90, $p \leq 0.001$). The mean difference between serum and plasma concentrations was statistically significant for 25(OH)D_3 (7.38 nmol/L; 95% CI 5.28, 9.48, $p \leq 0.001$) and Epi-25(OH)D_3 (0.39 nmol/L; 95% CI 0.14, 0.64, $p = 0.014$). Of 114 participants, 28% were classified as vitamin D deficient (<50 nmol/L) or insufficient (<75 nmol/L) based on plasma sample and 36% based on serum sample. Nineteen (16.7%) participants changed vitamin D status category based on sample type. 25-hydroxyvitamin D quantification using LC-MS/MS methodology differed significantly between serum and plasma, yielding a higher value in plasma; this influenced vitamin D status based on accepted cut-points, which may have implications in clinical and research settings.

Keywords: vitamin D; pregnancy; quantification; clinical; 25OHD; asthma; analytes; spectrophotometry; sample; plasma; serum; LC-MS/MS

1. Introduction

The circulating concentration of 25-hydroxyvitamin D (25(OH)D) is considered the accepted clinical biomarker of vitamin D status [1]. With increasing awareness of the importance of vitamin D

for maintaining optimal health by healthcare professionals, researchers and the public, requests for vitamin D quantification in human samples has increased in recent years [2,3], therefore ensuring the accuracy of analyses is highly relevant and important.

Testing is most commonly conducted through measurement of 25(OH)D via assay and quantification of 25(OH)D is routinely outsourced to be completed in external laboratories for clinical and research requirements. Previous studies have examined vitamin D in serum vs. plasma in small sample sizes, using assay techniques, and found serum and plasma to be mostly agreeable [4–7], but with evidence that 25(OH)D concentration may be higher in heparinised plasma compared to serum or ethylenediamine tetraacetic acid (EDTA) plasma [5]. However, this methodology has significant limitations to the usefulness and interpretation of the results, as assays have high result variability due to issues with standardisation, precision and accuracy [8]. Unlike immunoassays or high-pressure liquid chromatography (HPLC) methods, liquid chromatography tandem mass spectrophotometry (LC-MS/MS) offers higher specificity of detection, lower matrix interferences and high detectability of molecules present in low concentrations. This method is considered the gold standard method for vitamin D analysis, with the capability to measure $25(OH)D_2$, $25(OH)D_3$, and Epi-$25(OH)D_3$ separately [9–13].

Four previous studies have compared sample type for 25(OH)D quantification using LC-MS/MS; however, the samples were collected from non-pregnant populations of small sample sizes. Zhang et al. ($n = 25$) and Abu Kassim et al. ($n = 10$) did not find a significant difference between EDTA plasma, heparin plasma and serum for $25(OH)D_2$ and $25(OH)D_3$ concentrations in healthy adults [14,15]. Differences between sample type for Epi-$25(OH)D_3$ or total 25(OH)D were not examined. Albarhani et al. examined the usefulness of diluted plasma for quantification of $25(OH)D_3$ and Epi-$25(OH)D_3$ compared to serum in umbilical cord blood samples ($n = 20$), and although their findings demonstrated close agreement for $25(OH)D_3$ in serum and plasma across two independent laboratories (r = 0.983) issues with analytical sensitivity in regards to limits of detection (LoD) for Epi-$25(OH)D_3$ quantification highlighted issues with the use of diluted plasma instead of serum in other analytes of vitamin D [16]; $25(OH)D_2$ and total vitamin D were not reported. In a study of 13 healthy adults, Mena-Bravo et al. found that plasma and serum provided similar levels for $24,25(OH)D_3$, $25(OH)D_3$ and cholecalciferol (D_3), while significantly higher concentrations of $1,25(OH)D_3$ were detected in plasma versus serum [17]. However, no study has compared quantification of vitamin D analytes in plasma and serum samples collected during pregnancy. Comparing these sample types is of benefit to the Vitamin D Standardisation Program (VDSP) that aims to promote 25(OH)D concentration measurements that are accurate (precise and true) and comparable over time, location and laboratory to improve clinical and public health practice world-wide [18].

It is noted that none of the previous studies examined comparability of total 25(OH)D serum and plasma concentrations, and no study has examined changes in clinical vitamin D status based on sample type during pregnancy. The effect of the sample type on resulting concentrations of 25(OH)D and its analytes during pregnancy needs to be identified clearly to ascertain the suitability of using serum and plasma interchangeably in clinical and research settings. Pregnancy is associated with various hormonal and physiological changes in the body, and with vitamin D also acting as a hormone, adaptive changes of vitamin D homeostasis and metabolite concentrations in pregnancy may have implications for the systemic circulation of total 25(OH)D and its analytes [19]. Vitamin D-Binding Protein levels increase drastically during pregnancy, and this can influence the concentration of free 25(OH)D as well as other analytes [20]. A 2019 study examined serum samples in pregnant ($n = 88$) and non-pregnant women ($n = 20$) and found differences in vitamin D metabolism across a range of analytes in pregnancy, as well as across gestation [21]. Whether there are important differences in vitamin D analyte concentrations, as well as clinical vitamin D status, based on sample type, is unknown.

The primary aim of this study was to investigate the comparability of vitamin D quantification using LC-MS/MS between serum and plasma samples collected during pregnancy in a large well-defined

cohort of women enrolled in a clinical asthma trial. The secondary aim was to examine the proportion of women who change vitamin D category based on sample type.

2. Materials and Methods

Serum and plasma samples were collected from pregnant women who were enrolled in a clinical trial of asthma management during pregnancy, conducted in Newcastle, Australia. Details of the trial are previously described [22]. Briefly, women with current asthma, aged 18 years and older, were enrolled via antenatal clinics at the John Hunter Hospital in Newcastle, Australia. Written consent was obtained from participants before trial participation and ethics approval was granted by Hunter New England Health Human Research Ethics Committee (12/10/17/3.04, NSW HREC Reference No: HREC/12/HNE/357).

A non-fasting peripheral blood sample was collected by venepuncture (by a research nurse trained in phlebotomy) into a 6ml EDTA (1.8 mg/mL) plasma tube and 6mL plain serum tube at enrolment (between 12–25 weeks gestation), and processed within 60 min. All samples were centrifuged (3000 rpm) at 4 °C for ten minutes, aliquoted into Eppendorf tubes, and stored at −80 °C. All samples were collected between 2017–2019 and analysed in 2019. Samples were transported by courier on dry ice to the Centre for Microscopy, Characterisation & Analysis in Western Australia, for quantification of vitamin D via LC-MS/MS; this laboratory is certified by the Vitamin D Standardisation Program (VDSP) [18]. LC-MS/MS has been previously described [12]; briefly, samples were extracted using liquid-liquid extraction then separated using a 2D liquid chromatography UPLC system, followed by detection using tandem mass spectrometry. Total vitamin D was comprised of $25(OH)D_2$, $25(OH)D_3$ and Epi-$25(OH)D_3$. The LoD for both $25(OH)D_3$ and Epi-$25(OH)D_3$ was 2.0 nmol/L, and 3.0 nmol/L for $25(OH)D_2$. For blood samples with values below the LoD, a level equal to the detection limit was used, then divided by the square root of 2 (equal to 1.4 nmol/L for Epi-$25(OH)D_3$ and 2.12 nmol/L for $25(OH)D_2$) [23]. Vitamin D sufficiency was defined as total 25OHD ≥75 nmol/L. Cut points for vitamin D insufficiency and deficiency were 50- <75 nmol/L and <50 nmol/L of total $25(OH)D$, respectively [24].

Statistical Analysis

The Shapiro Wilks test was used to determine normality of the data. Bland-Altman plots were used to compare the difference between plasma and serum concentrations of total $25(OH)D$, $25(OH)D_2$, $25(OH)D_3$, epi-$25(OH)D_3$ and detect proportional bias [25]. Pearson correlation (r) and intraclass correlation coefficient (ICC) was used to examine the agreement between the concentrations. The difference in mean values for total $25(OH)D$, $25(OH)D_2$, $25(OH)D_3$ and Epi-$25(OH)D_3$ were examined using the paired t-test with 95% confidence intervals (CI). A *p*-value < 0.05 was considered statistically significant. The mean, range, standard deviation (SD) and coefficient of variation (CV) were reported for each analyte by sample type. The proportion of participants that were vitamin D sufficient, insufficient and deficient were determined based on sample type, as well as any participants that changed category based on the use of plasma compared to serum for total $25(OH)D$ quantification. Cohen's Kappa test was used to explore interrater reliability between participants vitamin D status and sample type. Statistics were computed with STATA IC v15.1 (StataCorp, College Station, TX, USA), and Microsoft Excel (v16.0.5083.1000, Microsoft Corporation, Santa Rosa, CA, USA).

3. Results

There were 114 matching serum and plasma samples. In total, 96.9% (221/228) samples were below the LoD for $25(OH)D_2$ and 11.8% (27/228) samples were below the LoD for Epi-$25(OH)D_3$ (Table 1). The mean (±SD) for total $25(OH)D$ in serum was 86.77 ± 24.91 nmol/L (range 33.8–169.8 nmol/L). The mean for total $25(OH)D$ in plasma was 94.4 ± 28.8 nmol/L (range 28.6–211.2 nmol/L) (Table 1).

Table 1. Mean, range, standard deviation and coefficient of variation for $n = 114$ matched serum and plasma samples collected from pregnant women.

Total 25(OH)D	Minimum (nmol/L)	Maximum (nmol/L)	Mean (nmol/L)	SD (nmol/L)	CV%	*p*-Value *
PLASMA	28.60	211.2	94.40	28.80	30.51	
SERUM	33.80	169.8	86.77	24.91	28.71	<0.001
25(OH)D$_3$						
PLASMA	24.50	200.80	87.83	27.10	30.9	
SERUM	30.3	161.7	80.45	23.65	29.4	<0.001
25(OH)D$_2$ ‡						
PLASMA	<3.0	6.3	2.23	0.54	24.22	
SERUM	<3.0	4.5	2.15	0.24	11.16	0.112
Epi-25(OH)D$_3$ ‡						
PLASMA	<2.0	20.2	4.44	2.81	63.29	
SERUM	<2.0	16.8	4.05	2.28	56.30	0.003

* Paired *t*-test *p*-value comparing mean difference between serum and plasma for each analyte and total 25-hydroxyvitamin D (25(OH). Dusing liquid chromatography-tandem mass-spectrometry. Minimum, maximum, mean and SD are expressed in nmol/L. CV, coefficient of variation, expressed as percentage. SD, standard deviation. ‡: 96.9% of 25-hydroxyvitamin D$_2$ (25(OH)D$_2$) and 11.8% of Epi-25-hydroxyvitamin D$_3$ (25(OH)D$_3$) results were below the LoD's and therefore imputed values were used.

Figure 1 illustrates the agreement between plasma and serum concentrations of total 25(OH)D ($r^2 = 0.903$), 25(OH)D$_3$ ($r^2 = 0.910$) and Epi-25(OH)D$_3$ ($r^2 = 0.862$); correlation analyses were not conducted for 25OHD$_2$ given the high percentage of samples below the LoD.
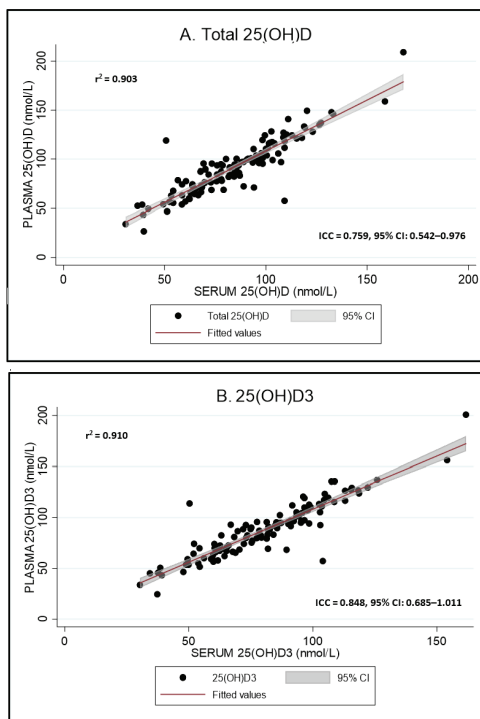
Figure 1. *Cont.*
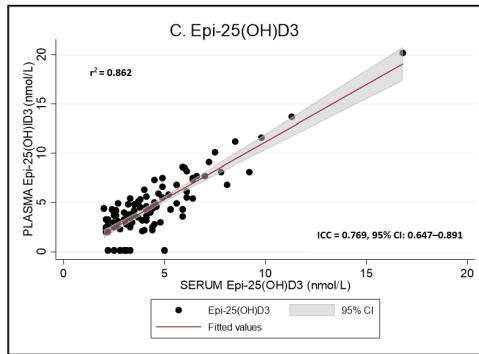
Figure 1. 25(OH)D: 25-hydroxyvitamin D. (**A**–**C**) Scatter plot with Pearson (r^2) and intraclass correlation coefficients (ICC) between serum and plasma concentrations of vitamin D analogues, quantified using LC-MS/MS. (**A**) Total 25(OH)D, (**B**) 25(OH)D$_3$ and (**C**): Epi-25(OH)D$_3$. 95% CI, 95% confidence interval. All results expressed as nmol/L.

The Bland-Altman plot shows a mean total concentration bias for serum vs. plasma of 7.63 mol/L (95% CI 5.36, 9.90, $p = < 0.001$) for total 25(OH)D, 7.38 nmol/L (95% CI 5.28, 9.48 $p = < 0.001$) for 25(OH)D$_3$, and 0.39 nmol/L (95% CI 0.14, 0.64, $p = 0.003$) for Epi-25(OH)D$_3$ (Figure 2).
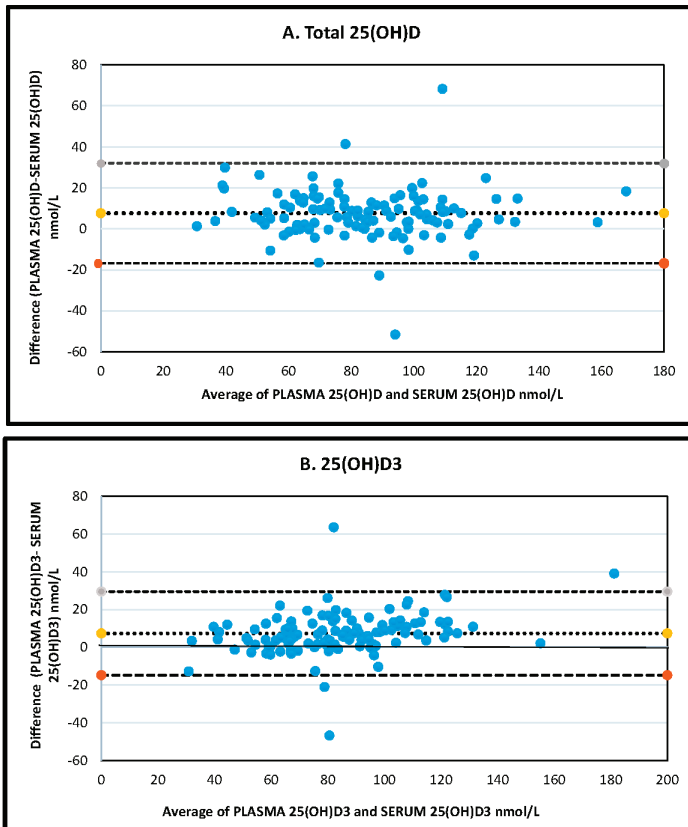
Figure 2. *Cont.*
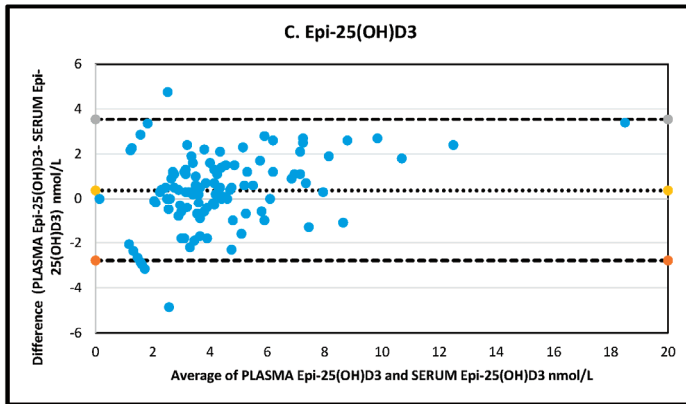
Figure 2. 25(OH)D: 25-hydroxyvitamin D. (**A**–**C**) Bland-Altman Plot comparison between plasma and serum concentrations of vitamin D analytes quantified using liquid chromatography-tandem mass-spectrometry. (**A**) Total 25-hydroxyvitamin D (25(OH)D), (**B**) 25-hydroxyvitamin D_3 (25(OH)D_3) and (**C**) Epi-25-hydroxyvitamin D_3 (25(OH)D_3). Dotted line: mean difference (bias); dashed line: upper and lower 95% confidence intervals. All results expressed as nmol/L.

In regards to vitamin D status based on sample type, for total 25(OH)D; results are shown in Figure 3 for serum and plasma. Cohen's Kappa (κ) statistic showed moderate agreement between the participants' vitamin D category based on serum compared to plasma ($\kappa = 0.639$, 95% CI 0.48, 0.80, $p \leq 0.001$) with 83.3% agreement found. In total, 16.7% ($n = 19$) of participants changed vitamin D status category based on which sample type was used. Of the $n = 19$ that changed vitamin D status based on serum vs plasma samples; $n = 12$ participants changed from insufficient (<75 nmol/L) to sufficient (≥ 75 nmol/L), $n = 3$ changed from deficient (<50 nmol/L) to insufficient (50- <75 nmol/L), $n = 1$ changed from insufficient to deficient and $n = 3$ changed from sufficient to insufficient.

Figure 3. Vitamin D status category based on serum and plasma samples from women between 12–25 weeks gestation using LC-MS/MS.

4. Discussion

This study is the first to examine the comparability of vitamin D quantification by LC-MS/MS in serum and plasma samples collected during pregnancy, and the first to examine the impact of sample type on vitamin D adequacy. Despite a relatively high Pearson and intraclass correlation between serum and plasma concentrations, examination of the concentration bias revealed a signific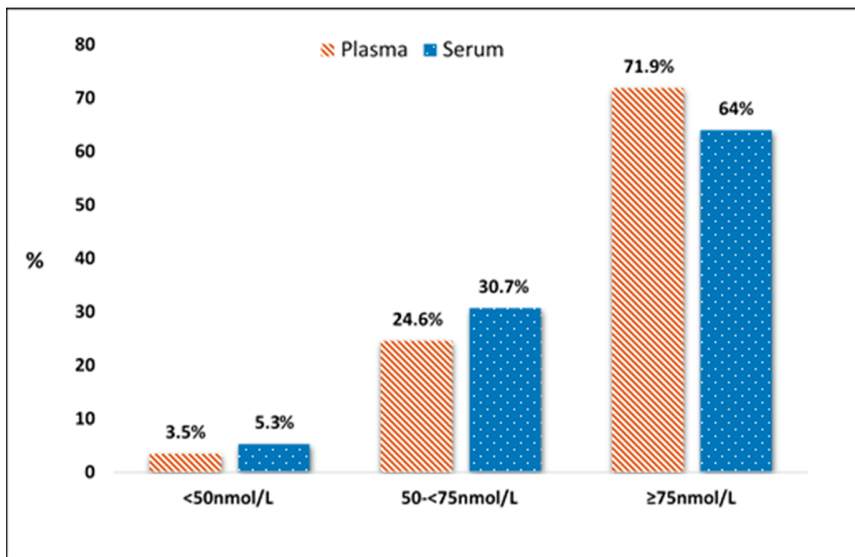ant difference in the mean concentrations of total 25(OH)D in serum vs. plasma. Furthermore, we found 16.7% of participants changed category based on sample type, with 13% changing from deficient to insufficient or insufficient to sufficient with the use of plasma instead of serum.

These results are of concern in a clinical context, especially for pregnant women, as even small differences for patients close to cut-off points for vitamin D deficiency or insufficiency may result in misclassification of their vitamin D status, and may influence subsequent treatment decisions. The current national Australian pregnancy guidelines state that vitamin D supplementation may be considered for women with vitamin D levels <50 nmol/L, highlighting the impact this may have on recommended maternal supplementation, based on the vitamin D test result obtained [26]. Cut-points for vitamin D status and appropriate levels for optimal health have been highly controversial in recent years [27,28]. The Institute of Medicine recommends optimal levels of serum 25(OH)D to be >50 nmol/L based on requirements for bone health [29]. The Endocrine Society has opposed this as an adequate target level, and has identified a target 25(OH)D concentration for optimal health to be >75 nmol/L, based on a large body of evidence highlighting associated outcomes [27], with clinical guidelines recommending supplementation in children and adults to obtain this level [24].

Due to our large sample size, we were able to use Kappa statistic to explore participant's vitamin D status category based on sample type [30,31]. Our resulting Kappa can be interpreted as moderate agreement between the participants resulting vitamin D status (deficient, insufficient or sufficient) based on serum or plasma sample type [31]. Whether a moderate level of agreement is adequate for the use of plasma and serum interchangeably has not been established, but our result does elucidate possible issues, as 13% of participants results could be categorised as false negatives for vitamin D insufficiency or deficiency when plasma was used instead of serum. A 2009 paper examined numerical specifications for trueness and analytical precision for routine analysis of serum/plasma 25(OH)D via immunoassay and LC-MS/MS for establishment of a reference measurement system [32]. Running several models for stringency and practical achievability; they found that, assuming a maximum tolerable limit of 20% clinical misclassifications, the quality goal for bias must be significantly less than 10% [32]. It is noted that the rate of 20% was chosen on an arbitrary basis and data are currently inadequate to ascertain a rate that could be considered acceptable to limit misclassification risk to the population [32]. Further research into acceptable agreement rates of vitamin D category based on sample type is warranted.

Assessing the comparability of sample types for results is important for future research and to assist in the VDSP's goal of standardising vitamin D analysis. These results provide evidence of an important difference in vitamin D quantification, and assessment of vitamin D status, during pregnancy, based on sample type. These results are applicable to LC-MS/MS methods; whether there are differences with nonchromatographic methods based on radioimmunoassay's, such as antibody assays, is unknown. Whether the results would be altered with different anticoagulants used is unknown and requires further investigation. Although serum and plasma are commonly used blood specimen types; they are not equivalent biological matrices [33]. Serum and plasma are used interchangeably for the quantification of vitamin D in some clinical and research settings, and this is the first study to supply evidence that there may be a significant difference in resulting vitamin D concentration using LC-MS/MS and status during pregnancy based on sample type. These results are applicable to pregnant populations; whether these differences in total 25(OH)D quantification by sample type, and subsequent categorisation of vitamin D status, are also seen in non-pregnant populations would require further research. Whilst evidence based recommendations are in place supporting the use of serum and plasma for other nutrients, such as vitamin A and E [16,34,35], similar recommendations

are not in place for vitamin D. Serum is considered the appropriate sample type to use for vitamin D analysis and vitamin D reference ranges are based on serum levels, not plasma levels [24]. Our data further support this recommendation; however, where the use of plasma is unavoidable, development of a conversion factor would allow serum and plasma to be used interchangeably with more confidence in regards to the accuracy and precision of the results.

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Diet and Healthy Lifestyle in the Management of Gestational Diabetes Mellitus

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Abstract: Gestational diabetes mellitus (GDM) among pregnant women increases the risk of both short-term and long-term complications, such as birth complications, babies large for gestational age (LGA), and type 2 diabetes in both mother and offspring. Lifestyle changes are essential in the management of GDM. In this review, we seek to provide an overview of the lifestyle changes which can be recommended in the management of GDM. The diet recommended for women with GDM should contain sufficient macronutrients and micronutrients to support the growth of the foetus and, at the same time, limit postprandial glucose excursions and encourage appropriate maternal gestational weight gain. Blood glucose excursions and hyperglycaemic episodes depend on carbohydrate-intake. Therefore, nutritional counselling should focus on the type, amount, and distribution of carbohydrates in the diet. Further, physical activity has beneficial effects on glucose and insulin levels and it can contribute to a better glycaemic control.

Keywords: gestational diabetes mellitus; GDM; pregnancy; lifestyle; diet; nutrition; weight management; physical activity

1. Introduction

Pregnant women gradually develop insulin resistance during pregnancy, thereby ensuring sufficient nutrient supply for the growing foetus [1]. In women with gestational diabetes mellitus (GDM), the insulin resistance leads to hyperglycaemia [2,3]. The definition of GDM is glucose intolerance with onset or first recognition during pregnancy [3]. Glucose passes through the placenta to the foetus and increases foetal insulin production, which, in turn, stimulates foetal growth, causing macrosomia and children large for gestational age (LGA) [4]. In the short-term, GDM is associated with increased risk of adverse pregnancy outcomes with a following long-term risk of childhood obesity and type 2 diabetes in mother and offspring [5]. The prevalence of GDM is rising [4], and so is the need for treatment.

Lifestyle changes are essential in the management of gestational diabetes. First-line treatment in GDM is medical nutrition therapy, together with weight management and physical activity [6,7]. It has been suggested that lifestyle modification alone is sufficient to control blood glucose in 70–85% of the women that were diagnosed with GDM [7]. How the diet should be composed for women with GDM is a complex matter and still not completely settled. In this review, we seek to provide an overview of the most important dietary interventions and components and how to treat and guide each woman with GDM during pregnancy.

2. Energy Requirements

2.1. Optimal Weight Gain

The recommended weight gain during pregnancy in women with GDM is the same when considering normal glucose tolerance pregnancies (NGTP). Gestational weight gain (GWG) should maintain the growth and development of the foetus [8]. The weight recommendations vary slightly from country-to-country. However, many countries refer to the recommendations for GWG that were made in 1990 by the Institute of Medicine (IOM) of National Academies, which were updated in 2009 based on pre-pregnancy Body Mass Index (BMI) (See Table 1) [8].

Table 1. Recommendations for total weight gain during singleton pregnancy.

Pre-Pregnancy BMI	Total Weight Gain (Range in kg)
Underweight (<18.5 kg/m^2)	12.5–C18
Normal weight (18.5–24.9 kg/m^2)	11.5–16
Overweight (25.0–29.9 kg/m^2)	7–11.5
Obese($\geq$30 kg/m^2)	5–9

Modified from Table S1 in the IOM report by Rasmussen & Yaktine, "Weight Gain During Pregnancy: Reexamining the Guidelines (2009)" [8]. BMI, body mass index.

These weight gain guidelines are based on studies that indicated that women, whose weight gains are outside the recommended ranges, are at increased risk of adverse maternal and neonatal outcomes, such as pregnancy complications, maternal postpartum weight retention, and obesity, in the offspring [9].

In the guidance of pregnant women, a recommended rate of weight gain during 2nd and 3rd trimester can be helpful. Hence, women with a BMI of less than 18.5 kg/m^2 should be recommended a weight gain between 0.44–0.58 kg/week. Women with a BMI between 18.5 to 24.9 kg/m^2 should be recommended a weight gain between 0.35–0.50 kg/week. Women with a BMI between 25.0 to 29.9 kg/m^2 should be recommended a weight gain between 0.23–0.33 kg/week and, finally, women with a BMI of 30 kg/m^2 or above should be recommended a weight gain between 0.17–0.27 kg/week [8].

2.2. Energy Requirements for Normal or Underweight Women

There is not sufficient evidence to suggest that the energy requirements for women with GDM should be different from normoglycemic women or suggest a specific optimal calorie intake for women with GDM [10]. In the clinic, the energy expenditure can be calculated using the equation by Henry multiplied by a factor of physical activity level (PAL) or the equations that were recommended by the IOM (see Table 2).

Table 2. Equations to calculate estimated energy requirement for nonpregnant women.

NNR		IOM	
Age	MJ/d	Age	kcal/d
11–18	(0.0393 W + 1.04 H + 1.93)*PAL	14–18	$135.3 - (30.8 \times$ age [y]) + PA $\times$ [(10.0 $\times$ weight [kg]) + (934 $\times$ height [m])] + 25
19–30	(0.0546 W + 2.33)*PAL	>19	$354 - (6.91 \times$ age [y]) + PA $\times$ (9.36 $\times$ weight [kg]) + (726 v height [m])]
31–60	(0.0433 W + 2.57 H − 1.180)*PAL		

NNR, Nordic Nutrition Recommendations; IOM, Institute of Medicine; PA, physical activity coefficient; PAL, physical activity level; MJ, mega Joule; W, weight in kilograms; H, height in meters, d, day. Modified from the IOM report by Rasmussen & Yaktine 2009, "Weight Gain During Pregnancy: Reexamining the Guidelines" and The Nordic Council of Ministers 2014 "Nordic Nutrition Recommendations: Integrating nutrition and physical activity" [8,11].

The physical activity coefficient or level (PA/PAL) can be determined by the reference values that were given by the Nordic Nutrition Recommendations (NNR) or the IOM (see Table 3).

Table 3. Physical activity level (PAL) for use in equations for energy requirement recommended by NNR and Physical Activity Coefficients (PA values) for use in equations for Energy requirement recommended by IOM.

PAL	NNR
1.1–1.2	Bed-bound or chair-bound
1.3–1.5	Seated work with none or only little physical activity
1.6–1.7	Seated work with some movement or some physical activity
1.8–1.9	Work including standing and moving around or seated work with some movement and with frequent activity
2.0–2.4	Very strenuous work or daily competitive physical training
PA, age ≥19 (ages 14–18)	**IOM**
1.0 (1.0)	Very low active level
1.12 (1.16)	Low active level
1.27 (1.31)	Active level
1.45 (1.56)	Highly active level

IOM, Institute of Medicine; NNR, Nordic Nutrition Recommendations; PA, physical activity coefficient; PAL, physical activity level. Modified from Table 8.7 chapter 8 in the Nordic Council of Ministers 2014 guideline "Nordic Nutrition Recommendations: Integrating nutrition and physical activity" [8] and Table B-1C from the IOM report by Rasmussen & Yaktine, "Weight Gain During Pregnancy: Reexamining the Guidelines (2009)" [11].

An additional assessment of daily energy requirements during pregnancy is based on trimesters, although there is no international agreement on the exact calorie requirements during the three trimesters (see Table 4). There may be considerable variance in the total energy requirement among women with GDM as in NGTP [12], and each patient should be regularly weighed during pregnancy.

Table 4. Additional daily calorie requirements during pregnancy.

Trimester	NNR	IOM
1st trimester	103 kcal	0 kcal
2nd trimester	329 kcal	340 kcal
3rd trimester	537 kcal	452 kcal

IOM, Institute of Medicine; NNR, Nordic Nutrition Recommendations [8,11].

2.3. Energy Requirements for Women with Overweight or with Excessive Gestational Weight Gain

In women with GDM, excessive weight gain has been associated with an increased risk of hypertensive disorders of pregnancy, caesarean section, and LGA-babies [13,14]. Additionally, a meta-analysis concludes that it is extremely important to prevent excessive weight gain in GDM pregnancies [14].

In women with GDM, who have already accomplished a recommended weight gain, weight stabilization is the goal and calorie restriction can be necessary. In women with obesity and GDM, a 30–33% calorie restriction has been shown to reduce hyperglycaemia and plasma triglyceride levels [15]. In a retrospective cohort by Kurtzhals et al., the women with GDM who had the best dietary adherence to an energy-restricted "diabetes diet" and the lowest weight gain had lower foetal growth (infants with a birth weight-SD (standard deviation) score of 0.15 ± 1.1 in contrast to a birth weight-SD score of 0.59 ± 1.6) and decreased HbA1c, as compared to women with GDM with the highest GWG and poor dietary adherence [5].

2.4. Summary, Energy Requirements

The general recommendations for weight gain and the calculation of energy requirements for NGTP are also appropriate for women with GDM. Furthermore, particular attention should be given

in order to avoid excessive weight gain. In women with obesity, or women who have already reached the recommended weight gain, a calorie restriction of 30–33% may be advisable.

3. Carbohydrates

In women with GDM, carbohydrates are the most important macronutrient. The digestion and absorption of carbohydrates cause an increase in blood glucose levels, and postprandial hyperglycaemia is primarily dependent on carbohydrate-intake [16]. The amount and the type of carbohydrate will both impact glucose levels [7]. Thus, a high intake of carbohydrate in a meal can result in hyperglycaemia [16]. However, glucose is the principal energy substrate for the placenta and foetus, which is essential for normal foetal growth and metabolism [17]. The IOM recommends 46–65 Energy percent (E%) from carbohydrates and a minimum of 175 g of carbohydrate daily to ensure appropriate foetal growth and cerebral development and function [2,8,10]. Ketonemia and/or ketonuria should be avoided, as it has been associated with lower mental or motor function in the offspring [2]. Carbohydrates should predominantly consist of starchy foods, a low glycaemic index, and a naturally high content of dietary fibre, such as vegetables, legumes, fruits, and whole grains [2,18,19]. The intake of added sugars should be kept low. The IOM has not set a daily intake of added sugars that individuals should aim for, but recommends that the intake of added sugar is limited to no more than 25% of total energy during pregnancy [8].

3.1. Low-Carbohydrate Diets

There is no international agreement on an appropriate amount of daily carbohydrate intake for women with GDM. Some guidelines recommend that the daily carbohydrate intake should not exceed 40–50E% [20]. Other countries, like Denmark, follow the general recommendation for NGTP, which, in the Nordic countries, is 45–60E% [11]. Only few clinical trials comparing low-carbohydrate diets with higher-carbohydrate diets have been conducted. Hernandez et al. compared a 40% carbohydrate diet with a 60% carbohydrate diet in a randomized crossover study. The 60% carbohydrate diet consisted of higher-complex carbohydrate. The low-carbohydrate diet resulted in a lower postprandial glucose, lower daytime mean glucose concentrations, lower area under the curve of 2 h postprandial glucose, and lower 24 h total glucose area under the curve, when compared with the 60% carbohydrate diet [21]. However, in the group receiving a 60% carbohydrate diet, the postprandial glucose values were still below current targets: 1 h <140 mg/dL (7.8 mmol/L) and 2 h <120 mg/dL (6.7 mmol/L). No differences for fasting blood glucose was found [21]. Moreno-Castilla et al. did not find any differences between groups in insulin treatment or in pregnancy outcomes, such as caesarean sections, LGA-babies, macrosomia, or gestational age at delivery, when comparing a 40% carbohydrate-diet with a 55% carbohydrate diet in a non-crossover randomized study [3]. Thus, there are conflicting results and it should be pointed out that a lower carbohydrate intake will often lead to an increased intake of fat, which, outside pregnancy, has been associated with an increase in serum fatty acids, insulin resistance, and increased foetal fat accretion and infant adiposity in NGTP [21].

3.2. Dietary Fibres

Normally, simple carbohydrates result in higher postprandial excursions than complex carbohydrates. NNR recommends a minimum of 25 g dietary fibre for women in general [11], while the American Diabetes Association recommends a minimum of 28 g of fibre to women with GDM [10], which is similar to IOM recommendations for normoglycemic women during pregnancy [8]. These recommendations can be met by eating 600 g of fruit and vegetables a day with a minimum of 300 g vegetables, with focus on rough and fibrous vegetables and by choosing wholemeal bread, pasta, and rice.

3.3. Low Glycaemic Index Diets

Carbohydrate food can be classified in relation to its effect on postprandial blood glucose expressed as a percentage of the blood glucose response of a reference food (e.g., glucose solution or white bread).

The Glycaemic Index (GI) is a number from 0 to 100 that is assigned to a food, with pure glucose being arbitrarily assigned the value of 100, which represents the relative rise in the blood glucose level two hours after consumption [22].

Fast absorbable carbohydrates with a GI >70 are considered as high GI foods, while slowly absorbed carbohydrates with a GI ≤55 are considered low GI foods [22]. Moses et al. did show a reduced need for insulin in women with GDM, when they consumed a diet with a low GI in a RCT of 63 women with GDM. Even though Moses et al. compared with a diet high in fibre and a low sugar content, a lower GI diet significantly reduce insulin requirements in women with GDM [23]. In a meta-analysis of six RCTs and 532 women with GDM, Xu et al. found that a low-GI diet significantly reduced 2 h postprandial glucose concentrations, without any effect on fasting plasma glucose (FPG), birth weight, HbA1c, macrosomia, or insulin requirements [24]. Moreover, in a recent systematic Cochrane review that included 19 randomized trials and 1389 women with GDM, no effect of a low GI-diet on LGA or other primary neonatal outcomes was found [25].

In the case of GI, the amount of carbohydrate is not considered, which is also a strong factor in the prediction of the postprandial blood glucose response. Glycaemic load (GL), on the other hand, is the product of the total available carbohydrate content in a given amount of food and a given GI [22]. Low GL diet has been shown to improve glycaemic control in type 2 diabetes [26]. The results might also apply to GDM, as GDM and type 2 diabetes mellitus (T2DM) are both characterized by insulin resistance [27]. In a study by Bao et al. of healthy adults, the GL was a more powerful predictor of postprandial glycaemia and insulinemia when compared to the carbohydrate content [28]. In a recent study by Lv et al., 134 women with GDM were randomly allocated to either conventional nutritional nursing or specific nutritional nursing intervention based on GL. Significant differences in fasting blood glucose and the 2 h postprandial glucose levels between the two groups was found with lower levels in the group receiving intervention based on GL [29]. No statistically significant differences in the rates of adverse pregnancy outcomes, such as preterm delivery, foetal macrosomia, and foetal distress, was found; however, there was a lower incidence of premature delivery, eclampsia, pregnancy hypertension syndrome, and foetal macrosomia in the group receiving nutritional nursing based on GL [29].

3.4. Meal Frequency and Carbohydrate Distribution

A daily meal frequency of three main meals and 2–3 small meals or snacks is recommended to avoid excessive food intake at the same time, more specifically to avoid large quantities of carbohydrate and, thereby, reduce the postprandial blood glucose that is illustrated in Figure 1 [2,4,20,30].

It has been suggested that breakfast should only contain small amounts of slowly absorbed carbohydrates, because there is usually a higher postprandial increase in blood glucose in the morning [20]; some guidelines recommend a maximum of 30 g carbohydrate at breakfast [30]. However, these recommendations are primarily based on personal experience and the scientific evidence is limited. In a randomized crossover study with 12 women with GDM, Rasmussen et al. demonstrated a significantly lower mean glucose and fasting blood glucose on a diet with a high carbohydrate intake in the morning as compared with a low carbohydrate intake in the morning. During both intervention periods (high and low carbohydrate in the morning), the recommended total carbohydrate intake was 46E% ± 2E%. In the same study, insulin resistance (as measured by homeostatic model assessment for insulin resistance (HOMA-IR)) significantly decreased during the period with the high carbohydrate intake in the morning. However, Rasmussen et al. also found a higher mean amplitude of glucose excursions and coefficient of variation in the group receiving a high carbohydrate intake in the morning as compared with the low intake [31]. There is a lack of randomized clinical trials studying whether a high or low carbohydrate intake in the morning is preferential.
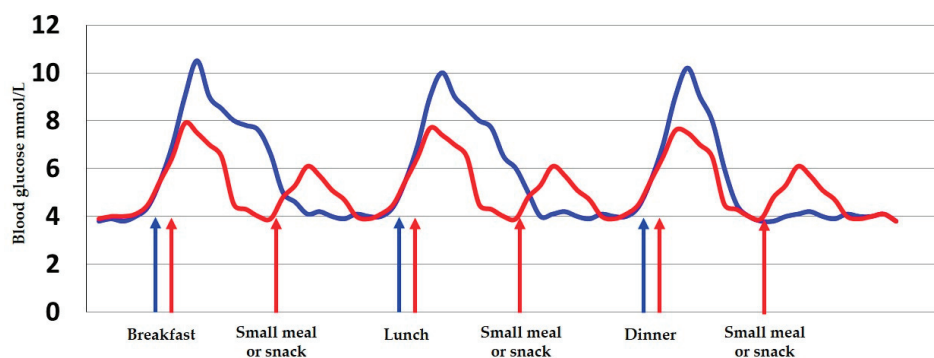
Figure 1. The blood glucose levels according to different strategies for daily food intake. Blue curve illustrates the normal meal pattern and red curve illustrates meal pattern in women with gestational diabetes mellitus (GDM) to avoid excessive blood glucose fluctuations and to preserve the planned number of calories to be ingested. Blue arrows: Three main meals. Red arrows: three main meals and three snacks.

3.5. Artificial Sweeteners

In the United States, the intake of artificial sweeteners (AS) during pregnancy has been increasing in recent years [32] and, in a study from Norway, it is reported that more than 40% of the pregnant women consumed artificially sweetened beverages (ASB) more frequently than once per week in early pregnancy [33]. It is conceivable that the intake of AS is particularly high in women with GDM, seeking to limit the intake of sugar and, to a greater extent, opt for "sugar-free" products and "No added sugar" products.

The Acceptable Daily Intake is defined as an estimate of the amount of food additive that can be ingested daily over a lifetime without health risk. The average use of AS, also called Non-Nutritive sweeteners (NNS), is usually below this limit and the US Food and Drug Administration and European Food Safety Authority, which regulates AS and NNS, has reported asulfame potassium, aspartame, saccharin, and steviol glycosides to be safe for use by the general public, including in pregnancy [34,35]. Observational human studies regarding AS and NNS exposure are often difficult to interpret because of heterogeneity and the lack of accuracy of self-reported intake of AS and NNS. In NGTP, some issues of concern, including increased infant BMI, childhood obesity, and small increase in preterm birth, have been observed [36]. Concerning preterm birth, the European Food Safety Authority has concluded that there is no evidence available to support a causal relationship between the consumption of ASBs and preterm delivery [37].

In a prospective study from the Danish National Birth Cohort, it was shown that approximately half of the women with GDM reported consuming ASB during pregnancy and 9% consumed it daily. When compared to no consumption, daily ASB intake during pregnancy was positively associated with an 1.57-fold increase in LGA risk in offspring, positively associated with an 0.59 SD increase in BMI z-scores at seven years and a 1.93-fold increased risk of overweight/obesity at seven years. The substitution of ASBs with water during pregnancy was associated with a 17% reduced risk for overweight/obesity at seven years, whereas sugar-sweetened beverages (SSB) substitution with ASBs was not related to a lower risk, but with an 1.14-fold increased risk of offspring overweight at seven years [38].

More studies, especially RCTs, on ASB and data with longer follow-up time are wanted.

3.6. Summary, Carbohydrates

Carbohydrate is the macronutrient that has the greatest impact on postprandial hyperglycaemia. Despite some studies suggesting a beneficial effect of low-carbohydrate diets, there is currently no

evidence to recommend a carbohydrate intake that is lower than in NGTP and a minimum of 175 g of carbohydrate should be ensured. The exact amount of carbohydrate should be individualized, and the focus should be on the types of carbohydrate. Carbohydrates should predominantly consist of starchy foods with a naturally high content of dietary fibre, such as vegetables, legumes, fruits, and whole grains. Furthermore, carbohydrate intake should be distributed throughout the day in order to avoid excessive amounts that result in postprandial hyperglycaemia.

4. Protein

During pregnancy, there is an increased requirement of protein due to its role in the synthesis of maternal (blood, uterus, and breasts), foetal, and placental tissues [11]. The recommended amount of protein in the dietary treatment of GDM is similar to the general nutrition advice for normal pregnancies. The IOM recommends 10–35E% from protein during pregnancy, and an estimated average requirement of 0.88 g/kg/d with a minimum recommended daily intake of 71 g protein [8]. NNR recommends a protein intake of 10–20E% for non-pregnant adult women, corresponding to approximately 0.8–1.5 g protein/kg/d based on a PAL of 1.6 for an intake of 10E% and a PAL of 1.4, for an intake of about 20E%, respectively. Further, NNR recommends an additional safe intake of protein for healthy women during pregnancy gaining 13.8 kg of 0.7, 9.6, and 31.2 g/d during first, second, and third trimester, respectively [11]. In general, most pregnant women are able to cover their protein needs, as the increased requirement of protein is met by consuming a normal diet in a quantity that allows a weight gain within the recommended limits [11].

4.1. Protein Metabolism in GDM

The antepartum loss of nitrogen is lower than the postpartum loss, which suggests a reduction in protein catabolism to accrete more nitrogen to support maternal and foetal growth [39]. The loss of nitrogen is similar in GDM pregnancies and normal pregnancies [39,40]. In early GDM, when patients have less metabolic decompensation, there appears to be no difference in leucine kinetics/rate of protein turnover [41]. Later in gestation, when insulin resistance is more pronounced and antidiabetic treatment may be intensified with diet and sometimes insulin, the rate of protein turnover is increased in women with insulin treated GDM [40]. The increased protein breakdown, together with the normal urea excretion, suggests an increased pool of amino acids (AA) available to the placenta and thereby the foetus. The increased pool of AA in GDM and the association with macrosomia is unclear, as the results are often conflicting. One study found no correlation between AA and birth weight in GDM [40]; another found a correlation between leucine and birth weight for both GDM and NGTP controls [41].

4.2. Protein, the Placenta and GDM

A study in Chinese women with GDM found an inverse relationship between protein intake and placental size without any association with birth weight [42]. AA are carried across the placenta through an active transport system providing a greater concentration of AA in the foetus when compared to the mother [43]. In GDM, the transfer of AA across the placenta has been shown to be both decreased [44], unchanged [45], and increased [46]. A study showed elevated levels of branch chained amino acids (BCAA) in GDM as compared to pregnant women with normal glucose tolerance [47]. It has been suggested that the flux of insulinotropic AA (e.g., BCAA) over the placenta affects the beta cell of the foetus creating hyperinsulinemia affecting foetal growth [48]. Studies using metabolomics on cord blood, including both normal and GDM pregnancies, found no association between BCAA and increased insulin/c-peptide levels, thus not supporting BCAA as a cause of foetal hyperinsulinemia [49,50]. However, there was an association with birth weight, but not with the sum of skinfolds [49] or infants being LGA [50]. These findings suggest an association with lean body mass, but not with fat mass.

4.3. Plant vs. Animal Protein

Animal proteins are considered to be complete proteins, as they contain all nine essential AA while plant proteins are considered incomplete, as they can be deficient of one or more essential AA. However, a variety of plant based proteins consumed throughout the day provide sufficient essential AA [51]. A review including studies on vegetarian and vegan diets during pregnancies with sufficient energy and protein supply in the setting of no financial constraint concluded that vegetarian and vegan diets were safe during pregnancy if supplemented with iron and B12 [52]. However, vegans should plan their diets well, as they have an increased risk of not consuming enough protein when compared to omnivores and vegetarians [53]. An Australian study compared vegetarian and non-vegetarian women with GDM from South Asia in Australia found that the vegetarian GDM group received 14 ± 3% of their energy intake from protein as compared to 17 ± 4% in non-vegetarians, but remained within the range of the non-vegetarians supporting the feasibility of a vegetarian diet [54]. Another meta-analysis found that, overall, a vegetarian diet was not associated with birth weight, but that Asian women had a higher risk of delivering babies with low birth weight when compared to Caucasian women [55]. In poor rural areas of Asia, living a life as a vegetarian is more often a result of low income than a choice of lifestyle and lack of micronutrients e.g., vitamin B12 [56] may explain the association between vegetarianism and low birth weight.

A randomized clinical trial (RCT) of animal vs. soy protein applied for six weeks in 68 women with GDM showed lower fasting glucose, lower insulin levels, lower HOMA-IR, and lower triglyceride levels in the plant protein group. The women were randomized to receive protein from either 70% animal or 70% plant protein (half being from soy protein)—both arms were identical in the amount of protein received [57]. Another RCT on soy protein-based protein rich diet vs. high fibre complex diet in GDM showed a reduction in the need for exogenous insulin in the soy diet group. The arms of treatment were isocaloric. However, a low GI diet might explain the results rather than the protein itself [58].

4.4. High Protein Supplementation

Only one study on high protein supplementation during pregnancy has been performed. A RCT was performed in 1980 in poor African American women at risk of having infants with low birth weight. The high protein content of the supplementation (74.2 g/day) was associated with very early premature births, neonatal deaths, and growth retardation up to 37 weeks of gestational age [59]. It is unclear whether the adverse effects occurred because of the study population being unaccustomed to the high protein supplementation or if the results would have been different in populations of normal weight, well-nourished women, and women with GDM. However, the results of the study and lack of other studies of high protein intake during pregnancy implies that one should be reluctant regarding diets exceeding the recommend intake of protein during pregnancy—NGTP or diabetic pregnancies.

4.5. Pre-Meals and GDM

Pre-meals of protein administered prior to a meal have shown promising results on the postprandial blood glucose in non-pregnant healthy individuals and individuals with T2DM [60,61]. In a RCT of 52 women with GDM receiving either 8.5 g of casein hydrolysate (*n* = 26) or placebo (*n* = 26) prior to breakfast and dinner for eight days, the average blood glucose was decreased in the casein group [62]. Milk protein consists of 80% casein and 20% whey. Pre-meal whey protein has shown promising results with lower postprandial blood glucose in both healthy subjects, subjects with metabolic syndrome, and T2DM [60,61,63,64]. T2DM and GDM share similarities in their pathophysiology and, hence, women with GDM may display the same beneficial effect of whey pre-meals on blood glucose.

4.6. Summary, Protein

The current evidence suggests that increased protein intake from plants, lean meat and fish, and reduced intake of red and processed meat are beneficial in the treatment of GDM and may improve insulin sensitivity. The beneficial effect of plant protein on GDM might not be directly attributable to the source of protein, but rather to the reduction of other nutrients that are associated with an increased risk of GDM, such as carbohydrate [65] and saturated fat [66]. Furthermore, the results might not be generalizable to all ethnicities, as the majority of studies only investigated Asian and Middle Eastern women.

5. Fat

The recommended amount of fat in the dietary treatment of GDM is similar to the general nutrition advice for NGTP. The IOM recommends 20–35E% from fat [8], while the recommendation by NNR is the same as in non-pregnancy; 25–40E% [11]. A high intake of fat should be avoided, because this has been associated with infant adiposity, increased maternal inflammation and oxidative stress, and impaired muscle glucose uptake. Further, high fat diets might cause placental dysfunction [21].

5.1. Saturated Fatty Acids

The IOM recommends keeping the intake of trans fatty acids and saturated fatty acids as low as possible while consuming a nutritionally adequate diet during pregnancy [8]. NNR recommends, in general, that adults intake of saturated fat should not exceed 10E% [11]. To meet these recommendations, women with GDM can be instructed in choosing meat and meat products with a maximum of 10% fat, to choose low-fat dairy products, including choosing sour milk products with a maximum of 1.5% fat and limit intake of fatty dairy products, such as cream and butter.

5.2. Monounsaturated Fatty Acids

The recommendation for Cis-Monounsaturated fatty acids (MUFAs) by NNR is the same as in non-pregnancy; 10–20E%. In a study by Lauszus et al., 27 women with GDM were randomized to either high-carbohydrate diet or a high-MUFA diet. The 24 h diastolic blood pressure increased more in the carbohydrate group than in the MUFA-diet group. However, Lauszus et al. also found a significant difference in the intervention effect on insulin sensitivity in delta changes between groups, with a 15% increased insulin sensitivity in the high-carbohydrate diet and 34% decrease in the high-MUFA-diet [67]. More studies are needed if the recommendation for MUFA is to be changed in GDM as compared to a NGTP.

5.3. Polyunsaturated Fatty Acids

Long-chain polyunsaturated fatty acids (PUFAs) of the *n*-3 (α-linolenic acid) and *n*-6 series (linoleic acid) are the most important fatty acids for foetal growth and development [68,69]. *n*-3 and *n*-6 serve as essential components of cell membranes. Additionally, they are precursors for the synthesis of eicosanoids, which are important in the development of foetal nervous, immune, visual, and vascular systems [70–72]. The depletion of long-chain PUFAs in foetal tissues are associated with behavioural, cognitive, and visual abnormalities later in life in NGTP [68]. Furthermore, low levels of *n*-3 and *n*-6 during pregnancy have been shown to be correlated with preterm birth or foetal growth retardation in NGTP [73]. NNR recommends 5–10E% from PUFAs and a minimum of 1E% *n*-3 fatty acids in general for adults. A total intake of 2.7 g/day *n*-3 is considered to be safe during pregnancy [11].The IOM recommends 5–10E% *n*-6 and 0.6–1.2 E% *n*-3 with a minimum of 13 g/d of *n*-6 and a minimum of 1.4 g/day of *n*-3 during pregnancy [8]. An intake of a minimum of 350 g of fish per week, of which 200 g should be fatty fish, will ensure that the patients follow these recommendations. However, pregnant women should avoid predatory fish, due to the content of heavy metals, and salmon from the Baltic sea, due to pollution [74].

With regard to supplements with PUFAs, the evidence is not clear, as studies have shown conflicting results. These are plausibly reflecting the nature of long-chain PUFAs ingested, type of supplement, dose, and on the outcome evaluation. However, some studies with fish oil supplements have shown positive results in women with GDM. In an RCT by Jamilian et al., women with GDM were randomized to either 1000 mg omega-3 acids from flaxseed oil plus 400 IU vitamin E supplements or placebo for six weeks. A positive effect on biomarkers of oxidative stress and inflammation was found together with a significant rise in the total antioxidant capacity, nitric oxide, a significant decrease in plasma malondialdehyde, and a lower incidence of hyperbilirubinemia in new-borns. There was no effect on new-born outcomes (e.g., caesarean section, preterm delivery, or macrosomia >4000g) or C-reactive protein levels [75]. In another RCT by Jamilian et al., 40 women with GDM were randomly allocated to either 1000 mg fish oil capsules or placebo twice a day for six weeks. Fish oil capsules improved gene expression that was related to insulin, lipids, and inflammation; proliferator-activated receptor gamma was upregulated, and low-density lipoprotein receptor, Interleukin-1, and tumor necrosis factor alpha were downregulated. Fish oil supplement, as compared to placebo, also led to a significant reduction in FPG, serum triglycerides, and a significant increase in LDL- and HDL-cholesterol levels. Further, a significant reduction in high-sensitivity C-reactive protein, in those who received fish oil supplements, was found. However, Jamilian et al. did not find any effect on serum insulin, total cholesterol levels, or HOMA-IR [76]. In a study conducted by Samimi et al., a significant difference in changes in serum insulin and HOMA-IR was found in those women with GDM, who received fish oil supplements when compared to placebo. However, Samimi et al. did not find any effect on FPG [77]. Contrary to these results, a systematic review from 2016 did not find any effect of fish oil supplements on FPG, Homeostatic model assessment-Beta cell function, or lipid profiles. It was concluded that there is not enough evidence to support the routine use of fish oil supplements during pregnancy in the treatment of diabetes [78].

5.4. Summary, Fat

Women with GDM can be recommended an intake of 20–35E% from fat. The intake of saturated fat should be limited, and special focus should be placed on ensuring a sufficient intake of *n*-3 fatty acids. Despite some studies reporting a positive effect of fish oil supplementation, there are still conflicting results and, based on the current evidence, routine supplements of fish oil cannot be recommended or refuted, whereas women with GDM are recommended an intake of 350 g/week of fish as in NGTP.

6. Vitamins, Minerals and Tracers

During pregnancy, the need for vitamins and minerals increases [8,11,79]. There is not sufficient evidence to suggest that vitamin and mineral requirements for women with GDM should be different from normoglycaemic women or to suggest a specific optimal vitamins and minerals intake for women with GDM.

Well-nourished women may not need multiple-micronutrient supplements to satisfy daily requirements, but individual adjustments should be made upon the women's specific needs. If pregnant women do not consume an adequate diet, then the IOM recommends multiple-micronutrient supplements [80]. As a minimum, there are recommendations for supplementation with folic acid, vitamin D, and iron. Any need for calcium supplement must be based on intake of dairy products. These micronutrients are discussed in more detail below and Table 5 shows recommendations.

6.1. Vitamin B9/Folic Acid

Folates are important vitamins in pregnancy. Folate is critical for the synthesis of nucleic acids and, thus, cell division, therefore being important in the foetal growth. If the maternal folate level is low, then the risk of low birth weight and neural tube defects increases. Supplementation with folic acid (the synthetic structure of the folate family) during the periconceptional period has been shown to reduce the risk of these outcomes in NGTP [81–83]. The IOM recommends a daily intake of 600 μg/d during pregnancy [8], while the Nordic Council of Ministers 2014 has a lower recommendation of 500

µg/d in pregnancy [11]. A daily supplement of 400 µg folic acid/d may be recommended for all women of childbearing age and during the first 12 week of gestation to avoid low levels of folate in the mother at conception and ensure sufficient dietary intake.

Table 5. Recommendation of specific micronutrients in pregnancy.

Micronutrient	NNR	IOM
Folic acid, µg/day	500	600
25-Hydroxyvitamin D, µg/day	10	5
Calcium, mg/day	900	1000
Iron, mg/day	40	27

IOM, Institute of Medicine; NNR, Nordic Nutrition Recommendations [8,11].

Of notice, the form of folate substitution might be relevant to take into consideration. Common genetic variations in the genes encoding proteins that are involved in folate metabolism can lead to a lower conversion rate of folate to the active form, L-methylfolate. Recently, focus has been put on supplementation with L-methylfolate rather than folic acid. Apparently, women with such genetic mutations may benefit from direct supplementation with L-methylfolate [84].

Some studies have found that homocysteine levels, which are a marker of low folate or vitamin B12 status, are higher in women with GDM as compared to non-diabetic pregnant women. As an example, a cross-sectional study conducted by Guven et al. showed a higher homocysteine concentration in second trimester. However, folate and vitamin B12 levels did not differ between groups [85] and, at present, the same recommendations as for NGTP apply to women with GDM.

6.2. 25-Hydroxyvitamin D

The IOM recommends a dietary intake of 5.0 µg vitamin D/d during pregnancy [8], while NNR, which covers the Nordic countries, where serum 25(OH)D concentrations are often low in winter, recommends 10 µg/d during pregnancy [11]. These recommendations for NGTP are also currently applicable to women with GDM.

Increasing evidence suggests that vitamin D may play an important role in modifying the risk of diabetes [86], as vitamin D acts directly on the pancreatic beta cell by increasing insulin secretion, and indirectly by attenuating systemic inflammation that is associated with insulin resistance [87,88]. Many cross-sectional and prospective observational studies have shown an inverse association between vitamin D status and the prevalence or incidence of type 2 diabetes [86]. Therefore, vitamin D is also the micronutrient that has been studied most extensively in relation to GDM. Several studies indicate a significant inverse relation of serum 25OHD and the incidence of GDM, but it is not clear whether this association is causal [89] and large RCTs of the effects of vitamin D in women with GDM are sparse. However, in a RCT by Asemi et al., 54 women with GDM received either placebo capsules or vitamin D capsules (50.000 IU) twice during the six week study period and intake of vitamin D supplements led to a significant decrease in FPG and insulin resistance assessed by HOMA-IR [90]. In another RCT, women with GDM were randomized to either placebo or 200 IU, 2000 IU, or 4000 IU vitamin D daily. Insulin levels, HOMA-IR, and total cholesterol were significantly reduced in the group receiving 4000 IU of vitamin D [91]. In a recent meta-analysis, including six RCTs, it was found that vitamin D supplementations improved insulin resistance and LDL cholesterol, but had no beneficial effect on FPG, insulin, HbA1c, total-, HDL-cholesterol, and triglycerides concentrations [92].

The effects of vitamin D supplementation in GDM are equivocal and the available trials have been conducted in different settings with differences in subject populations, length of intervention, and forms of vitamin D supplementation. Confounding variables, such as ethnicity and seasonality, add to the complexity of vitamin D studies and vitamin D can be seen as a proxy for a healthy lifestyle with an active life outside being exposed to the sun. At present, it is therefore difficult to conclude

whether vitamin D can reduce the risk of developing GDM and/or improve glycaemic control in women with GDM and vitamin D deficiency/insufficiency, as there is a need for larger well-designed RCTs that evaluate interventions together with the evaluation of confounding factors.

6.3. Calcium

The requirement of calcium is increased during pregnancy [93]. However, the Nordic Council of Ministers 2014 did not find enough data to draw firm conclusions on potential association between calcium intake during pregnancy and bone health in the offspring. The recommended daily intake of 900 mg/day was kept unchanged from the 2004 to the 2012 updated version [11]. However, the IOM has a slightly higher recommendation during pregnancy of 1000 mg/day in women >19 years [8].

Whether supplementation is necessary depends on the woman's food intake. However, calcium supplementation might have a potential positive effect on glycaemic control in women with GDM. Asemi et al. demonstrated a significant reduction in FPG in women with GDM who received 1000 mg calcium/d plus 50.000 U vitamin D3 supplements twice during a six week intervention when compared to placebo. In the same study, Asemi et al. also found a significant reduction in the serum insulin levels and HOMA-IR. It was concluded that calcium plus vitamin D supplementation in women with GDM had beneficial effects on their metabolic profile [93].

In conclusion, it can be advocated to ensure a minimum intake of 900–1000 mg calcium per day during pregnancy in women with GDM. Therefore, it can be recommended that all pregnant women receive e.g., 0.5 L of milk product per day, less when supplemented with cheese, or that 900–1000 mg calcium is ingested daily from other sources of calcium. If the woman is unable to meet these recommendations, then there may be a need of a daily supplement of 500 mg of calcium throughout pregnancy.

6.4. Iron

Iron deficiency is the most common micronutrient deficiency in pregnancy and during childbearing years. Women have increased needs for iron due to the iron losses during menstrual bleeding [11]. Additionally, many women have small iron stores, when they become pregnant and are not gaining appropriate amounts of iron in their diet to cover the increased need during pregnancy. Because of this, some countries recommend iron supplements of 40 mg as early as week 10 of pregnancy [94]. Maternal iron need increases during pregnancy in order to accommodate the growth and maintenance of the foetus and uterus and the increased red blood cell count. Further, there is an expected iron loss when giving birth [11]. The IOM recommends a daily intake of 27 mg/d during pregnancy [8], while iron supplementation of 40 mg/d from week 18–20 of gestation has been suggested by the Nordic Council of Ministers 2014, in order to reduce the risk of low birth weight and preterm delivery [11,95].

However, whether iron supplementation during pregnancy is necessary or a toxic supplement is a controversial topic. The literature suggests that iron influences glucose metabolism [95]. In a cohort study conducted by Bo et al., an association between the intake of iron supplements and a higher oral glucose tolerance test glucose values in women with GDM was found [95]. Today, there is not enough evidence to suggest a different recommendation for iron intake in women with GDM than what applies to NGTP.

7. Probiotics

In recent years, the role of gut microbiota in regulating metabolism has become a hot topic of investigation. Thus, gut microbiota may play a significant role in the development of obesity and may also have an important impact on glucose homeostasis [96]. Moreover, the results indicate that, in pregnancy, the changes in gut microbiota from the first to the third trimester may contribute to the maternal metabolic changes [97]. In a Danish study, the gut microbiota profiles were investigated in 50 women with GDM and in 157 pregnant women with normal glucose tolerance and it was reported that, in the third trimester of pregnancy, GDM was associated with an altered gut microbiota as

compared to that of NGTP [98]. Accordingly, several studies have been performed to determine whether probiotics could be beneficial for the prevention or treatment of GDM. However, the results of the many available studies are equivocal. In a Finnish RCT study, 439 pregnant women with overweight or obesity were divided into four intervention groups with fish oil + placebo, probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium animalis* ssp *lactis*) + placebo, fish oil + probiotics, and placebo + placebo. The primary outcomes were incidence of GDM and change in fasting glucose in the intervention period, but no benefits in lowering the risk of GDM or improving glucose metabolism was found in any of the groups [99]. Callaway et al. performed a large double-blind RCT, including 411 women, in order to determine whether probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium animalis* ssp *lactis*) that were administered from the second trimester in women with overweight or obesity could prevent GDM. Unfortunately, GDM could not be prevented by the intervention [100]. In an Irish RCT, 149 women with GDM received either a probiotic capsule (*Lactobacillus salivarius*) or placebo once daily from diagnosis of GDM to delivery and no effect on glycaemic control was found [101].

However, two meta-analyses have shown that the use of probiotics was associated with an improved glucose and lipid metabolism in pregnant women, and could tentatively reduce the risk of gestational diabetes [102,103]. Another meta-analysis showed that supplementation with probiotic reduced insulin resistance (HOMA-IR) and fasting serum insulin in women with gestational diabetes significantly, as compared to pregnant women with normal glucose tolerance [104]. In a recent study conducted by Kijmanawat et al., women with GDM were randomized to probiotics (*Lactobacillus* and *Bifidobacterium*) or placebo for four consecutive weeks and a significant improvement in glucose metabolism in the probiotic group, regarding fasting glucose, insulin, and HOMA-IR was found [105]. Additionally, in a study conducted by Karamali et al., where 60 women with GDM were included to determine the effects of probiotic supplementation on glycaemic control and lipid profiles after six weeks and beneficial effects on glycaemic control, triglycerides, and VLDL cholesterol were reported. The study was a double blind RCT where the women either received a probiotic capsule (containing three viable freeze-dried strains: *Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium bifidum*) or a matching placebo [106].

Summary, Probiotics

The question of whether gut microbiota modification could be an effective tool in improving glycemic control and reducing insulin resistance in pregnant women with GDM is complicated. The results differ as the human gut houses a complex microbial ecosystem and the present studies have used different pre-or probiotics or multi-strain probiotics, making it difficult to compare studies and to make a final conclusion at this point.

8. Nutrition Counselling

In a recent meta-analysis, including 18 RCTs involving 1151 women with GDM, a moderate effect of dietary interventions on maternal glycaemic outcomes, including changes in fasting, post-breakfast, and postprandial glucose levels, and the need for medication treatment was found [6]. For neonatal outcomes, including 16 RCTs and 841 women with GDM, it was found that modified dietary interventions were associated with lower infant birth weight and less macrosomia [6]. These associations were found despite a high heterogeneity between studies [6], which indicated that several methods can be used and the dietary guidance should probably be adapted to the individual patient.

The American Diabetes Association recommends that women with GDM receive an individualized nutrition plan as a part of medical nutrition therapy. The nutrition plan should be developed in collaboration between the women and an experienced dietician [10]. The adjustment of the nutrition plan should be continuous and based upon self-glucose monitoring, appetite, and weight-gain patterns, as well as consideration for maternal dietary preferences and work, leisure, and exercise. If insulin therapy is added to nutrition therapy, a primary goal is to maintain carbohydrate consistency at meals and snacks in order to facilitate insulin adjustment.

9. Physical Activity

In non-pregnant individuals, it is well established that physical activity reduces insulin resistance by stimulating the glucose transporters on the surface of skeletal muscle cells and thereby improving glucose uptake [107–109]. Interestingly, whereas many studies have addressed the impact of physical activity on various outcomes in pregnancy in general, only a paucity of studies have addressed the impact of physical activity on maternal blood glucose levels and glycaemic control during pregnancy in women with GDM.

9.1. Short Term Effects of Physical Activity in Pregnancy on Maternal Blood Glucose Levels

Acute bouts of physical activity appear to influence maternal glucose levels on short term. Treadmill exercise for 30 min reduces blood glucose and insulin levels in healthy pregnant women [110]. Among women at risk of GDM 20 min of moderate intensity cycling after an oral glucose tolerance test reduced blood glucose excursions and insulin levels within one to two hours after glucose ingestion [111]. However, a long-term effect was not observed, when evaluating continuous glucose measurements for up to 48 h after physical activity [111]. Similar findings were observed after walking, i.e., women at risk of GDM had decreases in blood glucose levels that were associated with the duration and intensity of the exercise with glucose levels aligning within a few hours after physical activity [112].

Similar observations have been made among women with GDM. Light intensity walking after a meal reduced 1-h blood glucose levels, but not 2-h values [113]. Moderate intensity walking after a meal had slightly longer lasting effects on blood glucose levels with effects visible for two to three hours where after blood glucose levels again aligned [114]. Cycling at mild and moderate intensity yielded similar results as after walking, i.e., a short-lasting decreasing effect on blood glucose levels when compared to the resting condition in a "dose-dependent" matter, i.e., larger effects with more intensified physical activity [115].

In the above-mentioned studies, blood glucose levels after physical activity were comparable after minutes to hours. Thus, is appears reasonable that acute bouts of physical activity have short lasting effects on maternal glucose levels. A continuous program of physical activity appears to be necessary for longer-term effects to be seen.

9.2. Longer-Term Effects of Physical Activity

Longer-term effects of bouts of physical activity are more diverse, as the effects could be the direct influence upon glucose metabolism or it could be effects relating to pregnancy outcomes for which glucose metabolism plays a role, i.e., birth weight and a range of pregnancy complications, such as hypertensive disorders, macrosomia, shoulder dystocia, and neonatal hypoglycaemia and jaundice.

Resistance exercise has been reported to be effective in reducing the need for insulin in GDM pregnancy [116], and moderate intensity cycling three times weekly in combination with diet was able to yield weekly blood glucose levels that were comparable to insulin combined with diet [117]. Again, exercising women managed to stay without any need for insulin [117]. In contrast, combined cycling exercise at moderate intensity alternated by walking three to four times weekly did not induce changes in daily blood glucose measurements or in HbA1c values [118].

The effects of physical exercise during GDM pregnancy on pregnancy outcomes have not been thoroughly examined. Often, study protocols have combined physical activity with other lifestyle modifications, so that the individual contributions from diet, physical activity, coaching, or other included interventions on the study outcomes may be difficult to discern. In a 2018 Cochrane overview of reviews, it was concluded that, in general, only limited effects of exercise as the sole intervention in GDM pregnancy could be documented. Of the palette of interventions that could be explored, the best documentation was available for the combination of healthy eating, physical exercise, and self-monitoring of blood glucose levels. In combination, these efforts could reduce the risk of LGA-babies, but probably at the cost of more prevalent inductions of labour [119]. Thus, the beneficial

effects of lifestyle interventions in pregnancy could be accompanied by an introduction of side effects or potential harms in pregnancy [119].

9.3. Recommendations for Exercise in GDM Pregnancy

In Denmark, pregnant women are recommended at least 30 min of (unspecified) moderate intensity physical exercise daily. There are no specific recommendations for physical activity or exercise that addresses women with GDM, but women with GDM are encouraged to exercise more than the recommendations in NGTP [120]. Similar recommendations are found in the Canadian guidelines for physical activity throughout pregnancy [121], in which 150 min of moderate intensity physical activity each week on at least three separate days is recommended for women independent of GDM status.

Exercise three times a week for 40 to 60 min at 65 to 75% of the age-corrected heart rate maximum has been suggested for women with GDM [122]. Activities could be circuit training, walking, or cycling, but the need for studies testing the most optimal physical activity was acknowledged [122].

Thus, physical activity during pregnancies complicated with GDM is recommended, and moderate intensity activity appears to be the choice agreed upon. However, currently, there is no common agreement on the type, frequency, and duration of physical activity that would be beneficial or even most optimal. Further, the optimal gestational age or the optimal range of gestational weeks for intervention needs to be clarified.

9.4. Societal Interventions

The increased prevalence of diabetes mellitus in especially industrialized countries have led to considerations regarding possible societal interventions. The construction of urban environments aimed at facilitating physical activity has been considered. Easy access to minor and local sport facilities might be an opportunity to improve physical activity for some individuals; however, this strategy is dependent on whether the individuals will use such facilities. Urban planning may be a means to increase the level of physical activity on a population level, and it has been reported that increasing the "walkability" of a neighbourhood is associated with a lower incidence of diabetes [123]. Walking has been suggested to be an especially attractive means of physical activity during pregnancy [124]. In GDM, a single study recently reported on the relationship between neighbourhood walkability and variables that were related to GDM [125]. High neighbourhood walkability was, in general, associated to a lower pre-pregnant BMI and higher pre-pregnant levels of physical activity. In pregnancy, though, increasing walkability of neighbour surroundings was not associated to GWG, insulin sensitivity, glycaemia, or beta cell function [125] Additionally, no difference in GDM prevalence was observed across the different classes of walkable surroundings [125].

Despite low evidence for the time being of the effect of walking on the risk for GDM in pregnancy, walking that is facilitated on both the individual and societal levels may prove to be a simple and obtainable way to introduce more physical energy expenditure in pregnancy [124,125].

9.5. Hindrances to Exercise in Pregnancy

During pregnancy, certain conditions may limit physical activity. Pre-existing medical conditions may limit the amount of physical activity that can be performed. Musculoskeletal or cardiac diseases may decrease the daily level of physical activity and preclude any invigorated physical activity. Additionally, conditions that are related to pregnancy may lead to the recommendation of immobilization or even bed rest, e.g., short cervix conditions or imminent premature delivery. Despite the lack of evidence for promoting immobilization of women with such complications, clinical practice implies that some degree of immobilization is often instituted. In the case of threatening preterm delivery, the administration of corticoid therapy for foetal lung maturation may further exacerbate insulin resistance, at least for days [126]. Furthermore, common conditions, like pelvic joint laxity and pelvic girdle discomfort, will often lead to cautious movements and decreased levels of physical activity. More uncommon, lower

extremity varicose veins or even deep venous thrombosis may cause immobilization. Such conditions are primarily related to the third trimester of pregnancy, i.e., at the time of maximal insulin resistance.

9.6. Summary, Physical Activity

When GDM is present, single physical activities clearly has short term effects on blood glucose levels. However, sustainable effects are more complex to obtain. Long-lasting effects, be it on maternal blood glucose levels or on pregnancy outcomes in general, do with all likelihood depend on daily physical activity and may be further corroborated by a concomitant reduction in GWG. Measures to increase the daily level of physical activity and the strategy for exercise and physical activity in pregnancy with GDM still need further exploration.

10. Conclusions

A summary of the above recommendations is found in Table 6. All women with GDM should be offered dietary advice by a clinical dietitian, as dietary counselling the cornerstone in the treatment of GDM. Knowledge of the impact of diet on blood glucose is of great importance in preventing complications, such as birth complications, caesarean section, LGA-babies, and type 2 diabetes, later in life. The woman should receive guidance on how to construct a varied diet and how to avoid hyperglycaemia. Particular efforts should focus on carbohydrate intake as both type, amount and distribution of carbohydrate are of major importance for the postprandial blood glucose. In general, the same recommendations for minerals and vitamins apply to women with GDM as in NGTP. In addition, physical activity of moderate intensity for at least 30 min daily or 150 min weekly should be encouraged, as this may contribute to improved glycaemic control.

Table 6. Summary of recommendations.

Dietary Components	Recommendations
Energy	Excessive weight gain should be avoided and a calorie restriction of 30–33% is advisable in women with overweight or women who have already gained the recommended weight during pregnancy
Carbohydrates	Exact amount of carbohydrate should be individualized. A minimum of 175 g/d should be ensured. Patients should be guided to choose starchy foods such as vegetables, legumes, fruits, and whole grains.Carbohydrate intake should be distributed throughout the day.
Protein	Total amount of protein should be 10–35E% with a minimum of 71 g/d. Protein intake should primarily come from plants, lean meat, and fish.
Fat	Total amount of fat should be 20–40E% with a maximum of 10E% from saturated fat, a minimum of 10–20E% from MUFAs, and 5–10E% from PUFAs. An intake of a minimum 350 g of fish/week may be advisable.
Folic acid	500–600 µg/d is recommended. Daily supplement of 400 µg/d may be advisable for all women at childbearing age and during the first 12 week of gestation.
25-Hydroxyvitamin D	5–10 µg/d is recommended depending on how much sunlight the woman gets.
Calcium	900–1000 mg/d is recommended. Supplement may be advisable in women with a lack of intake of dairy products.
Iron	27–40 mg/d is recommended.
Probiotics	It remains unresolved whether probiotics have beneficial metabolic effects in women with GDM.

d, daily; E%, energy precent; GDM, gestational diabetes mellitus; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

Abbreviations

AA	Amino acids
AS	Artificial sweeteners
ASB	Artificially sweetened beverages
BCAA	Branch chained amino acids
BMI	Body mass index
E%	Energy percent
FPG	Fasting plasma glucose
GDM	Gestational diabetes mellitus
GI	Glycaemic index
GL	Glycaemic load
GWG	Gestational weight gain
HOMA-IR	Homeostatic Model Assessment for insulin Resistance
IOM	Institute of Medicine
LGA	Large for gestational age
MUFA	Monounsaturated fatty acid
NGTP	Normal glucose tolerance pregnancies
NNR	Nordic Nutrition Recommendations
NNS	Non-Nutritive sweeteners
PA	Physical activity coefficient
PAL	Physical activity level
PUFA	Polyunsaturated fatty acid
T2DM	Type 2 diabetes mellitus

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Maternal Arsenic Exposure and Gestational Diabetes: A Systematic Review and Meta-Analysis

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Abstract: Gestational diabetes mellitus (GDM) is a metabolic complication associated with adverse outcomes for mother and fetus. Arsenic (As) exposure has been suggested as a possible risk factor for its development. The aim of this meta-analysis was to provide a comprehensive overview of published evidence on the association between As and GDM. The systematic search from PubMed, MEDLINE, and Scopus was limited to full-length manuscripts published in peer-reviewed journals up to April 2020, identifying fifty articles. Ten studies met the inclusion criteria, nine for quantitative synthesis with a total of n = 1984 GDM cases. The overall pooled risk was 1.56 (95% Confidence Interval - CI = 1.23, 1.99) with moderate heterogeneity (χ^2 = 21.95; $I^2\%$ = 64). Several differences among the included studies that may account for heterogeneity were investigated. Stratification for exposure indicator confirmed a positive association for studies assessing urine As. A slightly higher risk was detected pooling studies based in Asia rather than in North America. Stratification for GDM diagnostic criteria showed higher risks when diagnosis was made according to the Canadian Diabetes Association (CDA-SOGC) or World Health Organization (WHO) criteria, whereas a lower risk was observed when adopting the American Diabetes Association (ADA) criteria. These results provide additional evidence for a possible association between As exposure and GDM, although the data need to be interpreted with caution due to heterogeneity.

Keywords: arsenic; arsenic exposure; arsenic toxicity; gestational diabetes mellitus; pregnancy

1. Introduction

Gestational diabetes mellitus (GDM), a common metabolic disease that affects up to 14% of pregnant women worldwide, is a glucose intolerance that develops during pregnancy and usually resolves after delivery [1,2]. This condition exposes both mother and fetus to multiple adverse outcomes including an increased likelihood of pre-eclampsia, early delivery, congenital malformations, intrauterine fetal death, fetal macrosomia, polyhydramnios and neonatal hypoglycemia [3–6]. Furthermore, both GDM mothers and their offspring have higher risk of developing type 2 diabetes mellitus (DM2) and cardiovascular diseases [7–10]. Since traditional well-known GDM risk factors such as maternal age, obesity, lifestyle and ethnicity [11–13] do not clearly explain the prevalence of the disease in pregnancy, there has been

a growing interest in the hypothesis that some environmental factors may be implicated in GDM pathogenesis. Among all the widespread naturally occurring pollutants, Arsenic (As) is one of the potential candidates [14–16]. Millions of people are chronically exposed to As, primarily through contaminated drinking water at concentrations above the World Health Organization (WHO) guideline limit of 10 μg/L [17,18] or by ingestion of some foods such as rice or seaweed. Inorganic As, largely consisting of arsenate and to a lesser extent arsenite [19], is either metabolized and methylated in the liver to both monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) or excreted unchanged in urine [20]. This metal seems to interfere with different processes including oxidative stress, signal transduction and gene expression, resulting in the growth hormone/insulin-like growth factor axis disruption and pancreatic beta-cell dysfunction [21–24].

Several studies have found an association between GDM and As levels in maternal blood, urine and meconium, supporting the possibility that a high level of As exposure might predispose to maternal GDM. However, the data obtained so far are quite inconsistent [14,25–33].

To offer an overview of the evidence available in the literature, we conducted a systematic review and meta-analysis on the plausible link between maternal As exposure and the risk of developing GDM.

2. Materials and Methods

This systematic review and meta-analysis were performed according to the Preferred Reporting Item for Systematic Reviews and Meta-analysis (PRISMA) guidelines [34]. The study protocol was registered and accepted in PROSPERO before starting the data extraction (ID CRD42020195667). No Institutional Review Board approval was needed.

2.1. Search Strategy and Study Selection

We performed an advanced, systematic search of the online medical databases PubMed, Medline and Scopus using the following keywords: "arsenic" and "arsenic exposure" in combination with "gestational diabetes mellitus" or "diabetes in pregnancy". Specific tools available in each database such as MeSH terms were used to optimize search output. Only manuscripts written in English and published in peer-reviewed journals up to April 2020 were included and duplicates were removed by using Endnote software version X9 (Clarivate Analytics, Philadelphia, USA, 2013). The potential eligibility of articles was first assessed by screening titles and abstracts. Then, full-text manuscripts were obtained and the final decision for inclusion was made after detailed examination of the articles. In order to identify any additional relevant citations, we also checked the reference lists of the eligible articles. The electronic search, the study selection and the eligibility for qualitative synthesis were independently assessed by two authors (R.V. and C.D). An independent author (N.S) assessed the eligibility for quantitative synthesis. Disagreements were resolved by discussion with a fourth reviewer (J.O.).

2.2. Inclusion Criteria

The following predefined inclusion criteria were used to screen citations for eligibility: (i) exposure to As was assessed through an appropriate exposure indicator (serum/plasma As, urinary As, toenail As, tap water As, meconium As); (ii) risk estimates were provided using odds ratio (OR) or relative risk (RR) with the corresponding 95% confidence interval (CI); (iii) study design limited to analytical studies (cross-sectional, case-control, cohort, ecologic or correlational); (iv) outcome of interest was GDM and diagnosis of GDM was confirmed by a positive glucose challenge test (GCT, 50 gr) and/or a positive oral glucose tolerance test (OGTT, 75/100 gr), according to the diagnostic criteria recommended by either the American Diabetes Association (ADA), the World Health Organization (WHO), the French National College of Obstetricians and Gynecologists (Collège National des Gynécologues et Obstétriciens Français, CNGOF), or the Canadian Diabetes Association and the Society of Obstetricians and Gynecologist of Canada (CDA-SOGC) [35–39].

We excluded descriptive studies (case-report and case-series) and studies not reporting original results (reviews, abstracts, editorials, comments) as well as those dealing with the pathological condition of altered blood glucose levels not satisfying the diagnostic criteria for GDM (i.e., impaired fasting glucose (IGT)). Finally, studies were excluded from the quantitative synthesis (meta-analysis) if a comparable estimation of effect size was not provided or in the sensitivity analyses.

2.3. Data Extraction

The following data from studies included in the quantitative synthesis were collected and tabulated by three independent reviewers (N.S., C.D. and R.V.) using a standardized data extraction form: (i) first author name, (ii) publication year, (iii) study country, (iv) study period, (v) study design, (vi) sample size, (vii) age and demographic data of the sample, (viii) number of cases, (ix) diagnostic method used to define cases, (x) exposure, (xi) exposure indicator (serum/plasma As, urinary As, toenails As, tap water As, meconium As), (xii) time of pregnancy when exposure was detected, (xiii) confounding variables in multivariate analysis, and (xiv) risk estimates with 95% CI.

2.4. Assessment of Risk of Bias

Two review authors (R.V. and C.D.) independently assessed the risk of bias by using the risk of bias tool for cohort studies developed by the Clarity Group (Supplementary Figure S1) [40].

We classified the possible sources of bias as definitely yes (low risk of bias), probably yes (moderate risk of bias), probably no (serious risk of bias), and definitely no (critical, high risk of bias), and then we assessed a comprehensive risk of bias judgment for each study included in our review.

In the case of disagreements, resolution was achieved by discussion with a third reviewer (J.O.).

2.5. Data Analysis

Risk estimates with 95% CI were extracted by an independent reviewer (N.S.) from the original works. Almost all the studies included in the quantitative analysis presented odds ratios (ORs) and their 95% CIs. Relative risks (RRs) were converted in ORs [41]. In studies reporting results for several confounding parameters, we used the data adjusted for the largest number of factors. In studies reporting risk estimates for tertiles/quartiles of exposure, we considered the data for the highest.

Multivariate-adjusted risk estimates were transformed into log ORs and were pooled together using the generic inverse-variance approach as the model estimator with both fixed and random effect analysis. To incorporate the estimate of the pooled effect measure in the between-study variance (τ^2), the random-effect model suggested by DerSimonian and Laird was preferred for the quantitative synthesis of all included studies [42]. A *p*-value < 0.05 was interpreted as statistically significant. Sensitivity analyses were conducted by omitting one study at a time to explore the weight of each work in estimating pooled risks.

Statistical heterogeneity of the intervention effects was assessed with χ^2 test and I^2 statistics. I^2 index values were interpreted as follows: insignificant heterogeneity if I^2 was 0–25%, low heterogeneity for I^2 25–50%, moderate heterogeneity when I^2 50–75% and high heterogeneity, whereas I^2 was greater than 75% [43]. A low *p*-value (<0.10) from the χ^2 test indicated heterogeneity [44].

Potential publication bias was investigated by plotting the natural logarithm of the estimated OR (lnOR) against its standard error (SE). Asymmetry of the funnel plot was verified using the linear regression method proposed by Egger et al. [45].

Subgroup analyses were performed following the guidelines suggested by Wang et al. [46]. Risk estimates were combined using both fixed and random effect models. An a priori-defined subgroup analysis based on study design (cross-sectional, case-control, cohort, correlational) was performed. Subgroup-analysis based on the exposure assessment method (serum/plasma As, urinary As, toenail As, tap water As, newborn meconium As), study country (North America, South America, Asia, Europe), and diagnostic criteria for GDM (ADA, WHO, CNGOF, CDA-SOGC) were then performed to

investigate the possible causes of statistical and clinical heterogeneity. All subgroup analyses were implemented when at least two studies could be included.

Statistical analysis was performed using RevMan software version 5.3 (Copenhagen: The Nordic Cochrane Center, The Cochrane Collaboration, 2014).

3. Results

3.1. Literature Search

The literature search identified 50 articles: among them, 10 met the inclusion criteria and the following characteristics were extracted [14,25–33]. The main characteristics of the included studies are summarized in Table 1. All the included studies were published recently, between 2015 and 2020. More than a half (six out of ten) were cohort studies, two were cross-sectional studies, one was a retrospective case-control study nested in a cohort and one was a correlational study.

The flowchart of the systematic review is available in Figure 1 (PRISMA template). The risk of bias of the included studies are summarized in Supplementary Figure S1.

3.2. Description of Studies

The plausible association between GDM and As exposure was assessed by the analysis of different human samples. Three studies collected blood samples, five papers analyzed urine samples, two evaluated arsenic concentration in home tap water, only one study measured As concentration in the meconium, and one paper in urine samples, home tap water, and toenails.

3.2.1. Arsenic in Blood Samples

Shapiro et al. used As in first trimester blood samples as an indicator of exposure, finding elevated odds of GDM in the highest quartile of As exposure in the adjusted analysis (adjusted odds ratio (aOR) = 3.7; 95% CI = 1.4, 9.6) [14].

Similar results were obtained by Xia et al., who evaluated As levels in blood samples in the first and second trimester and cord blood, finding an association between GDM and As levels only for the fourth quartile of the first trimester samples (aOR = 1.71; 95% CI = 1.23, 2.38). Stratified analyses showed the association was largely limited to normal maternal age (aOR = 1.90; 95% CI = 1.19, 3.04) and normal weight women (aOR = 1.77; 95% CI = 1.18, 2.66) [25].

The cohort study conducted by Wang et al., which evaluated blood samples taken the day after delivery, showed an increased risk of GDM for the second tertile (aOR = 1.49; 95% CI = 1.11, 2.01). This risk was even higher among women with low pre-pregnancy BMI (<18.5 kg/m^2) (aOR = 2.69; 95% CI = 1.04, 6.95) and high pre-pregnancy BMI (≥24 kg/m^2) (aOR = 2.68; 95% CI = 1.36, 5.27) in the second tertile [26].

Table 1. Main characteristics of the considered studies.

Author, Year	Study Country	Study Design	Study Period	Sample Size (Cases/ Controls)	Age (Cases/ Controls)	Definition of Cases	Exposure Indicator and When	As Exposure (Cut-Off or LOD)	Confounding Factors Considered
• **Blood samples (3 studies)**									
Shapiro et al., 2015 [14]	Canada	Cohort study	2008–2011	48/1167	18–29 yo: 12.5%/ 24.8% 30–34 yo: 45.8%/ 34.8% ≥35 yo: 41.7%/ 40.2%	CDA-SOGC Criteria [a] [38,39]	1st trimester blood samples [1]	LOD: 0.22 µg/L	Maternal age, race, pre-pregnancy BMI, education, parity, race
Xia et al., 2018 [25]	China	Cohort study	05/2013– 09/2014	419/2841	cases: 27.79 ± 4.25 yo controls: 26.18 ± 3.48 yo	ADA Diagnostic Criteria [b] [35]	1st, 2nd, 3rd trimester serum samples	LOD: 0.0047 µg/L	Maternal age, pre-pregnancy BMI, monthly income, gestational age, parity
Wang Y. et al., 2019 [26]	China	Cohort study	2012–2016	776/776	cases: 31.00 ± 4.53 yo controls: 30.97 ± 4.53 yo	ADA Diagnostic Criteria [b] [35]	Serum [2] samples the day before delivery	As level: a. Low <10.64 µg/L b. Middle 10.64–21.12 µg/L c. High ≥21.12 µg/L	Maternal age, pre-pregnancy BMI, gestational weight gain, physical activity, family history of diabetes, month of conception, residence, education, monthly income, smoking, fetal gender, parity, gestational age
• **Urine samples (5 studies)**									
Farzan et al., 2016 [27]	USA	Cohort study	01/2009– 05/2016	14/1032	cases: 32.2 yo controls: 30.9 yo	ADA Diagnostic Criteria [b,c] [35]	home tap water samples, urine samples at 24–28 gw, toenails samples	LOD (urine): 0.10–0.15 µg/L	Maternal age, pre-pregnancy BMI, pregnancy weight gain, smoking, secondhand smoke exposure, education, gestational week of glucose testing, urinary creatinine
Ashley-Martin et al., 2018 [28]	Canada	Cohort study	2008–2011	42/1049	<29 yo: 19.2%/ 30.3% 30–34 yo: 46.8%/ 36.3% ≥35 yo: 34.0%/ 33.7%	CDA-SOGC Diagnostic Criteria [a] [38,39]	1st trimester urinary concentrations of arsenite, arsenate, MMA, DMA and AsB	LOD: 0.75 µg/L	Maternal age, gravidity, race, education, parity, pre-pregnancy BMI, maternal first trimester blood Cd levels

Table 1. *Cont.*

Author, Year	Study Country	Study Design	Study Period	Sample Size (Cases/ Controls)	Age (Cases/ Controls)	Definition of Cases	Exposure Indicator and When	As Exposure (Cut-Off or LOD)	Confounding Factors Considered
Munoz et al., 2018 [29]	Chile	Cross-sectional study	06/2013– 10/2013	21/223	≤29 yo: 57.1%/ 74.4% 30–34 yo: 28.6%/ 15.7% ≥35 yo: 14.3%/ 9.9%	WHO Diagnostic Criteria [d,e] [36]	2nd trimester urinary levels of arsenite, arsenate, MMA, DMA, T-InAs (calculated by adding values of these species)	LOD: 0.1 µg/L	Maternal age, education, ethnicity, BMI
Khan et al., 2018 [30]	Bangladesh	Cross-sectional study	–	31/169	cases: 25.19 ± 4.28 yo controls: 23.95 ± 3.92 yo	WHO Diagnostic Criteria [d] [36]	urine samples (not said when)	Not As exposed: ≤0.100 mg/L As exposed: >0.100 mg/L	Maternal age, gestational age, parity, BMI
Wang X., et al., 2020 [31]	China	Cohort study	07/2014– 07/2016	241/1849	cases: 29.54 ± 4.13 yo controls: 28.25 ± 3.34 yo all sample: 28.40 ± 3.47 yo	ADA Diagnostic Criteria [b] [35]	urine samples [3] <20 gw	LOD: 0.009 µg/L CAU-As: a. Low <32.11 µg/L b. Middle 32.11–48.11 µg/L c. High ≥48.11 µg/L	Maternal age, pre-pregnancy BMI, gravidity, occupational status, smoking exposure, average personal monthly income, family history of diabetes, physical activity, fetal sex
• **Tap water samples (2 studies)**									
Farzan et al., 2016 [27]	USA	Cohort study	01/2009– 05/2016	14/1032	cases: 32.2 yo controls: 30.9 yo	ADA Diagnostic Criteria [b,c] [35]	home tap water samples, urine samples, toenails samples	LOD (water): 0.001–0.07 µg/L	Maternal age, pre-pregnancy BMI, pregnancy weight gain, smoking, secondhand smoke exposure, education, gestational week of glucose testing, urinary creatinine
Marie et al., 2018 [33]	France	Semi-ecological study (correlational)	2003 2006 2010	286/4767	all sample: 29.1 ± 5.6 yo	CNGOF Diagnostic criteria [g] [37]	water samples during the 12 months before pregnancy	Not As exposed: <10 µg/L As Exposed: a. Low 10–30 µg/L b. High ≥ 30µg/L	Maternal age, family situation, number of inhabitants in commune of residence, geographic origin, employment during pregnancy, paid employment, pre-pregnancy BMI, type of pregnancy, year of delivery

Table 1. *Cont.*

Author, Year	Study Country	Study Design	Study Period	Sample Size (Cases/Controls)	Age (Cases/Controls)	Definition of Cases	Exposure Indicator and When	As Exposure (Cut-Off or LOD)	Confounding Factors Considered
• Meconium samples (1 study)									
Peng et al., 2015 [32]	China	Case-control study nested in a cohort	06/2012–07/2012	137/190	cases: 27.85 ± 3.87 yo controls: 26.34± 2.64 yo	WHO Diagnostic Criteria d,f [36]	meconium samples during the first 2 postnatal days	LOD: 0.06 µg/L	Maternal age, pre-pregnancy BMI, gravidity, parity, HBV infection, newborn sex
• Toenails samples (1 study)									
Farzan et al., 2016 [27]	USA	Cohort study	01/2009–05/2016	14/1032	cases: 32.2 yo controls: 30.9 yo	ADA Diagnostic Criteria b,c [35]	home tap water samples, urine samples, toenails samples 2 weeks post-partum	Ln toenails As (µg/g) (not said LOD)	Maternal age, pre-pregnancy BMI, pregnancy weight gain, smoking, secondhand smoke exposure, education, gestational week of glucose testing, urinary creatinine

Abbreviations: As, Arsenic; LOD, limit of detection; GDM, Gestational diabetes mellitus; yo, years old; BMI, body max index; ADA, American Diabetes Association; CAU-As, creatinine-adjusted urinary arsenic; gw, gestational week; Cd, Cadmium; MMA, monomethylarsonic acid; DMA, dimethylarsinic acid; AsB, and arsenobetaine; T-InAs, Total inorganic arsenic; WHO, World Health Organization; CDA-SOGC, Canadian Diabetes Association-Society of Obstetricians and Gynecologist of Canada; CNGOF, French National College of Obstetricians and Gynecologists; HBV, Hepatitis B virus; Ln, Logarithm. Notes: ^{1} Arsenic, Cadmium, Mercury, Lead, Eleven phthalate metabolites and Total Bisphenol A. ^{2} Nickel, Arsenic, Cadmium, Antimony, Tallium, Mercury, and Lead. ^{3} Nickel, Arsenic, Antimony, Cadmium, Cobalt and Vanadium. ^{a} GCT 50 gr positive (After 1 h: >10.3 mmol/L) or OGTT 75/100 gr at least 2 altered values (Fasting: ≥5.3/5.8 mmol/L; After 1 h: ≥10.6 mmol/L; After 2 h: >8.9/9.2 mmol/L; After 3 h: -/8.0 mmol/L). ^{b} One step approach: OGTT 75 gr at 24–28 gw at least 1 altered value (Fasting: ≥5.1 mmol/L; After 1 h: ≥10.0 mmol/L; After 2 h: ≥8.5 mmol/L). ^{c} *Farzan* et al. (2016). Two step approach: GCT 50 gr at 24–28 gw high positive (After 1 h: >200 mg/dL) or GCT 50 gr at 24–28 gw borderline (After 1 h: 120–140 mg/dL)/positive (After 1 h: 140–200 mg/dL) and OGTT 100 gr at least 2 positive values (Fasting: ≥5.3 mmol/L; After 1 h: ≥10.0 mmol/L; After 2 h: ≥8.6 mmol/L; After 3 h: 7.8 mmol/L)/diagnosis of GDM in medical records. ^{d} GDM diagnosis: OGTT 75 gr at any time of pregnancy at least 1 altered value (Fasting: 5.1–6.9 mmol/L (92–125 mg/dL); After 1 h: 10.0 mmol/L (180 mg/dL); After 2 h: 8.5–11.0 mmol/L (153–199 mg/dL)). ^{e} *Munoz* et al. (2018). Criteria established in Pregnancy and Diabetes Guide by the Ministry of Health of Chile according to WHO diagnostic criteria for diabetes: Blood glucose at early pregnancy on 2 different days positive (Fasting glycemia: 100–125 mg/dL) and/or OGTT 75 gr at 24–28 gw positive (After 2 h: ≥140 mg/dL). ^{f} *Peng* et al. (2015). Diabetes in pregnancy diagnosis (more severe than GDM): OGTT 75 gr at any time of pregnancy at least 1 altered value (Fasting ≥7.0 mmol/L; After 2 h: ≥11.1 mmol/L). ^{g} GCT 50 gr positive (After 1 h: ≥2.0 g/L) or GCT 50 gr borderline (1.30–2 g/L) and OGTT 100 gr at least 2 positive values (Fasting: >0.95 g/L; After 1 h: >1.80 g/L; After 2 h: >1.55 g/L; After 3 h: >1.40 g/L).
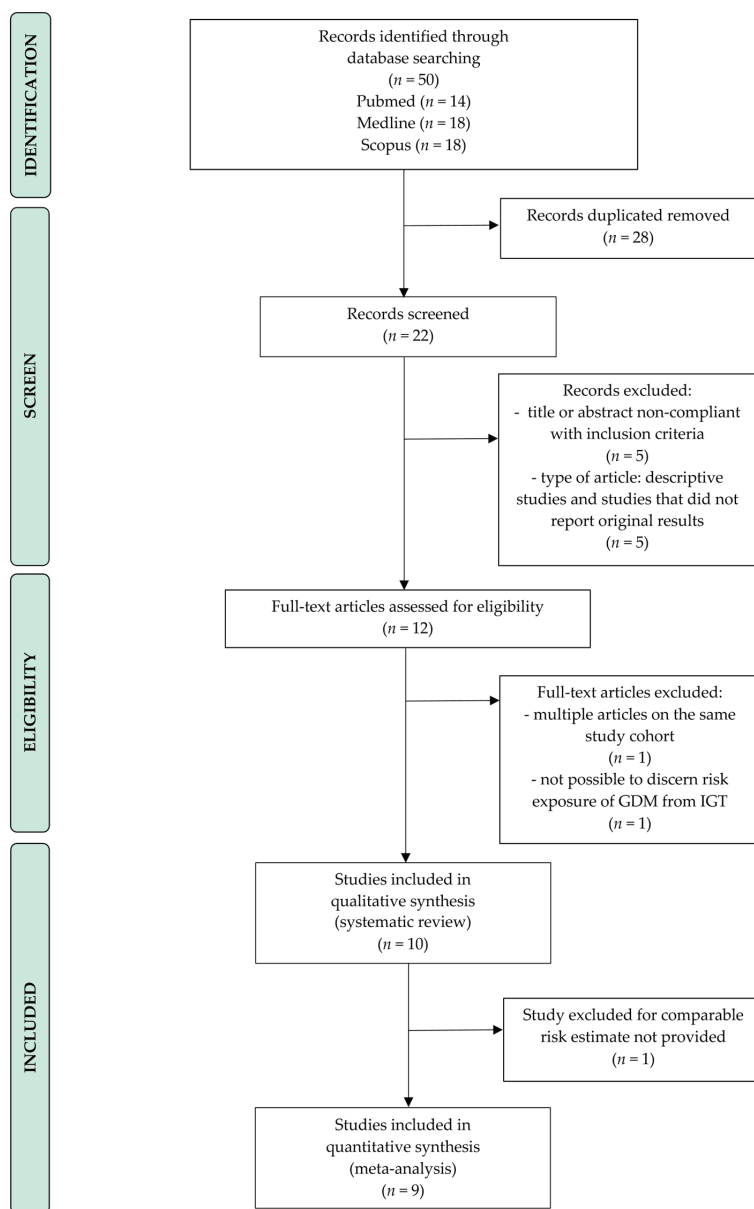
Figure 1. Flow diagram of the search strategy, screening, eligibility and inclusion criteria. Abbreviations: GDM, Gestational diabetes mellitus; IGT, impaired glucose tolerance.

3.2.2. Arsenic in Urine Samples

The prospective cohort study by Wang et al., which evaluated the exposure to multiple metals in pregnancy, showed a significant and positive association between creatinine-adjusted urinary arsenic.

Levels and GDM (p = 0.026). However, a significant association between arsenic concentration and risk of GDM was found only in the single metal model (p = 0.019) without any validation in the multiple-metals model analysis (including urinary nickel, antimony, cadmium, cobalt, vanadium) [31].

Ashley-Martin et al. analyzed urinary metabolites (DMA and arsenobetaine) of As, stratifying results for urinary specific gravity. They found a significantly increased risk of GDM (aOR = 3.86; 95% CI = 1.18, 12.57) in women with DMA concentration higher than 3.52 μg As/L (third tertile). Interestingly, the aOR was even higher when the analysis was restricted to women carrying male infants (aOR = 4.71; 95% CI = 1.05, 21.10) [28].

The study conducted by Khan et al. demonstrated that As level in urine might predict the likelihood of having GDM [30]. However, both Farzan et al. and Munoz and colleagues did not draw similar conclusions, finding no association between urinary As concentrations and GDM [27,29].

3.2.3. Arsenic in Tap Water Samples

The findings from Farzan et al. found a close relationship between As exposure via home well water and risk of GDM: each 5 μg/L increase in As concentration in home well water was associated with a 10% increased odd of GDM (aOR = 1.1; 95% CI = 1.0, 1.2). This association was largely limited to obese women (BMI $\geq$ 30 kg/m^2) (aOR = 1.7; 95% CI = 1.0, 2.8) [27].

The French correlational study carried out by Marie and colleagues [33] provided additional evidence on the association between As concentration in tap water samples and incidence of GDM. Women exposed to As level $\geq$10 μg/L (As + group) had a higher risk of developing GDM than those exposed to As level $\leq$10 μg/L (As − group) (aOR = 1.62; 95% CI = 1.01, 2.53). Stratified analysis of pre-pregnancy BMI showed a positive association only for obese or overweight women (BMI $\geq$ 25 kg/m^2) (aOR = 2.30; 95% CI = 1.13, 4.50).

3.2.4. Arsenic in Meconium Samples

Only one study investigated the link between GDM and As exposure in meconium, finding a higher concentration of the metal in studied cases when compared to controls. Arsenic levels were positively associated with maternal GDM with aORs of 3.28 (95% CI = 1.24, 8.71), 3.35 (95% CI = 1.28, 8.75) and 5.25 (95% CI = 1.99, 13.86) for the second, third, and fourth quartiles, respectively [32].

3.2.5. Arsenic in Toenail Samples

One of the included studies investigated the association between As exposure and the risk of GDM measuring As concentrations in toenails. A positive and statistically significant association was observed: each 100% increase in toenail As was associated with a nearly four-fold increased risk of GDM (aOR = 4.5), despite the wide confidence interval (95% CI = 1.2, 16.6) [27].

3.3. Meta-Analysis

The forest plot of the meta-analysis including all studies for As exposure and the risk of GDM is reported in Figure 2. Funnel plot for publication bias is illustrated in Figure 3. The study conducted by Khan et al. was excluded from the quantitative synthesis as it was not possible to obtain a comparable estimation of effect size [30].

For all the included studies (n = 9) the pooled OR calculated according to the random effect model was 1.56 (95% CI = 1.23, 1.99), with obvious moderate heterogeneity (χ^2 = 21.95; p = 0.005; I^2% = 64) and slightly high publication bias (Egger's test: t = 3.00; p = 0.02) [14,25–29,31–33]. The positive association of maternal As exposure with GDM yielded a statistically significant result (p for effect = 0.0003). The meta-analysis performed using the fixed effect model showed quite similar results (OR = 1.34; 95% CI = 1.20, 1.51; p for effect <0.00001). Sensitivity analysis conducted by omitting one study at time (n = 8) revealed that the result of the pooled analysis was quite robust.
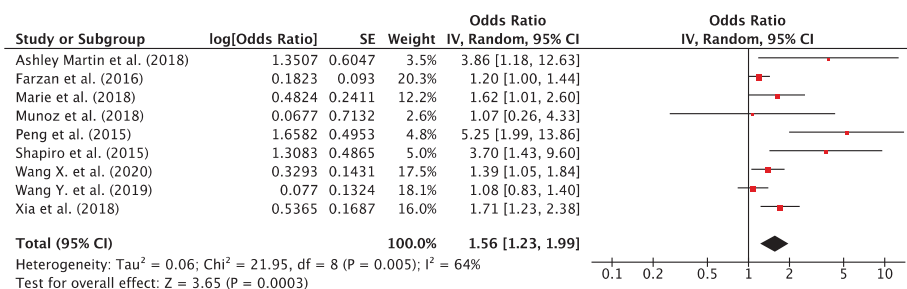
				Odds Ratio	Odds Ratio
Study or Subgroup	log[Odds Ratio]	SE	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Ashley Martin et al. (2018)	1.3507	0.6047	3.5%	3.86 [1.18, 12.63]	
Farzan et al. (2016)	0.1823	0.093	20.3%	1.20 [1.00, 1.44]	
Marie et al. (2018)	0.4824	0.2411	12.2%	1.62 [1.01, 2.60]	
Munoz et al. (2018)	0.0677	0.7132	2.6%	1.07 [0.26, 4.33]	
Peng et al. (2015)	1.6582	0.4953	4.8%	5.25 [1.99, 13.86]	
Shapiro et al. (2015)	1.3083	0.4865	5.0%	3.70 [1.43, 9.60]	
Wang X. et al. (2020)	0.3293	0.1431	17.5%	1.39 [1.05, 1.84]	
Wang Y. et al. (2019)	0.077	0.1324	18.1%	1.08 [0.83, 1.40]	
Xia et al. (2018)	0.5365	0.1687	16.0%	1.71 [1.23, 2.38]	
Total (95% CI)			100.0%	1.56 [1.23, 1.99]	

Heterogeneity: Tau^2 = 0.06; Chi^2 = 21.95, df = 8 (P = 0.005); I^2 = 64%
Test for overall effect: Z = 3.65 (P = 0.0003)

Figure 2. Forest plot of all studies included in the quantitative-synthesis (*n* = 9). The point estimate for each study is represented by a red square where the size of the square is proportional to the weight of the study in the meta-analysis and the 95% CI is symbolized by an horizontal line. The total effect with 95% CI is represented by a black diamond. The results of the pooled analysis demonstrate that As exposure increased the risk of developing GDM (OR = 1.59; 95% CI = 1.23, 1.99). Abbreviations: CI, confidence interval; df, degrees of freedom; IV, inverse variance; SE, standard error.
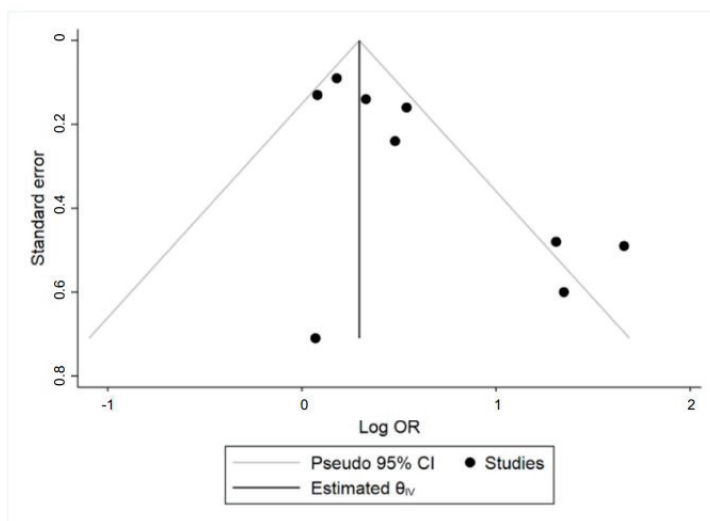

Figure 3. Funnel plot of all studies included in the quantitative-synthesis (*n* = 9). Visual inspection demonstrates slightly high publication bias, as confirmed by Egger's test (t =3.00; *p* = 0.02). Abbreviations: CI, confidence interval; OR, odds ratio.

Subgroups Analyses

The results of the different meta-analyses performed are reported in Table 2. An a priori-defined subgroup analysis based on study design showed less inconsistency/ heterogeneity (χ^2 = 1.86; *p* = 0.17; I^2% = 46) and high pooled risk (OR = 2.28, 95% CI = 0.92, 5.64) when combining data from cross-sectional studies rather than when pooling data from the cohort studies (heterogeneity: χ^2 = 13.73; *p* = 0.008; I^2% = 71; effect estimate: OR = 1.16; 95% CI = 1.07, 1.26).

Table 2. Stratified meta-analysis of maternal as exposure and the risk of developing GDM.

Stratifications	N. Studies	Effect Estimates		Heterogeneity		
		OR	(95% CI)	χ^2	*p*	I^2
All included studies [a,b] [14,25–33]	9	1.56	(1.23, 1.99)	21.95	0.005	64%
All studies less Peng et al. (2015)	8	1.43	(1.17, 1.74)	14.28	0.05	51%
All studies less Farzan et al. (2016)	8	1.73	(1.27, 2.43)	19.46	0.007	64%
All studies less Wang Y. et al. (2019)	8	1.72	(1.30, 2.27)	18.55	0.01	62%
All studies less Ashley Martin et al. (2018)	8	1.50	(1.19, 1.89)	18.88	0.009	63%
All studies less Marie et al. (2018)	8	1.57	(1.20, 2.05)	21.32	0.003	67%
All studies less Munoz et al. (2018)	8	1.59	(1.24, 2.04)	21.85	0.003	68%
All studies less Shapiro et al. (2015)	8	1.47	(1.17, 1.84)	17.56	0.01	60%
All studies less Wang X. et al. (2020)	8	1.66	(1.23, 2.23)	21.89	0.003	68%
All studies less Xia et al. (2018)	8	1.55	(1.18, 2.04)	19.63	0.006	64%
Study design						
Cohort studies	5	1.16	(1.07, 1.26)	13.73	0.008	71%
Cross-sectional studies	2	2.28	(0.92, 5.64)	1.86	0.17	46%
Nested case-control studies	1	/	/	/	/	/
Correlational studies	1	/	/	/	/	/
Exposure indicator						
Blood samples	3	1.35	(1.11, 1.65)	8.87	0.01	77%
Urine samples	4	1.39	(1.07, 1.82)	4.20	0.24	29%
Tap water samples	2	1.11	(1.02, 1.21)	2.49	0.11	60%
Meconium samples	1	/	/	/	/	/
Toenails samples	1	/	/	/	/	/
Study country						
North America	3	1.28	(1.07, 1.53)	8.57	0.01	77%
North America less *Shapiro* et al. *(2015)* [c]	2	1.23	(1.03, 1.48)	3.65	0.06	73%
Asia	4	1.37	(1.17, 1.62)	12.32	0.006	76%
Asia less *Peng* et al. *(2015)* [d]	3	1.32	(1.12, 1.56)	4.78	0.09	58%
South America	1	/	/	/	/	/
Europe	1	/	/	/	/	/
Diagnostic criteria						
ADA	4	1.27	(1.12, 1.43)	5.37	0.15	44%
ADA less *Farzan* et al. *(2016)* [e]	3	1.32	(1.12, 1.56)	4.78	0.09	58%
WHO	2	3.13	(1.41, 6.95)	3.36	0.07	70%
CDA-SOGC [c]	2	3.76	(1.79, 7.91)	0.00	0.96	0%
CNGOF	1	/	/	/	/	/

Abbreviations: As, Arsenic; GDM, Gestational Diabetes Mellitus; N. studies, Number of studies; OR, Odds Ratio; 95% CI, 95% Confidence Interval; ADA, American Diabetes Association; WHO, World Health Organization; CDA-SOGC, Canadian Diabetes Association-Society of Obstetricians and Gynecologist of Canada; CNGOF, French National College of Obstetricians and Gynecologists. Notes: [a] Forest plot in Figure 2. Funnel plot in Figure 3. [b] Sensitivity analyses were conducted by omitting one study at time. [c] Shapiro et al. (2015) and Ashley-Martin et al. (2018) extracted study participants from the Maternal-Infant Research on Environmental Chemicals (MIREC) longitudinal birth cohort, Canada. Because of possible redundancy between some data, stratified analysis according to study country (North America) was also performed by omitting the study Shapiro et al. (2015), whereas stratification according to diagnostic criteria of GDM (CDA-SOGC diagnostic criteria) needs to be interpreted with caution. [d] Peng et al. (2015) conducted a retrospective case-control study nested within a cohort using newborns' meconium as exposure indicator. The study designs of Wang X. et al. (2020), Wang Y. et al. (2019), and Xia et al. (2018) were all prospective cohort studies based on maternal samples (respectively urine, blood, blood) as exposure assessment mode. In light of these methodological differences, analysis was also performed by omitting Peng et al. (2015). [e] Farzan et al. (2016) defined cases based on ADA diagnostic criteria according to the one step or the two step approaches. As all the other studies where diagnosis of GDM was made according to these criteria [25,26,31] considered only the one step approach, analysis was also performed by omitting Farzan et al. (2016).

Further analyses were performed to investigate the possible causes of heterogeneity, stratifying studies according to exposure indicator, study country, and diagnostic criteria for GDM.

We found low heterogeneity when combining studies assessing urine As (χ^2 = 4.20; *p* = 0.24; I^2% = 29), moderate heterogeneity when pooling studies measuring tap water As (χ^2 = 2.49; *p* = 0.11; I^2% = 60), and high heterogeneity when studies based on blood As were combined together (χ^2 = 8.87; *p* = 0.01; I^2% = 77). The pooled effect estimates according to stratification by exposure indicator carried quite similar results for urine and blood As (urine As: OR = 1.39; 95% CI = 1.07, 1.82; blood As: OR = 1.35; 95% CI = 1.11, 1.65), whereas a minor association was found for tap water As (OR = 1.11; 95% CI = 1.02, 1.21).

When combining data from different study countries, we found a similar high heterogeneity for studies conducted in North America ($\chi^2 = 8.57$; $p = 0.01$; $I^2\% = 77$) and in Asia ($\chi^2 = 12.32$; $p = 0.006$; $I^2\% = 76$). The pooled risk estimate was slightly higher for studies based in Asia (OR = 1.37; 95% CI = 1.17, 1.62), rather than in North America (OR = 1.28; 95% CI = 1.07, 1.53). For studies based in North America, a sensitivity analysis was conducted by omitting Shapiro et al., since redundancy of data between Shapiro et al. and Ashley-Martin et al. could not be excluded [14,28].

For studies based in Asia, a sensitivity analysis was conducted by omitting Peng et al., in light of the methodological differences in study design and exposure indicator from the other studies included in the analysis [25,26,31,32]. Sensitivity analyses reduced heterogeneity, confirming that results were quite robust.

Stratification by diagnostic criteria of GDM showed higher pooled risk estimates when diagnosis of the disease was made according to CDA-SOGC criteria (OR = 3.76; 95% CI = 1.79, 7.91) or WHO criteria (OR = 3.13; 95% CI = 1.41, 6.95) rather than with ADA criteria (OR = 1.27; 95% CI = 1.12, 1.43). We found no heterogeneity when combining studies where GDM diagnosis was established with CDA-SOGC criteria ($\chi^2 = 0.00$; $p = 0.96$; $I^2\% = 0$), low heterogeneity when pooling studies adopting ADA diagnostic criteria ($\chi^2 = 5.37$; $p = 0.15$; $I^2\% = 44$), and moderate heterogeneity when studies defining cases according to WHO diagnostic criteria were combined together ($\chi^2 = 3.36$; $p = 0.07$; $I^2\% = 70$). For studies where diagnosis of GDM was based on ADA criteria, a sensitivity analysis was performed by omitting Farzan et al. [27] since this was the only study where cases were identified with both the one-step and the two-step approaches of ADA diagnostic criteria [35]. The sensitivity analysis showed that the result was quite robust.

For all the subgroup analysis performed, visual inspection of funnel plots did not detect substantial asymmetries and yielded little evidence of publication bias (Supplementary Figure S2). However, due to the low number of publications, such bias could not be entirely ruled out.

4. Discussion

The overall results from this meta-analysis provide evidence for an association between exposure to As and GDM, underlining the possible disrupting role of As in glucose metabolism. However, the few number of studies available and the strong heterogeneity existing among them suggests caution in the interpretation of the data.

Gestational diabetes mellitus is a common complication of pregnancy characterized by a dysfunction of pancreatic β-cells on a background of chronic insulin resistance [47]. In normal pregnancy, insulin sensitivity physiologically changes depending on gestational age; in early gestation, the sensitivity increases, promoting glucose uptake in adipocytes in order to store energy for later pregnancy [48]. In the second half of pregnancy, the insulin sensitivity decreases, improving circulating glucose levels for fetal growth requests [49]. In the case of GDM, the β-cells became dysfunctional, losing the ability to adequately control glucose blood concentration. According to the most recent International Diabetes Federation (IDF) estimates, GDM affects approximately one out of seven pregnancies [2]. Since traditional risk factors do not clearly explain the worldwide increasing incidence of the disease, there is a growing interest in the exposure to untraditional risk factors such as environmental contaminants. Among them, the interference with critical steps in glucose metabolism induced by As metabolites has been quite extensively investigated [50].

Arsenic environmental pervasiveness makes its exposure a daily event [51]. As it is comprised of numerous inorganic and organic species, each of them induces a heterogeneous degree and type of toxicity [52]. Arsenate and arsenite are the two most common forms of inorganic As found in drinking water, rice, and seaweed. The components of organic As (mainly found in seafood) such as arsenosugars, arsenolipids, and arsenobetaine (AsB) have historically been thought to be relatively nontoxic and excreted largely unchanged in urine [19,52]. The inorganic As compound has multiple properties that may adversely affect glucose homeostasis [50]. Arsenate can substitute phosphates in the synthesis of adenosine triphosphate (ATP), altering the ATP-dependent insulin secretion. It can form covalent bonds

with the disulfide bridges of insulin, insulin receptors, glucose transporters (GLUTs), and enzymes involved in glucose metabolism (e.g., pyruvate dehydrogenase and α-ketoglutarate dehydrogenase). Moreover, it can alter the expression of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor involved in insulin activation. However, the pancreatic β-cell dysfunction induced by oxidative stress and by interferences in signal transduction or gene expression seems to be the main molecular mechanisms responsible for arsenic-induced diabetes mellitus. As exposure induces the formation of superoxide that, through the interaction with uncoupling protein 2 (UCP2), theoretically impair insulin secretion and create a state of oxidative stress that leads to amyloid deposition in β-cells, causing their progressive destruction [24].

On the basis of these observations, recently, several studies have tested the hypothesis that maternal As exposure may also increase the risk of developing adverse maternal metabolic outcomes such as GDM [14,25–29,31–33].

Three studies reported statistical support to the relationship between As exposure and risk of GDM using total As in blood as the exposure variable [14,25,26]. The assessment of total blood As may represent an overestimation of the exposure because of the different toxicity of inorganic and organic As species [15,53]. Moreover, As levels in blood have a short half-life, possibly leading to mistakes in the assessment of exposure [54]. On the other hand, blood As can reach a steady-state status in chronically exposed people, also reflecting long-term exposure levels [55]. The current meta-analysis showed a significant association between blood As level and GDM only for the highest levels of exposure (OR = 1.35; 95% CI = 1.11, 1.65). However, some factors may have influenced these results such as the different study populations, different pregnancy trimesters of sampling, stratification of level of exposure, confounding factors considered, and the inability to rule out the contributions of organic As to total As. In any case, a considerable heterogeneity was detected among the studies (I^2% = 77).

Five studies used urine samples in order to assess the association between exposure to As and GDM [27–31]. Urinary As levels reflect the As exposure over the past 2–3 days, representing a short-term measure of both inorganic and organic As species [55]. Three studies out of five showed a significant and positive association between As concentration and GDM [28,30,31]. An increased risk of GDM in women with urinary metabolite DMA concentrations higher than 3.52 μg As/L during the first trimester was found by Ashely-Martin and coworkers [28]. However, it is likely that those results were influenced by several issues including the different timing of urine sampling, different stratification of level of exposure, different confounding factors, and urinary markers of exposure considered. The main finding of this meta-analysis revealed a significant association between urinary As level and GDM (OR = 1.39; 95% CI = 1.07, 1.82) with a low heterogeneity among the included studies (I^2% = 29), suggesting a possible more accurate assessment of As exposure when using urine As as the exposure indicator.

The association between exposure to As in tap water and GDM was assessed in two studies, which reported a significant association [27,33]. In line, the current meta-analysis showed a significant moderate association between tap water As level and the disease (OR= 1.11; 95% CI = 1.02, 1.21), although lower than that of the other sources, with a moderate heterogeneity among the included studies (I^2 % = 60). Water As level represents a valid exposure measure for inorganic As if it is the primary source of exposure and individual water intake levels are known. However, it might underestimate the exposure among people with high inorganic As intake from foods (e.g., rice, poultry, fruits, and dairy product), leading to altered exposure assessment [56,57].

Arsenic levels in maternal toenail samples and fetal meconium were also analyzed by two different studies that found a significant association [27,32]. Toenail As is a valid biomarker of inorganic As exposure since it reflects the exposition of 6–12 months prior to sample collection, providing a more long-term exposure measure compared to urine samples [58]. Furthermore, the use of meconium offers even more interesting advantages such as its production from the 12th week of gestation to childbirth (the longest term exposure indicator), the non-invasive sampling, and its capability to reflect maternal and fetal exposure simultaneously [59].

Grouping of the studies by study country did not reduce heterogeneity either for studies conducted in North America ($I^2\% = 77$) or when pooling Asian studies together ($I^2\% = 76$). Moreover, a substantial reduction in heterogeneity among Asian studies ($I^2\% = 58$) was observed when the analysis was performed by omitting the study by Peng et al. [32] because of its intrinsic methodological differences from the other studies included in the stratification, confirming that the results were quite robust. A significant positive association was detected both in North American and in Asian studies, however, with a slightly higher risk estimate for studies based in Asia rather than in North America (OR = 1.37; 95% CI = 1.17, 1.62 and OR = 1.28; 95% CI = 1.07, 1.53, respectively). These findings could be explained by different ethnic, geographic, and dietary arsenic exposures among countries [60]. Moreover, the frequencies of different genetic polymorphisms of the main enzymes involved in the arsenic metabolism such as purine nucleoside phosphorylase (PNP), arsenic methyltransferase (AS3MT), and glutathione-S-transferases (GSTs) vary worldwide, depending on ethnicity/race [61–63]. In any case, the low number of studies included in the stratifications led to not very accurate risk estimates in those analyses.

Combining studies according to the different criteria adopted for GDM diagnosis, we found no heterogeneity among studies based on CDA-SOGC diagnostic criteria ($I^2\% = 0$). Nevertheless, both studies included according to this stratification [14,28] extracted study participants from the Maternal-Infant Research on Environmental Chemicals (MIREC) longitudinal birth cohort of Canada, with the consequence that the result of this analysis needs to be interpreted with caution. Indeed, a substantial reduction in heterogeneity was also observed when pooling studies adopting ADA diagnostic criteria ($I^2\% = 44$), while the main source of heterogeneity came from studies based on WHO diagnostic criteria ($I^2\% = 70$). This finding could be explained by a different definition of cases in the study based in Chile [29], which actually adopted a modified version of the WHO diagnostic criteria established by the Ministry of Health, Chile [64]. A significant strong association was observed when diagnosis of the disease was made by the CDA-SOGC criteria (OR = 3.76; 95% CI = 1.79, 7.91) or WHO criteria (OR = 3.13; 95% CI = 1.41, 6.95), whereas a lower yet still positive association was observed among studies defining GDM cases according to ADA criteria (OR = 1.27; 95% CI = 1.12, 1.43). These findings could be explained by the marked differences among these criteria in blood glucose assessment tests (GCT 50 g, OGTT 75 g, OGTT 100 g) and thresholds, the period of pregnancy in which the test is recommended, the screening approach (universal or selective), and the screening steps (one or two step) to confirm GDM diagnosis [35–39]. Indeed, an internationally consistent definition of GDM remains elusive despite the attempts at building a consensus [65]. The lack of consistency in screening and diagnosis of GDM within and between countries leads to a substantial difficulty in estimating GDM prevalence worldwide. As a matter of fact, identification of potential environmental risk factors linked to the disease remains challenging [66].

The major strength of the current meta-analysis is that it offers an up-to-date overview for those who approach this topic. Indeed, a significant association between As exposure and diabetes has been already established in the non-pregnant population [24,50]. In recent years, only a few studies investigating the link between As exposure and the risk of GDM have been published. The present study is, to our knowledge, the first comprehensive overview of available evidence on the association between As and GDM.

To properly interpret the results, it needs to be emphasized that a causal relationship between As exposure and GDM could be demonstrated only if the occurrence of As exposure was prior to the development of GDM. As already mentioned, the various As biomarkers have several strengths and limitations and reflect a different time of exposure to As. Therefore, considering that the half-life of As in blood is short (several hours) [54], we included in our meta-analysis the data from one study that collected samples during the first trimester [14], and only the data from the first trimester samples of the study assessing As levels in all trimesters of gestation [25]. The third of the studies included in the analysis [26] collected samples the day before delivery, so after the diagnosis of GDM. Moreover, since the main sources for blood As are drinking water and food and the authors declared a relatively stable

consumption of them by women during pregnancy [26], we considered women included in this study as chronically exposed to As. Since the steady-state status reached by those women reflects a long-term exposure [55], it should be plausible to consider the causal relationship between prior As exposure and GDM development. Urinary As is a short-term biomarker (2–3 days) [55], thus we excluded from our meta-analysis one article where no timing of exposure was provided [30]. All the selected articles assessed As exposure by urinary levels in the first [28] or in the early second trimester [27,29,31] (so prior to GDM diagnosis), making a causal relationship possible between prior As exposure and subsequent GDM development. For both articles assessing tap water As, a relationship between earlier As exposure and later GDM diagnosis could be supposed. One study enrolled women at 24–28 gestational weeks, who reported using the same water at their residence since their last menstrual period [27], whereas in the other included study, the period of exposure for each woman was the entire year preceding the date of delivery, thus comprising the periconceptional period and all the trimesters of pregnancy [33]. Both toenail As and meconium As are long-term exposure indicators, since the first reflects As exposure of 6 to 12 months prior to sample collection [58] and the second is produced from the 12th week of gestation [59]. Therefore, measurement of As in both samples could be a reliable source of exposure prior to GDM development.

The major limitation of this meta-analysis is the strong heterogeneity and degree of inconsistency existing between the nine individual risk estimates. Several differences between the included studies that may account for this heterogeneity were analyzed including study design, exposure indicator, study country, and diagnostic criteria of GDM. The random model estimator analysis did not substantially change the risk estimates and no reduction in heterogeneity among the included studies was observed when adopting this model. As a matter of fact, a fixed meta-analysis has natural complements that provide heterogeneity (i.e., Cochran's Q), thus measures of heterogeneity should not be used to determine if this model could be appropriate [67,68]. Indeed, stratified analysis partially helped in understanding possible sources of heterogeneity. Nevertheless, the low number of studies included in the stratified analyses led to restricted statistical power and less precise risk estimates. Another limitation of this study is the significant publication bias indicated by the Funnel plot as a consequence of the exclusion of evidence from unpublished (i.e., grey literature) and non-English language studies. To assess the association between As exposure and risk of developing GDM, we deemed it more appropriate not to include studies providing poor replicable evidence. Additionally, it is known that scientific literature is predominantly biased toward positive results, of which many are unlikely to correspond to the reality and to be applicable worldwide [69]. These limitations suggest that the results should be interpreted with caution until validated by future research projects providing more detailed, well designed, and standardized data collection.

5. Conclusions

In summary, the results of this systematic review and meta-analysis provide additional evidence for a possible association between As exposure and the risk of GDM. To improve and confirm the available data, future study designs might benefit from the inclusion of standardized methods with more sensitive limits of exposure detection in order to evaluate the effects of inorganic and organic As on glucose homeostasis during early pregnancy, hence prior to GDM diagnosis. Additionally, as controversy still surrounds the diagnosis of GDM, a universally endorsed diagnostic criteria could help in confirming the potential role of As in contributing to the onset of this disease, hopefully implying new prevention strategies to reduce the burden of GDM worldwide.

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Article

Maternal Dietary Carbohydrate Intake and Newborn Aortic Wall Thickness

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Abstract: Evidence from animal models indicates that maternal diet during pregnancy affects offspring cardiometabolic health. Improving carbohydrate quality during high-risk pregnancies reduces aortic intima-medial thickness; a marker for early atherosclerosis; in the infant offspring. We sought to determine whether maternal carbohydrate quantity and quality are associated with newborn aortic intima-medial thickness in healthy pregnancies. Maternal diet throughout pregnancy was evaluated in 139 mother–child dyads using a validated food frequency questionnaire. Carbohydrate intake was expressed as quantity (% total energy), quality (fibre, glycaemic index), and glycaemic burden (glycaemic load). Aortic intima-medial thickness was measured by high-frequency ultrasound of the neonatal abdominal aorta. Neither quantity nor quality of maternal carbohydrate intake during pregnancy was associated with meaningful differences in offspring maximum aortic intima-medial thickness with the exception of fibre intake in women with overweight or obesity which was inversely associated (-8 μm [95% CI -14, -1] per g fibre, $p = 0.04$). In healthy pregnancy, the quantity and quality of maternal carbohydrate intake is likely not a meaningful modifiable lifestyle factor for influencing offspring vascular health. The effect of carbohydrate quality may only be evident in high-risk pregnancies, consistent with previous findings. These findings may be confirmed in prospective dietary trials in pregnancy.

Keywords: cardiovascular disease; aortic intima-media thickness; maternal diet

1. Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality globally. Atherosclerosis is the underlying cause for the majority of heart attacks and ischemic strokes [1]. Despite most clinical CVD events occurring in the 5th decade of life and later, the pathogenesis of atherosclerosis is initiated in utero [2]. Accordingly, a life course approach to reduce CVD risk has marked potential yet remains poorly understood. Pre-clinical markers of atherosclerosis, such as arterial intima-media thickness (IMT), are an established means by which to study cardiovascular risk and interventions [3], and may be particularly relevant for identifying early-life risk factors.

Nutrition-related characteristics, including impaired foetal growth and maternal obesity, are key early-life risk factors for later life cardiovascular disease and increased arterial IMT in childhood [4,5]. Maternal dietary risk factors remain poorly characterised.

Carbohydrate intake comprises a significant component of most Westernised diets [6]. Given that glucose is the primary energy substrate towards fetal growth, maternal dietary carbohydrate intake and glycaemic burden are important fetal exposures [7]. Both dietary glycaemic index (GI), a measure of carbohydrate quality, and glycaemic load (GL), a measure of overall glycaemic burden, are strong determinants of glucose levels throughout pregnancy [8]. We have previously shown that the infant offspring of women with a high-risk pregnancy who were randomly allocated to consume a low GI during pregnancy had lower aortic IMT [9], and that higher maternal glycaemic index and lower fibre intake in women with healthy pregnancies are associated with poorer measures of cardiovascular control in their newborn offspring [10]. It is not known whether maternal carbohydrate quantity or quality are associated with aortic IMT infants from healthy pregnancies.

Accordingly, we sought to determine whether the quantity of maternal carbohydrate intake, measured as percentage total energy intake, the quality of maternal carbohydrate, measured as GI and fibre intake, and overall glycaemic burden, measured as GL, in healthy pregnancies are associated with aortic IMT in their newborn offspring.

2. Materials and Methods

2.1. Participant Characteristics

The cohort in this manuscript was part of a larger study exploring the associations of infant body fatness with offspring cardiovascular risk [5]. Mothers and their newborns were recruited from the postnatal wards of Royal Prince Alfred Hospital (Sydney, Australia). Singleton newborns with gestational age greater than 34 weeks and who had undergone a body composition measurement shortly after birth were eligible for the study. Newborns from multiple birth pregnancy, those with significant congenital abnormalities and those requiring ongoing intensive care were excluded from the study. This study was conducted in accordance with ethical standards and ethical approval was granted from the Sydney Local Health District Human Research Ethics Committee (HREC/14/RPAH/478). Participation was voluntary and informed written consent was obtained from all mothers.

Of the 224 newborns recruited, maternal dietary data was available for 214 and of those aortic IMT was available from 179 infants. Mothers with diabetes ($n = 3$), gestational diabetes mellitus (GDM) ($n = 35$), preeclampsia ($n = 8$) and hypertension of pregnancy ($n = 6$) were excluded from this analysis, leaving 139 participants.

Maternal demographic and perinatal characteristics were collected using a self-administered questionnaire and confirmed using health records. An electronic food frequency questionnaire, the Cancer Council Victoria Dietary Questionnaire for Epidemiological Studies Version 2 (DQESV2), was used to capture maternal dietary intake during pregnancy. The DQESV2 covers 74 food and beverage items typically consumed in Australia, grouped according to several categories including cereal foods, sweets and snacks, dairy products, meats and fish, and fruit and vegetables. Nutrient intakes are derived using the Australian Food Composition Database (NUTTAB95) [11,12]. When completing the dietary questionnaire, women were requested to consider their dietary intake throughout their pregnancy, which we have validated using dietary biomarkers [13].

Physical activity during pregnancy was assessed using a self-administered validated questionnaire which instructs respondents to report time spent doing a particular activity [14]. Total activity was calculated as metabolic equivalent (MET) x hours per week as per the protocol described in Chasan-Taber et al. (2004) [14].

Other birth and pregnancy data were collected as part of routine clinical care, these were then obtained by the study team from health records. Aortic IMT was assessed as per best practice guidelines [15]. The far-wall of the neonatal abdominal aorta was imaged using high-frequency B-mode ultrasonography (EPIQ 5, Phillips Medical Systems, Bothell, WA, USA) using a linear array probe (18–5 MHz). Aortic IMT was subsequently measured off-line using a validated semi-automated edge-detection software, Carotid Analyzer for Research (Version 5, Medical Imaging Applications, Coralville, IA, USA), by a blinded assessor (Y.K.). Maximum aortic IMT was used for all analyses as it has been shown to

have the strongest associations with risk factors in early life [15]. The final IMT value was the mean maximum thickness from a minimum three end-diastolic frames as previously described [5].

2.2. Statistical Analysis

Descriptive data are presented as mean (SD) for continuous variables and *n* (%) for categorical variables, unless otherwise stated. Visual assessment and Kolmogorov–Smirnov tests were used to assess data for normality and non-parametric data were log-transformed.

Absolute maternal carbohydrate intake during pregnancy (g/d) was converted to energy content (kJ/d) using a conversion factor of 17 kJ per gram of carbohydrate [16], and subsequently converted to a percentage of daily energy intake (%) for statistical analysis. Total fat (and fatty acids) and protein were similarly converted to percentage daily energy intake with a conversation factor of 37 kJ and 17 kJ per gram, respectively [16]. Maternal carbohydrate intake, GI, GL and fibre were analysed both as continuous variables and as categorical variables based on quartiles. The range and cut-offs for quartiles were as follows: carbohydrate intake (minimum 30.3% total energy intake; 25th percentile 40.5%; 50th percentile 42.9%; 75th percentile 47.4%; maximum 62.8%); GI: (minimum 41.7; 25th percentile 47.0; 50th percentile 49.8; 75th percentile 52.0; maximum 59.8); fibre: minimum 3.5 g/d; 25th percentile 17.3 g/d; 50th percentile 20.8 g/d; 75th percentile 26.8 g/d; maximum 65.3 g/d. Quartiles for GL were calculated using the residual method, adjusted for maternal total energy intake [17].

Statistical analysis was performed with SPSS Statistics (Version 26; IBM Corp., Somers, NY, USA). Results were considered significant at $2p < 0.05$. Unadjusted correlations were undertaken using Pearson's and Spearman's correlation for parametric and non-parametric data, respectively. Multivariable linear regression was performed to evaluate associations between maternal dietary characteristics and infant aortic IMT. Analyses were adjusted for maternal total energy intake during pregnancy, maternal age and newborn sex. An a priori power calculation had been carried out as part of the larger study based on infant body fatness [5]. For this cohort, the sample size (*n* = 139 mother–child dyads) provided 85% power to detect a correlation coefficient of 0.25 at $2p < 0.05$.

3. Results

3.1. Demographics

Maternal and neonatal characteristics are summarised in Table 1. Mothers who participated in the study had a mean age of 33.6 years [SD 4.4]. On average, women obtained 43.5% (SD 5.4) of their total energy intake from carbohydrates. While GI was relatively low, fibre intake was below the current recommended intake for pregnant women in Australia [18]. The mean macronutrient proportions (Carbohydrate:Fat:Protein) when stratified by quartiles of carbohydrate intake were: Q1 37:42:22; Q2 41:39:20; Q 3 45:37:19 and Q4 50:33:18.

Table 1. Maternal, including diet, and neonatal characteristics.

Characteristic	
Maternal Demographics	
Age (years)	33.6 (4.4)
Height (cm)	164.9 (6.5)
Pre-pregnancy BMI (kg/m^2)	22.8 (3.9)
Highest level of education completed (*n* (%))	
High School	15 (10.8)
More than High School	124 (89.2)

Table 1. *Cont.*

Characteristic	
Ethnicity (*n* (%))	
Caucasian	89 (64.0)
Asian	30 (21.6)
South Asian	11 (7.9)
Middle Eastern	5 (3.6)
Other	4 (3.7)
Maternal smoking (*n* (%))	
Current	5 (3.6)
Never	128 (92.1)
Previous	6 (4.3)
Maternal Diet	
Total energy intake (kJ/d)	7786 (3828)
Carbohydrate intake (% total energy)	43.5 (5.4)
Carbohydrate (g/d)	197.5 (96.4)
Sugars (g/d)	89.7 (39.5)
Fat intake (% total energy)	37.7 (4.4)
Fat (g/d)	79.7 (41.3)
Protein intake (% total energy)	19.5 (2.8)
Protein (g/d)	90.0 (50.7)
Fibre (g/d)	22.7 (9.7)
Glycaemic Index	49.7 (5.1)
Glycaemic Load	99.0 (39.0)
Total energy expenditure (MET.hours/week)	284.5 (127.4)
Newborn	
Female/Male (*n* (%))	74 (55)/65 (45)
Gestational age (weeks)	38.7 (1.6)
Birth weight (g)	3339.6 (566.4)
Birth length (cm)	49.4 (2.6)
Head circumference (cm)	34.6 (1.5)
Maximum aortic IMT (µm)	618 (83)

Values are mean (SD) for continuous variables and *n* (%) for categorical variables. Glycaemic load was not normally distributed and is expressed as median (interquartile range). BMI, Body Mass Index; MET, metabolic equivalent; IMT, intima-media thickness.

3.2. Infant Aortic Intima-Medial Thickness and Maternal Carbohydrate Intake

On univariate analysis, maternal fibre intake (r = 0.219, p = 0.010; Figure 1) was positively associated with offspring aortic IMT whilst carbohydrate intake (r = 0.089, p = 0.30), glycaemic index (r = 0.040, p = 0.64) and glycaemic load (r = 0.131, p = 0.12) were not. In multivariable models adjusted for total energy intake, maternal age and newborn sex, neither the quality nor quantity of maternal carbohydrate intake was associated with meaningful differences in offspring aortic IMT. These findings were similar when the carbohydrate characteristics were expressed as continuous outcomes (9 µm (−4, 22) per 5% energy from carbohydrate, p = 0.19; 1 µm (−20, 22) per 5 units GI, p = 0.91; 48 µm (−18, 114) per unit log-GL, p = 0.18; 2 µm (−1, 5) per g fibre, p = 0.17), or in quartiles of intake (Table 2). Further adjustment for maternal BMI, maternal physical activity during third trimester, and infant aortic diameter did not modify these associations (results not shown).

In analyses stratified by maternal BMI, there was a positive association of dietary fibre intake with offspring aortic IMT in mothers with heathy BMI (<25 kg/m^2) although this did not reach statistical significance (3 µm (−0, 6) per g fibre, p = 0.10); whereas there was evidence for an inverse association of fibre with aortic IMT in women with overweight or obesity (*n* = 27; −8 µm (−14, −1) per g fibre, p = 0.04).
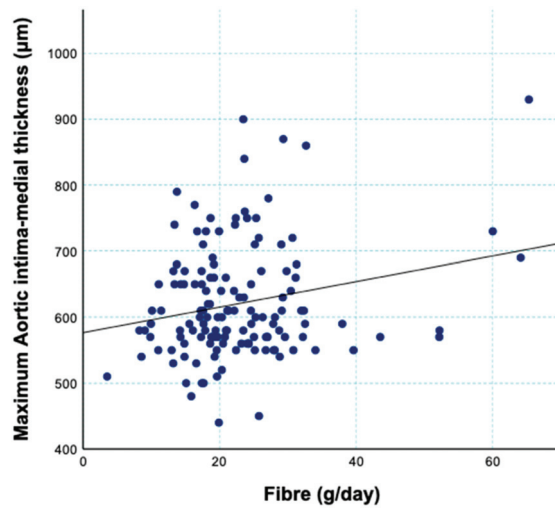
Figure 1. Correlation between maternal fibre (g/d) intake and offspring maximum aortic intima-medial thickness.

Table 2. Associations between maternal carbohydrate intake, both quantity and quality, with newborn aortic intima-medial thickness (IMT).

	Aortic IMT (µm) *n* = 139	
	β (95% CI)	*p* Value
Carbohydrate		
Q_1	Reference	
Q_2	−11 (−52, 30)	0.59
Q_3	28 (−13, 69)	0.17
Q_4	15 (−26, 55)	0.48
Glycaemic Index		
Q_1	Reference	
Q_2	11 (−30, 52)	0.61
Q_3	−13 (−56, 29)	0.54
Q_4	10 (−31, 51)	0.37
Glycaemic Load		
Q_1	Reference	
Q_2	−11 (−52, 30)	0.61
Q_3	−12 (−52, 29)	0.58
Q_4	18 (−23, 58)	0.39
Fibre		
Q_1	Reference	
Q_2	−6 (−46, 35)	0.79
Q_3	21 (−22, 64)	0.34
Q_4	7 (−44, 59)	0.78

Values are unstandardized β-regression coefficients (95% CI) from multivariable regression analyses and represent the differences in newborn maximum aortic MT (µm), adjusted for total energy intake, maternal age and newborn sex.

In post hoc analysis of carbohydrate intake expressed as grams per day, there was a strong association with aortic IMT (0.634 (0.166, 1.101), *p* = 0.008; adjusted for total energy

intake, maternal age and newborn sex). This association remained significant after further adjustment for maternal intake of sugars (0.638 (0.063, 1.214), *p* = 0.030).

In additional post hoc analysis, total fat, fatty acids classes (saturated, monounsaturated and polyunsaturated acids) and protein % daily energy intake were explored as dietary exposures. Neither total fat (r = −0.123, *p* = 0.151) nor protein (r = 0.018, *p* = 0.833) were significantly correlated with infant aortic IMT in crude correlation analysis, nor in multivariable regression (3 μm (−6, 1) per % energy from total fat, *p* = 0.11; 0 μm (−5, 5) per % energy from protein, *p* = 0.94; adjusted for total energy intake, maternal age and newborn sex). Associations of fatty acid classes with aortic IMT were not significant (results not shown).

4. Discussion

Our findings indicate that predominantly neither the quantity nor quality of maternal carbohydrate intake are associated with meaningful differences in aortic IMT in the offspring of women with a metabolically healthy pregnancy. However, there was some evidence that dietary fibre intake was associated with lower offspring aortic IMT in women with overweight or obesity.

Carbohydrates are the major source of energy in most diets [19]. Both the quantity of carbohydrates in the diet and their quality are associated with maternal blood glucose levels and pregnancy outcomes [20]. It has been previously demonstrated that the infants of women with a high-risk pregnancy and who were randomly assigned to a low glycaemic index diet, consistent with higher quality carbohydrates, showed no difference in newborn body fatness or birth weight, compared to controls. However, at 1 year of age, these infants of women assigned to the low glycaemic index diet had reduced aortic IMT [21], suggesting that carbohydrate quality may impact infant vascular development. Interestingly, the control group in this trial was assigned a high fibre diet. Our current finding of a direct association of fibre with aortic IMT in unadjusted correlation analysis is consistent with this previous finding, and may suggest a counterintuitive adverse effect of maternal fibre intake on the onset and early progression of atherosclerosis in the offspring.

We previously demonstrated that maternal carbohydrate intake during pregnancy was not significantly correlated with newborn body fatness or infant birth weight, although there is a weak association of carbohydrate quality, as measured by fibre and GI, with offspring cardiac autonomic function [10]. This highlighted a novel putative link between maternal diet and infant cardiovascular risk. In this study, we aimed to further explore this link by measuring offspring aortic IMT, an age-appropriate surrogate marker for atherosclerotic burden [15]. While we did not observe any meaningful associations with aortic IMT in multivariable models adjusted for appropriate covariates, it has been proposed that a longer time-course may be required for the development of aortic IMT in response to specific exposures [5]. This may at least partially explain the divergent results observed in the associations of cardiac autonomic activity and aortic IMT with carbohydrate quality, with the former being more rapidly affected by risk exposures.

In a post hoc analysis in which maternal carbohydrate intake was expressed in grams per day, adjusting for energy intake as a covariate, we did find a meaningful positive association with offspring aortic IMT. It may be that our a priori analysis of carbohydrate intake as a percentage of energy intake, with additional adjustment for energy as a covariate, over adjusts for energy intake.

It may be that any effects of carbohydrate quality on offspring vascular health are only evident in higher risk pregnancies, consistent with changes in dietary quality countering the vascular effects of poor metabolic health. Indeed, we have previously shown that the infant offspring of women with a high-risk pregnancy who were randomly allocated to consume a low GI diet during pregnancy had lower aortic IMT [9]. This is consistent with our subgroup analyses in women with overweight or obesity, in whom fibre is inversely associated with aortic IMT. Our main findings, that there are no meaningful association of maternal carbohydrate quality or quantity with offspring arterial wall thickness, may

provide reassurance to women with healthy pregnancies, that their carbohydrate intake (within normal ranges) is unlikely to have a meaningful direct impact on their offspring's cardiovascular health.

There are several strengths and limitations to this study. We used an FFQ validated in pregnant women [13], and to minimise the effect of mis-reporting of overall nutrient quantities we used measures that are proportionate to energy intake and analyses adjusted for total energy intake. The use of aortic IMT is the most age-appropriate method for assessing subclinical atherosclerosis during infancy and childhood [15], consistent with post-mortem studies showing that the abdominal aorta is the first site to develop atherosclerotic lesions [22]. As this is a cross sectional sample, we have not been able to assess potential longer-term programming of offspring cardiometabolic health, although this should be a priority for long term pregnancy and birth cohorts, which would also have greater statistical power than our current analysis. Carbohydrate characteristics were the focus of this manuscript and given the implications for modelling in an isocaloric setting and the small sample size, models were not adjusted for other macronutrients (i.e., fat and protein). Whilst our post hoc analysis of total fat, fatty acid classes and protein did not produce any meaningful associations with infant aortic IMT in crude correlations and multivariable regression, exploration of overall diet composition, including food-based analyses and complex nutrient interactions, are an area that requires future exploration. Psychosocial characteristics such as stress, anxiety and social support were not collected in this cohort, although they are known to affect health behaviors during pregnancy, including dietary intake [23]. In their study, Hurley et al. (2005) [23] showed that women who reported higher stress and anxiety levels during pregnancy increased their carbohydrate and fat intake, respectively. The association between psychosocial factors and diet in pregnancy is similar to what is otherwise observed in adults [24] and their influence should be considered in future research linking maternal diet with offspring cardiovascular outcomes. Gestational weight gain was not measured, and therefore we are unable to determine whether it is a potential mediator of these associations, or a confounder. We excluded women with gestational diabetes from our current analysis, due to the potential that their clinical dietary advice received during pregnancy may result in spurious associations. Our sample was recruited from a single site, with a diverse inner-city population albeit small and relatively affluent. Finally, our a priori sample size calculation was based on infant body fatness as the exposure. As such, our study may be potentially underpowered to detect weaker associations of maternal dietary exposures with offspring aortic IMT.

In conclusion, we find that quality and quantity of maternal carbohydrate intake are not meaningfully associated with newborn aortic IMT, with the exception of maternal fibre intake in women with overweight or obesity. Accordingly, the effects of maternal carbohydrate quality on offspring vascular health may only be evident in high-risk pregnancies. Future dietary trials and cohort studies applying validated and standardized methodologies could look to determine causality and longer-term associations, respectively.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available as participants of this did not consent for their data to be shared publicly.

Conflicts of Interest: M.R.S. is employed by The University of Sydney as the Maurice Blackmore Principal Research Fellow in Integrative Medicine. This position was established through a gift from the Blackmores Institute. M.R.S. receives no research funding or in-kind support from Blackmores Limited. The authors declare no other conflict of interest.

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