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Special Issue Reprint

Diet and Nutrition

Metabolic Diseases (2nd Edition)

Edited by
Iñaki Elío

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Diet and Nutrition: Metabolic Diseases (2nd Edition)

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Guest Editor

Iñaki Elío

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About the Editor

Iñaki Elío

Iñaki Elío is Academic Director of the Degree in Human Nutrition and Dietetics at Universidad Europea del Atlántico and serves as the Editor-in-Chief of MLS Health and Nutrition Research. He holds a Doctorate in Health, Nutrition, and Food Projects; a Master's in Nutrition and Metabolism; and a Degree in Human Nutrition and Dietetics. Professor Elío has extensive experience as a registered dietitian–nutritionist, having worked in both clinical settings, such as the Hospital Universitari de Bellvitge, and in academia, where he has contributed to the development and supervision of clinical nutrition, digestive pathology, head and neck cancer patients, neurology, and metabolic disease curricula. His primary research interests focus on the complex relationships between diet, nutrition, and metabolic diseases, with special emphasis on prevention and management strategies for obesity, diabetes, and metabolic syndrome. Professor Elío has led multidisciplinary projects exploring the health benefits of the Mediterranean diet, the impact of ultra-processed foods, and the role of functional foods and nut consumption in elderly populations. Currently, Professor Elío is involved in projects that utilize advanced methodologies such as multi-omics, computational models, eating disorders, and patient-derived organoids to better understand nutritional pathophysiology and develop more effective dietary interventions. He is also the leader of the E+DIETing_LAB project, which has been awarded for its innovative approach to nutrition education through digital simulation and international collaboration. Professor Elío's work is driven by a passion for translating scientific discoveries into practical dietary recommendations and public health strategies. He has received recognition for his commitment to research, education, and interdisciplinary collaboration, and continues to inspire the next generation of nutrition professionals through his editorial and teaching roles.

Preface

It is our pleasure to present this Reprint, which brings together a curated collection of scientific contributions focused on the complex and evolving relationship between diet, nutrition, and metabolic diseases. The increasing global prevalence of conditions such as obesity, type 2 diabetes, metabolic syndrome, and related cardiometabolic disorders underscores the urgent need for effective, evidence-based strategies grounded in nutritional science.

The scope of this Reprint is intentionally comprehensive, encompassing both original research articles and review papers that address key aspects of metabolic health. The contributions included in this volume explore the role of dietary patterns, specific nutrients, and lifestyle factors in the onset, progression, and the management of metabolic diseases. In particular, the Reprint highlights current evidence on widely studied dietary approaches, such as the Mediterranean and plant-based diets, alongside emerging areas such as personalized nutrition, functional foods, and nutraceuticals.

A central aim of this Reprint is to provide an integrated perspective that bridges mechanistic insights with clinical and public health applications. The studies presented offer valuable information on biological pathways involved in metabolic dysfunction, including inflammation, oxidative stress, insulin resistance, and gut microbiota modulation. At the same time, they contribute practical knowledge that can support clinical decision-making and the design of effective interventions.

This Reprint is addressed to a broad audience, including researchers, clinicians, dietitians, and public health professionals who are engaged in the prevention and management of metabolic diseases. By compiling diverse methodological approaches, from clinical trials to epidemiological and translational studies, this collection aims to facilitate the dissemination of up-to-date knowledge and to promote interdisciplinary collaboration.

The motivation for compiling this Reprint arises from the growing recognition that nutrition plays a central role in addressing the global burden of metabolic diseases. Despite the advances achieved in recent years, significant challenges remain in translating scientific evidence into sustainable and accessible solutions. We hope that this Reprint will contribute to advancing the field, supporting evidence-based practice, and encouraging further research and innovation in nutrition and metabolic health.

Iñaki Elío
Guest Editor

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Article

Trends in HDL Cholesterol and Their Association with Dietary Carbohydrate Reduction Among Korean Adults: A Serial Cross-Sectional Trend Analysis Using KNHANES 2014–2023

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Abstract

Purpose: Low HDL cholesterol (HDL-C) is a common lipid abnormality among East Asian populations, including Koreans, and is closely associated with increased cardiovascular disease risk. This has traditionally been linked to high-carbohydrate dietary patterns. Therefore, it is essential to assess how recent changes in dietary and lifestyle habits in Korea have influenced HDL-C levels. **Methods:** This study utilized data from the Korea National Health and Nutrition Examination Survey (KNHANES), conducted from 2014 to 2023. Using a complex sampling method, we analyzed annual trends in carbohydrate intake and HDL-C levels in Korean adults (aged 20–59 years), stratified by gender (male and female) and age (20–39 and 40–59 years), and identified determinants such as demographic factors, health behaviors, and dietary intake. **Results:** The primary factor in the increase in HDL-C levels was a reduction in carbohydrate intake. When analyzed by age and gender, this significant upward trend in HDL-C levels was consistently observed across all four groups: young men, middle-aged men, young women, and middle-aged women. However, the increase in obesity, indicated by increased BMI and waist circumference, had a negative impact on the improvement in HDL-C levels. The recent improvement in HDL-C levels in Korean adults can be attributed to the successful national dietary policy (reducing carbohydrate intake and increasing healthy fat intake). **Conclusions:** Public health policies should thus continue emphasizing healthy dietary practices, strengthening smoking cessation initiatives, and managing obesity. These findings suggest similar dietary and lifestyle interventions could effectively reduce cardiovascular disease risk in other Asian populations undergoing comparable dietary transitions.

Keywords: HDL cholesterol; dietary habits; lifestyle; risk factors; public health

1. Introduction

Historically, high-density lipoprotein cholesterol (HDL-C) has been colloquially termed 'good cholesterol' due to its inverse epidemiological associations with atherosclerotic cardiovascular disease (CVD) [1]. However, the causal role of HDL-C mass in directly preventing CVD has been heavily scrutinized. While HDL particles participate in reverse cholesterol transport and exhibit anti-inflammatory and antioxidant properties in vitro, the direct translation of these functions to clinical cardiovascular protection remains complex. Epidemiologically, rather than being a direct causal determinant, low HDL-C is

now primarily recognized as a key component of metabolic syndrome and a powerful biomarker for poor cardiometabolic health [1,2]. However, recent large-scale randomized controlled trials and Mendelian randomization studies have prompted a paradigm shift, demonstrating that pharmacologically raising HDL-C levels does not directly translate to improved cardiovascular outcomes [3]. Consequently, contemporary evidence highlights that the quality, composition, and functionality of HDL particles—assessed via proteomic and lipidomic profiling—are more critical determinants of cardiovascular protection than HDL-C quantity alone [4]. Nevertheless, at the population level, low HDL-C mass remains a robust and highly accessible proxy marker for adverse metabolic phenotypes, insulin resistance, and poor lifestyle habits.

In East Asian populations, including South Korea, a phenotype of low HDL-C has traditionally been prevalent, largely attributed to diets rich in refined carbohydrates and low in healthy fats [5]. However, South Korea has recently undergone rapid lifestyle and nutritional transitions. Notably, smoking rates have declined, and national health campaigns have successfully promoted weight management and shifting dietary patterns toward reduced refined carbohydrates and increased protein and unsaturated fats [6–11]. These changes would be expected to raise HDL-C levels at the population level, reflecting broader metabolic improvements, since higher unsaturated fat intake and reduced refined carbohydrates can favorably increase HDL-C [12,13]. On the other hand, rising trends in obesity and physical inactivity—potentially exacerbated by recent societal changes—could negatively impact HDL [14]. Understanding how HDL-C levels have evolved in the Korean population during this period of transition is important for evaluating the net impact of health policies and lifestyle shifts and for guiding future interventions.

In this study, we analyzed national big data from 2014 to 2023 to examine trends in HDL-C levels among Korean adults and to investigate the determinants of HDL-C in this population. We hypothesized that HDL-C has increased over time and that this increase is associated with improvements in diet (lower carbohydrate intake) and lifestyle (less smoking), outweighing adverse factors like weight gain. We also sought to quantify the contributions of various factors—such as demographic characteristics, diet, and physical activity—to HDL-C variation. By framing these findings in a public health context, we aim to explore how tracking HDL-C as a population-level indicator can inform strategies for metabolic health management and cardiovascular risk reduction in Korea and similar settings.

2. Methods

Study Design and Data Source

We conducted a trend analysis and cross-sectional determinant study using data from the Korea National Health and Nutrition Examination Survey (KNHANES) [15] for the years 2014–2023. KNHANES is an ongoing nationwide surveillance program that uses a stratified, multistage probability sampling design to select a representative sample of the non-institutionalized Korean population each year [16,17]. The survey consists of three components: a health interview, a health examination, and a nutrition survey. For this analysis, we pooled 10 consecutive annual surveys to assess temporal trends. Each year's survey employs new sample households drawn from census units, allowing cross-sectional estimates for that year; combining years enabled us to increase statistical power for determinants [16,17].

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3. Population and Variables

3.1. Study Population

This study analyzed data from the KNHANES 2014–2023. From the total participants, we excluded individuals aged under 20 or over 60 years, pregnant women, and those with missing data on lipid profiles, anthropometric measurements, or key covariates. Consequently, a total of 28,101 adults were included in the final analysis. To address the distinct physiological and lifestyle characteristics associated with gender and life stages, as requested by rigorous methodological standards, we stratified the study population into four subgroups: men aged 20–39 (n = 5016), women aged 20–39 (n = 6434), men aged 40–59 (n = 6693), and women aged 40–59 (n = 9958). Detailed demographic and physical characteristics of the participants are presented in Section 5.

3.2. Criteria of Inclusion and Exclusion

The inclusion criteria for this study were participants aged 20 to 59 years who participated in the KNHANES 2014–2023 survey. The exclusion criteria were as follows:

(1) Individuals aged under 20 years or over 60 years.
(2) Individuals with missing data on the dependent variable (HDL-C).
(3) Individuals with missing data on major covariates.

Consequently, out of the initial 74,952 participants, 46,851 were excluded, leaving 28,101 participants for the final analysis.

3.3. Variable Selection

The primary outcome of interest was serum HDL cholesterol, measured enzymatically after an 8–12 h fast. Consistent measurement techniques and quality control were applied over the years by the central laboratory according to standardized protocols. Key exposure variables for determinant analysis were chosen based on known or hypothesized influences on HDL-C. Demographic factors included sex, age, education level, and household income. Lifestyle factors included smoking status (current smoker vs. non-smoker), alcohol drinking status (yes vs. no, defined as regular alcohol consumption), and physical activity. Physical activity was assessed via self-report questionnaires; for this study, we focused on leisure-time moderate-intensity physical activity hours per week (average hours of moderate exercise such as brisk walking, cycling, etc.), as well as vigorous activity and walking hours for correlation analyses. Sedentary time (daily sitting) was also recorded. Dietary factors were assessed by 24 h dietary recall conducted by trained dietitians in each participant's home. We extracted total energy intake (kcal per day) and intake of macronutrients (grams per day of carbohydrate, fat, and protein) as well as selected micronutrients (including dietary fiber, calcium, iron, vitamins and others that emerged from preliminary analyses). Anthropometric measurements (body weight, height, and waist circumference) were taken by trained examiners using standardized methods; BMI was calculated as weight (kg)/height (m^2).

4. Statistical Analysis

All analyses accounted for the complex sampling survey designed to produce representative population estimates and correct standard errors. To ensure the national representativeness of the findings and to account for the complex, multi-stage probability sampling design of KNHANES, all statistical analyses were conducted using survey-weighted estimation methods. This approach strictly incorporates sampling weights, stratification (strata), and primary sampling units (clusters) to calculate accurate population-level estimates and unbiased standard errors.

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To account for physiological differences and lifestyle patterns, all major statistical analyses were stratified by age and gender. The study subjects were strictly divided into four subgroups based on gender (male and female) and age (younger age group: 20–39 years; middle-aged age group: 40–59 years).

Means with standard errors (SE) were computed for continuous variables and proportions for categorical variables, using weighted survey commands. Differences between groups were tested with chi-square tests as appropriate.

To assess trends over time, we calculated the yearly weighted mean HDL-C from 2014 to 2023 and performed a survey linear regression of HDL-C on survey year (treated as an ordinal variable) to test for a linear trend. We also used one-way complex-sample ANOVA to compare mean HDL-C across the ten survey years. Specifically, for comparing continuous variables across different survey years or demographic groups, we utilized a survey-weighted analysis of variance (ANOVA) employing the design-adjusted Wald F-test. This method is specifically intended for complex survey data, effectively replacing the standard ANOVA, which assumes simple random sampling and would otherwise inappropriately underestimate standard errors. followed by Bonferroni-adjusted pairwise comparisons (post hoc) to identify which years differed significantly from others. Similar trend analyses were conducted for key determinants: mean energy intake, carbohydrate intake, and smoking prevalence by year.

We determined partial correlation coefficients adjusted for age to account for confounding by age distribution. Variables correlated with HDL-C at $p < 0.05$ in age-adjusted analysis were considered for inclusion in multivariable modeling. We then performed a complex-sample multivariable linear regression with HDL-C as the outcome. All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant. Analyses were conducted using R Studio (R version 4.4.1) [18] in accordance with the KNHANES analytical guidelines [19].

5. Results

5.1. General Characteristics of Study Subjects and HDL-C Levels According to Complex Sampling

In the young male group (aged 20–39 years), significant differences in HDL-C levels were observed according to socioeconomic factors, specifically education level ($p = 0.024$) and individual income ($p = 0.023$). Regarding health behavioral factors, alcohol consumption, smoking, and perceived stress all demonstrated strong associations with HDL-C. Alcohol drinkers exhibited significantly higher HDL-C levels (50.49 mg/dL) compared to non-drinkers (47.41 mg/dL) ($p < 0.001$), whereas current smokers had significantly lower HDL-C levels than non-smokers (48.52 vs. 50.30 mg/dL, $p < 0.001$). Additionally, the group with high perceived stress showed lower HDL-C levels than the low-stress group ($p = 0.002$). Notably, participants taking medication for dyslipidemia presented with lower HDL-C levels compared to non-users (42.75 vs. 49.74 mg/dL, $p < 0.001$), which likely reflects the impact of their underlying metabolic conditions.

In the young female group (aged 20–39 years), a clear positive correlation was observed between socioeconomic status (SES) and HDL-C levels. Higher education levels, individual income, and household income were all associated with significantly higher HDL-C levels (all $p < 0.001$). Regarding employment status, the employed group demonstrated significantly higher HDL-C levels (60.39 mg/dL) than the unemployed group (58.43 mg/dL) ($p < 0.001$). In terms of health behaviors, similar to men, alcohol drinkers had higher HDL-C levels than non-drinkers ($p < 0.001$); however, unlike men, no significant difference was observed according to smoking status ($p = 0.458$). Furthermore, the low-stress group exhibited significantly higher HDL-C levels compared to the high-stress group ($p = 0.001$).

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In the middle-aged male group (aged 40–59 years), unlike their younger counterparts, no statistically significant differences were found based on education level ($p = 0.234$) or individual income ($p = 0.413$). However, significant differences were observed according to household income ($p = 0.049$) and employment status ($p = 0.034$). Lifestyle factors showed a pattern similar to the younger group: alcohol drinkers had higher HDL-C than non-drinkers ($p < 0.001$), smokers had lower HDL-C than non-smokers ($p < 0.001$), and the high-stress group exhibited lower HDL-C levels ($p = 0.004$). Meanwhile, in this age group of men, the difference in HDL-C according to dyslipidemia medication use was not statistically significant ($p = 0.197$).

In the middle-aged female group (aged 40–59 years), similar to younger women, socioeconomic factors were shown to have a strong impact on HDL-C levels. Higher education levels, individual income, and household income were consistently associated with significantly higher HDL-C levels (all $p < 0.001$). Among lifestyle factors, alcohol consumption showed a significant difference ($p < 0.001$), whereas smoking ($p = 0.356$) and perceived stress ($p = 0.402$) did not show significant associations with HDL-C levels. Additionally, participants taking dyslipidemia medication had significantly lower HDL-C levels compared to non-users (55.63 vs. 57.75 mg/dL, $p < 0.001$) (Table 1).

Table 1. General characteristics of study subjects and HDL-C levels according to Complex Sampling.

Variable	Categories	Male 20–39							Female 20–39						
		HDL-C			Weighted		Unweighted		HDL-C			Weighted		Unweighted	
		M	SE	*p*	*n*	%	*n*	%	M	SE	*p*	*n*	%	*n*	%
Education	**College**	49.29	0.23	0.024	3,757,476	55.1	2855	56.9	60.32	0.22	<0.001	4,019,276	65.9	4310	67.0
	High	50.07	0.26		2,946,571	43.2	2078	41.4	58.56	0.34		1,946,767	31.9	1983	30.8
	Middle	51.28	1.52		90,766	1.3	69	1.4	54.27	1.31		102,030	1.7	104	1.6
	Elementary	48.16	3.95		18,830	0.3	14	0.3	48.98	2.01		31,059	0.5	37	0.6
Income	**High**	50.54	0.35	0.023	1,706,253	25.0	1246	24.8	61.16	0.37	<0.001	1,528,412	25.1	1587	24.7
	Middle-High	49.59	0.33		1,685,804	24.7	1265	25.2	59.77	0.37		1,540,444	25.3	1637	25.4
	Middle-Low	49.43	0.35		1,746,009	25.6	1272	25.4	59.29	0.37		1,518,515	24.9	1637	25.4
	Low	49.05	0.37		1,675,577	24.6	1233	24.6	58.17	0.37		1,511,761	24.8	1573	24.4
Household Income	**High**	50.08	0.29	0.227	2,541,396	37.3	1685	33.6	60.92	0.33	<0.001	2,142,811	35.1	2192	34.1
	Middle-High	49.55	0.30		2,320,595	34.1	1720	34.3	59.32	0.32		2,096,040	34.4	2259	35.1
	Middle-Low	49.08	0.40		1,410,202	20.7	1038	20.7	58.14	0.38		1,430,816	23.5	1561	24.3
	Low	49.61	0.64		541,450	7.9	393	7.8	59.26	0.76		429,465	7.0	422	6.6
Occupation	**Yes**	49.55	0.20	0.317	4,974,041	73.0	3777	75.3	60.39	60.39	<0.001	3,648,225	59.8	3819	59.4
	No	49.94	0.34		1,839,603	27.0	1239	24.7	58.43	58.43		2,450,976	40.2	2615	40.6
Alcohol Drinking	**Yes**	50.49	2.21	<0.001	4,967,888	72.9	3685	73.5	61.20	61.20	<0.001	3,641,063	59.7	3785	58.8
	No	47.41	0.30		1,845,755	27.1	1331	26.5	57.24	57.24		2,458,069	40.3	2649	41.2
Cigarette Smoking	**Yes**	48.52	0.29	<0.001	2,460,524	36.1	1834	36.6	59.10	59.10	0.458	502,032	8.2	485	7.5
	No	50.30	0.22		4,353,120	63.9	3182	63.4	59.65	59.65		5,597,100	91.8	5949	92.5
Stress	**High**	48.84	0.31	0.002	2,161,706	31.7	1617	32.2	58.84	58.84	0.001	2,371,250	38.9	2462	38.3
	Low	50.03	0.21		4,651,937	68.3	3399	67.8	60.09	60.09		3,727,882	61.1	3972	61.7
Medication for dyslipidemia	**Yes**	42.75	1.07	<0.001	77,833	1.1	60	1.2	53.10	2.79	0.019	26,466	0.4	37	0.6
	No	49.74	0.18		6,735,810	98.9	4956	98.8	59.63	0.19		6,069,666	99.5	6397	99.4
Total		49.44	0.26		6,813,643	100.0	5016	100.0	59.47	59.47		6,099,132	100.0	6434	100.0

Table 1. *Cont.*

Variable	Categories	Male 40–59							Female 40–59						
		HDL-C			Weighted		Unweighted		HDL-C			Weighted		Unweighted	
		M	SE	*p*	*n*	%	*n*	%	M	SE	*p*	*n*	%	*n*	%
Education	**College**	47.73	0.21	0.234	4,014,670	53.1	3495	52.2	59.37	0.28	<0.001	3,120,571	41.5	4110	41.3
	High	47.65	0.27		2,703,223	35.8	2374	35.5	56.96	0.24		3,330,634	44.3	4309	43.3
	Middle	47.86	0.58		547,370	7.2	526	7.9	54.35	0.56		640,717	8.5	907	9.1
	Elementary	46.23	0.72		294,131	3.9	298	4.5	53.22	0.58		425,144	5.7	632	6.3
Income	**High**	47.97	0.30	0.413	1,897,101	25.1	1676	25.0	58.84	0.33	<0.001	1,922,028	25.6	2526	25.4
	Middle-High	47.82	0.30		1,956,111	25.9	1704	25.5	57.66	0.33		1,924,124	25.6	2520	25.3
	Middle-Low	47.47	0.32		1,904,770	25.2	1663	24.8	56.85	0.32		1,914,951	25.5	2504	25.1
	Low	47.33	0.31		1,801,412	23.8	1650	24.7	56.68	0.32		1,755,963	23.4	2408	24.2
Household Income	**High**	47.99	0.24	0.049	3,127,300	41.4	2750	41.1	58.49	0.28	<0.001	2,939,245	39.1	3837	38.5
	Middle-High	47.54	0.27		2,410,700	31.9	2087	31.2	57.38	0.29		2,358,091	31.4	3074	30.9
	Middle-Low	47.63	0.34		1,468,559	19.4	1329	19.9	56.79	0.34		1,644,293	21.9	2227	22.4
	Low	46.26	0.59		552,835	7.3	527	7.9	55.38	0.49		575,437	7.7	820	8.2
Occupation	**Yes**	47.76	0.16	0.034	6,879,507	91.0	6076	90.8	57.68	0.20	0.235	4,666,470	62.1	6309	63.4
	No	46.59	0.53		679,887	9.0	617	9.2	57.28	0.27		2,850,596	37.9	3649	36.6
Alcohol Drinking	**Yes**	48.63	0.19	<0.001	5,520,322	73.0	4885	73.0	59.87	0.25	<0.001	3,481,787	46.3	4540	45.6
	No	45.00	0.26		2,039,072	27.0	1808	27.0	55.51	0.20		4,035,279	53.7	5418	54.4
Cigarette Smoking	**Yes**	46.64	46.64	<0.001	2,888,498	38.2	2593	38.7	56.91	0.82	0.356	345,295	4.6	453	4.5
	No	48.28	48.28		4,670,896	61.8	4100	61.3	57.56	0.17		7,171,771	95.4	9505	95.5
Stress	**High**	46.92	46.92	0.004	2,058,797	27.2	1806	27.0	57.29	0.33	0.402	1,954,281	26.0	2653	26.6
	Low	47.93	47.93		5,500,597	72.8	4887	73.0	57.61	0.19		5,562,785	74.0	7305	73.4
Medication for dyslipidemia	**Yes**	48.18	0.44	0.197	905,415	12.0	835	12.5	55.63	0.50	<0.001	783,648	10.4	1070	10.7
	No	47.58	0.17		6,653,979	88.0	5858	87.5	57.75	0.18		6,733,418	89.6	8887	89.2
Total		47.43	47.43		7,559,394	100.0	6693	100.0	57.45	0.26		7,517,066	100.0	9958	100.0

5.2. Complex Sampling Correlation Analysis Focused on HDL-C

In all groups, waist circumference and body mass index (BMI) showed the strongest and most consistent negative correlations with HDL-C (all groups $p < 0.001$). Notably, these correlations were relatively more pronounced in young adults (aged 20–39 years) than in middle-aged adults (aged 40–59 years) (Male 20–39: waist circumference r = −0.354, BMI r = −0.328; Female 20–39: waist circumference r = −0.331, BMI r = −0.339). This demonstrates a clear inverse relationship, indicating that higher degrees of adiposity are associated with lower HDL-C levels.

The duration of high- and moderate-intensity leisure-time physical activity showed significant positive correlations with HDL-C in most groups, excluding women aged 20–39. Conversely, sedentary time exhibited significant negative correlations across both male groups (Male 20–39: r = −0.041, $p = 0.006$; Male 40–59: r = −0.048, $p < 0.001$), suggesting that longer sitting hours are associated with lower HDL-C levels in men. In women, however, a consistent inverse correlation between sedentary time and HDL-C was not observed.

Among dietary factors, the intake of fat, its subcategories (saturated, monounsaturated, and polyunsaturated fatty acids), and cholesterol showed significant positive correlations with HDL-C across all four groups. This trend was particularly prominent in women (both Female 20–39 and Female 40–59, $p < 0.001$).

Notably, carbohydrate intake exhibited different patterns depending on the age group. In middle-aged adults (40–59 years), both men and women showed significant negative correlations, indicating that higher carbohydrate intake is associated with decreased HDL-C levels (Male: r = −0.046, Female: r = −0.056, both $p < 0.001$). In contrast, young men (20–39 years) showed a weak positive correlation (r = 0.039, $p = 0.005$), while the association was not significant in young women (20–39 years). Regarding other micronutrients, the

intakes of water, calcium, and vitamin B2 generally demonstrated significant positive correlations with HDL-C across multiple groups (Table 2).

Table 2. Complex Sampling Correlation Analysis Focused on HDL-C.

Variables	Male 20–39				Female 20–39			
	M	**SE**	**r**	***p***	**M**	**SE**	**r**	***p***
HDL-C (mg/dL)	49.66	0.17	1	1	59.6	0.19	1	1
High Physical Activity—Work (minutes)	3.92	0.38	0.009	0.493	0.98	0.18	0.007	0.605
Moderate Physical Activity—Work (minutes)	16.11	0.86	0.016	0.275	11.73	0.72	0.010	0.464
Location Move Physical Activity (minutes)	25.64	0.5	0.028	0.052	26.39	0.46	−0.018	0.000
High Physical Activity—Leisure (minutes)	15.12	0.55	0.077	<0.001	5.38	0.24	0.045	0.184
Moderate Physical Activity—Leisure (minutes)	19.92	0.54	0.073	<0.001	13.84	0.37	0.046	0.003
Walk (minutes)	46.63	0.78	0.007	0.618	48.79	0.72	−0.003	0.839
Sedentary (minutes)	551.91	3.51	−0.041	0.006	544.94	3.16	0.033	0.023
Waist circumference (cm)	86.16	0.17	−0.354	<0.001	74.62	0.15	−0.331	<0.001
Body Mass Index (kg/m^2)	24.94	0.06	−0.328	<0.001	22.32	0.06	−0.339	<0.001
Energy intake (kcal)	2462.53	17.2	0.030	0.039	1754.87	10.36	0.047	0.001
Water intake (g)	1208.28	11.48	−0.041	0.008	967.77	7.77	0.073	<0.001
Carbohydrate (g)	95.53	0.8	0.039	0.005	65.74	0.49	−0.020	0.152
Protein intake (g)	70.26	0.73	0.021	0.152	50.78	0.47	0.047	0.001
Fat intake (g)	22.37	0.25	0.035	0.023	16.79	0.18	0.075	<0.001
Saturated fatty acids intake (g)	23.53	0.29	0.037	0.019	16.39	0.17	0.069	<0.001
Monounsaturated fatty acids intake (g)	16.87	0.19	0.040	0.011	12.12	0.13	0.066	<0.001
Polyunsaturated fatty acids intake (g)	2.11	0.03	0.024	0.092	1.61	0.02	0.080	<0.001
*n*3 fatty acid (g)	14.74	0.17	−0.002	0.878	10.49	0.11	0.055	<0.001
*n*6 fatty acid (g)	357.61	4.24	0.027	0.060	267.33	2.93	0.079	<0.001
Cholesterol intake (mg)	318.86	2.17	0.024	0.109	241.83	1.46	0.049	<0.001
Total Dietary Fiber intake (g)	23.85	0.21	−0.015	0.320	18.64	0.14	0.006	0.647
Calcium intake (mg)	541.4	4.84	0.005	0.752	450.6	3.82	0.065	<0.001
Phosphorus intake (mg)	1278.16	9.03	0.009	0.570	943.78	6.01	0.042	0.004
Sodium intake (mg)	4252.81	38.41	−0.011	0.423	2964.06	24.69	0.007	0.614
Potassium intake (mg)	3015.95	22.77	−0.008	0.597	2351.85	15.89	0.018	0.216
Iron intake (mg)	13.84	0.16	−0.015	0.342	10.35	0.1	−0.004	0.754
Carotene intake (μg)	2853.99	59.49	0.018	0.564	2329.45	60.74	0.002	0.863
Retinol intake (mg)	213.4	8.54	0.015	0.108	174.91	5.04	0.045	0.010
Vitamin B1 (mg)	1.76	0.02	−0.019	0.196	1.22	0.01	−0.035	0.027
Vitamin B2 (mg)	1.99	0.02	0.028	0.062	1.48	0.01	0.066	<0.001
Niacin (mg)	17.98	0.18	0.021	0.171	12.59	0.11	0.019	0.243
Vitamin C (mg)	69.88	1.87	0.007	0.671	63.16	1.15	0.021	0.151

Table 2. *Cont.*

Variables	Male 40–59				Female 40–59			
	M	**SE**	**r**	***p***	**M**	**SE**	**r**	***p***
HDL-C (mg/dL)	47.65	0.16	1	1	57.53	0.17	1	1
High Physical Activity—Work (minutes)	3.24	0.38	0.008	0.419	0.67	0.11	0.034	0.055
Moderate Physical Activity—Work (minutes)	11.66	0.63	0.024	0.090	6.82	0.46	0.022	0.025
Location Move Physical Activity (minutes)	20.35	0.44	0.005	0.684	23.71	0.36	−0.011	0.327
High Physical Activity—Leisure (minutes)	9.59	0.4	0.058	<0.001	4.07	0.2	0.062	<0.001
Moderate Physical Activity—Leisure (minutes)	17.94	0.48	0.040	0.002	13.33	0.35	0.083	<0.001
Walk (minutes)	48.6	0.8	0.038	0.010	51.27	0.6	0.009	0.463
Sedentary (minutes)	500.71	3.33	−0.048	<0.001	460.86	2.57	0.010	0.383
Waist circumference (cm)	87.82	0.12	−0.276	<0.001	78.99	0.12	−0.279	<0.001
Body Mass Index (kg/m^2)	24.93	0.04	−0.267	<0.001	23.45	0.04	−0.279	<0.001
Energy intake (kcal)	2362.67	13.36	0.040	0.004	1670.29	7.57	0.020	0.071
Water intake (g)	1242.39	10.11	0.044	0.002	1040.55	7.2	0.055	<0.001
Carbohydrate (g)	85.54	0.62	−0.046	<0.001	61.39	0.34	−0.056	<0.001
Protein intake (g)	55.19	0.52	0.022	0.063	40.78	0.3	0.043	<0.001
Fat intake (g)	17.18	0.18	0.033	0.013	12.55	0.11	0.090	<0.001
Saturated fatty acids intake (g)	17.99	0.2	0.029	0.036	13	0.11	0.085	<0.001
Monounsaturated fatty acids intake (g)	14.02	0.14	0.034	0.013	10.76	0.09	0.090	<0.001
Polyunsaturated fatty acids intake (g)	2.12	0.03	0.036	0.006	1.74	0.02	0.073	<0.001
*n*3 fatty acid (g)	11.89	0.12	0.033	0.012	9.01	0.08	0.024	0.124
*n*6 fatty acid (g)	305.17	3.58	0.034	0.012	227	2.23	0.075	<0.001
Cholesterol intake (mg)	328.85	1.79	0.046	<0.001	256.79	1.27	0.080	<0.001
Total Dietary Fiber intake (g)	28.39	0.19	0.006	0.656	24.82	0.17	−0.005	0.656
Calcium intake (mg)	582.18	4.45	0.044	0.002	482.47	3.36	0.041	<0.001
Phosphorus intake (mg)	1258.37	7.68	0.024	0.065	967.39	4.97	0.041	<0.001
Sodium intake (mg)	4356.41	38.98	0.010	0.300	2978.36	20.71	−0.020	0.067
Potassium intake (mg)	3293.13	20.48	0.011	0.447	2759.29	18.38	−0.007	0.553
Iron intake (mg)	14.24	0.13	−0.008	0.542	11.1	0.08	−0.043	<0.001
Carotene intake (μg)	3393.71	46.22	0.007	0.640	3046.38	38.41	−0.011	0.317
Retinol intake (mg)	157.36	5.01	0.029	0.155	136.83	3.05	0.048	<0.001
Vitamin B1 (mg)	1.66	0.01	−0.006	0.663	1.23	0.01	−0.044	<0.001
Vitamin B2 (mg)	1.84	0.01	0.040	0.005	1.43	0.01	0.066	<0.001
Niacin (mg)	16.32	0.14	−0.004	0.785	12.16	0.08	0.002	0.884
Vitamin C (mg)	78.69	1.69	−0.013	0.287	79.65	1.31	0.005	0.686

5.3. Factors Affecting HDL Cholesterol

Table 3 presents the results of the complex-sample multiple linear regression analysis conducted to identify the independent determinants affecting HDL-C levels by sex and

age group. The explanatory power of the models (R^2) ranged from 14.1% to 17.6% across the groups.

Table 3. Factors Affecting HDL Cholesterol.

Variable	Male 20–39			Female 20–39		
	B	**β**	***p***	**B**	**β**	***p***
(Intercept)	82.578		<0.001	80.004		<0.001
Education	−0.463	−0.022	0.139	1.184	0.049	<0.001
Income	0.110	0.004	0.687	1.304	0.043	0.173
Household Income	−0.043	−0.001	0.769	−2.011	−0.071	0.054
Occupation	0.002	0.000	0.996	0.833	0.031	0.020
Alcohol Drinking	2.945	0.115	<0.001	2.959	0.110	<0.001
Cigarette Smoking	−2.038	−0.086	<0.001	−0.021	0.000	0.974
Stress	−0.208	−0.008	0.547	−0.725	−0.027	0.032
Medication for dyslipidemia	−3.177	−0.030	0.005	0.185	0.001	0.941
High Physical Activity Hour—Work	−0.001	−0.001	0.914	0.010	0.009	0.515
Moderate Physical Activity Hour—Work	0.004	0.022	0.119	0.005	0.020	0.123
Location Move Physical Activity Hour	0.000	0.001	0.971	0.001	0.002	0.889
High Physical Activity Hour—Leisure	0.012	0.036	0.029	0.021	0.027	0.050
Moderate Physical Activity Hour—Leisure	0.020	0.056	<0.001	0.009	0.018	0.187
Walk Hour	0.001	0.002	0.867	0.001	0.005	0.727
Sedentary Hour	−0.002	−0.032	0.029	0.000	0.008	0.569
Waist circumference	−0.352	−0.328	<0.001	−0.142	−0.106	0.001
Body Mass Index	−0.067	−0.024	0.541	−0.757	−0.230	<0.001
Energy intake	0.002	0.153	0.002	0.002	0.141	0.019
Water intake	0.001	0.051	0.011	0.001	0.055	0.008
Carbohydrate	−0.011	−0.131	<0.001	−0.017	−0.134	0.001
Protein intake	0.009	0.042	0.332	0.020	0.051	0.195
Fat intake	−0.158	−0.657	<0.001	−0.078	−0.198	0.236
Saturated fatty acids intake	0.154	0.220	0.004	0.066	0.062	0.416
Monounsaturated fatty acids intake	0.191	0.305	<0.001	0.087	0.079	0.272
Polyunsaturated fatty acids intake	0.842	0.914	0.083	1.466	1.017	0.071
*n*3 fatty acid	−0.922	−0.150	0.069	−1.492	−0.177	0.068
*n*6 fatty acid	−0.654	−0.628	0.174	−1.317	−0.797	0.104
Cholesterol intake	0.000	0.005	0.828	0.000	0.004	0.850
Total Dietary Fiber intake	0.035	0.040	0.092	0.042	0.032	0.135
Calcium intake	0.000	0.010	0.686	0.003	0.059	0.007
Phosphorus intake	−0.001	−0.029	0.570	−0.002	−0.059	0.212
Sodium intake	0.000	0.003	0.865	0.000	−0.004	0.807
Potassium intake	−0.001	−0.075	0.026	0.000	−0.014	0.644
Iron intake	−0.025	−0.022	0.294	−0.049	−0.026	0.148
Carotene intake	0.000	0.034	0.300	0.000	0.012	0.525
Retinol intake	0.000	0.011	0.209	0.001	0.018	0.310
Vitamin B1	−0.674	−0.066	0.003	−1.374	−0.083	<0.001
Vitamin B2	0.172	0.017	0.534	0.427	0.027	0.234
Niacin	0.003	0.003	0.914	−0.061	−0.035	0.178
Vitamin C	0.001	0.008	0.632	0.005	0.029	0.042
R^2 Score	R^2 = 0.176			R^2 = 0.167		

Table 3. *Cont.*

Variable	Male 40–59			Female 40–59		
	B	**β**	***p***	**B**	**β**	***p***
(Intercept)	79.272		<0.001	81.676		<0.001
Education	0.138	0.009	0.478	0.799	0.047	<0.001
Income	−0.465	−0.017	0.646	−0.691	−0.022	0.422
Household Income	0.461	0.017	0.625	0.849	0.028	0.302
Occupation	0.595	0.015	0.317	0.211	0.007	0.507
Alcohol Drinking	3.494	0.133	<0.001	3.806	0.136	<0.001
Cigarette Smoking	−2.330	−0.097	<0.001	−0.944	−0.014	0.238
Stress	−0.446	−0.017	0.173	0.035	0.001	0.919
Medication for dyslipidemia	1.936	0.054	<0.001	0.542	0.012	0.283
High Physical Activity Hour—Work	0.002	0.005	0.634	0.047	0.033	0.058
Moderate Physical Activity Hour—Work	0.008	0.030	0.023	0.003	0.008	0.429
Location Move Physical Activity Hour	−0.007	−0.020	0.115	−0.002	−0.004	0.682
High Physical Activity Hour—Leisure	0.015	0.037	0.005	0.021	0.024	0.064
Moderate Physical Activity Hour—Leisure	0.004	0.012	0.359	0.021	0.040	0.001
Walk Hour	0.005	0.025	0.086	0.001	0.005	0.669
Sedentary Hour	−0.001	−0.024	0.068	0.000	−0.005	0.629
Waist circumference	−0.268	−0.199	<0.001	−0.205	−0.136	<0.001
Body Mass Index	−0.425	−0.117	<0.001	−0.546	−0.138	<0.001
Energy intake	0.002	0.199	<0.001	0.007	0.350	<0.001
Water intake	0.000	0.025	0.190	0.001	0.044	0.009
Carbohydrate	−0.017	−0.187	<0.001	−0.038	−0.300	<0.001
Protein intake	0.004	0.014	0.763	−0.012	−0.025	0.430
Fat intake	−0.081	−0.260	0.066	−0.091	−0.172	0.187
Saturated fatty acids intake	0.076	0.084	0.202	0.032	0.021	0.696
Monounsaturated fatty acids intake	0.063	0.076	0.313	0.078	0.053	0.355
Polyunsaturated fatty acids intake	0.279	0.246	0.539	0.280	0.159	0.059
*n*3 fatty acid	−0.116	−0.025	0.798	−0.279	−0.040	0.001
*n*6 fatty acid	−0.181	−0.134	0.687	−0.167	−0.082	0.244
Cholesterol intake	0.001	0.020	0.345	0.001	0.018	0.292
Total Dietary Fiber intake	0.034	0.041	0.054	0.066	0.064	0.001
Calcium intake	0.002	0.051	0.018	0.001	0.031	0.099
Phosphorus intake	−0.001	−0.056	0.292	0.001	0.023	0.554
Sodium intake	0.000	0.001	0.955	0.000	−0.033	0.011
Potassium intake	0.000	0.011	0.684	0.000	−0.050	0.051
Iron intake	−0.048	−0.039	0.039	−0.121	−0.062	<0.001
Carotene intake	0.000	−0.007	0.651	0.000	−0.001	0.909
Retinol intake	0.000	0.002	0.908	0.001	0.013	0.294
Vitamin B1	−0.176	−0.014	0.430	−0.764	−0.041	0.011
Vitamin B2	0.434	0.037	0.113	0.274	0.015	0.473
Niacin	−0.082	−0.075	0.006	−0.128	−0.061	0.002
Vitamin C	−0.001	−0.007	0.567	0.003	0.023	0.069
R^2 Score	$R^2 = 0.141$			$R^2 = 0.142$		

As a central finding of this study, carbohydrate intake was identified as a robust factor independently lowering HDL-C levels across all four groups. Even after multivariable

adjustment, increased carbohydrate intake was significantly associated with decreased HDL-C (Male 20–39: $\beta = -0.131$, $p < 0.001$; Female 20–39: $\beta = -0.134$, $p = 0.001$; Male 40–59: $\beta = -0.187$, $p < 0.001$), and this impact was particularly pronounced in the middle-aged female group (40–59 years) ($\beta = -0.300$, $p < 0.001$). Conversely, total energy intake demonstrated an independent positive association with HDL-C in all groups. Among micronutrients, total dietary fiber intake was found to significantly contribute to the elevation of HDL-C in women aged 40–59 ($\beta = 0.064$, $p = 0.001$).

Obesity indicators were major factors exerting a consistent negative impact on HDL-C levels. Waist circumference was an independent factor significantly reducing HDL-C in all four groups (all groups $p < 0.001$). Body mass index (BMI) also demonstrated a strong and significant association with reduced HDL-C in three groups, excluding men aged 20–39 ($p < 0.001$). This suggests that, even when controlling all dietary and lifestyle factors, both abdominal and general obesity independently contribute to the reduction in HDL-C.

Among lifestyle factors, alcohol drinking was a common determinant that significantly increased HDL-C across all age and sex groups (all groups $p < 0.001$). In contrast, cigarette smoking acted as a strong risk factor, independently lowering HDL-C across all male groups (both 20–39 and 40–59 years) ($p < 0.001$). In women, no significant effect of smoking was observed; however, a consistent positive association was confirmed where higher educational levels led to significantly increased HDL-C levels ($p < 0.001$) in both young and middle-aged women. Regarding physical activity, moderate-intensity leisure-time physical activity (Moderate PA—Leisure) emerged as a significant factor contributing to HDL-C elevation in men aged 20–39 ($p < 0.001$) and women aged 40–59 ($p = 0.001$) (Table 3).

5.4. Trends in Major Factors Affecting HDL-C and Carbohydrate Intake

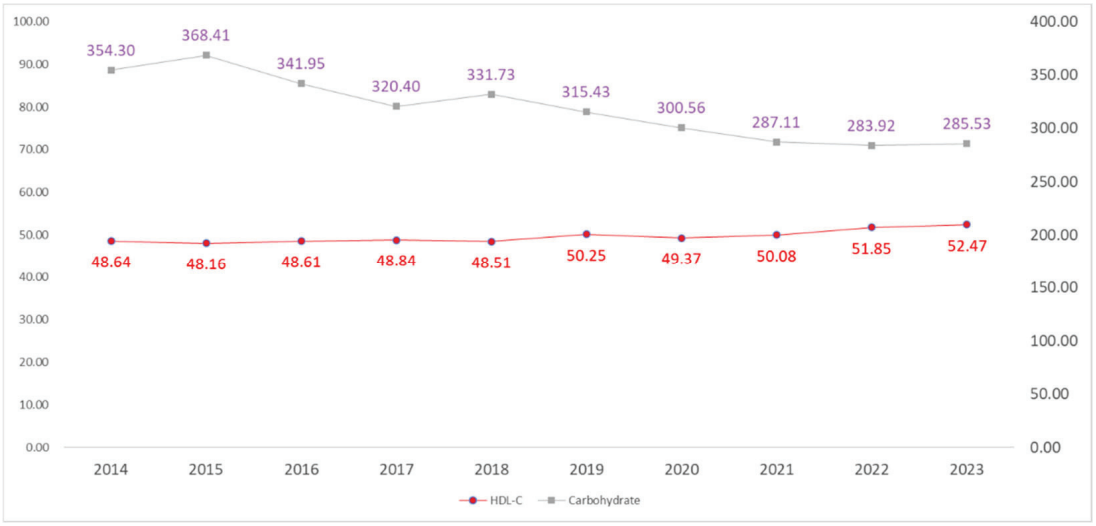
Figure 1a–d illustrates the 10-year temporal trends of carbohydrate intake and HDL-C levels from 2014 to 2023 across four subgroups stratified by sex and age (men and women aged 20–39 and 40–59 years). These graphs clearly demonstrate a consistent, macroscopic inverse trajectory between the two indicators across all demographic subgroups.

In all four groups, carbohydrate intake (gray line) depicted a distinct downward curve over the past decade. Statistical post hoc analysis (Bonferroni) revealed that carbohydrate intake was highest during the early years of the study (2014–2016; a, b, c) and exhibited a stepwise declining pattern toward the later period from 2019 onward (f–j), a trend consistently observed regardless of sex and age.

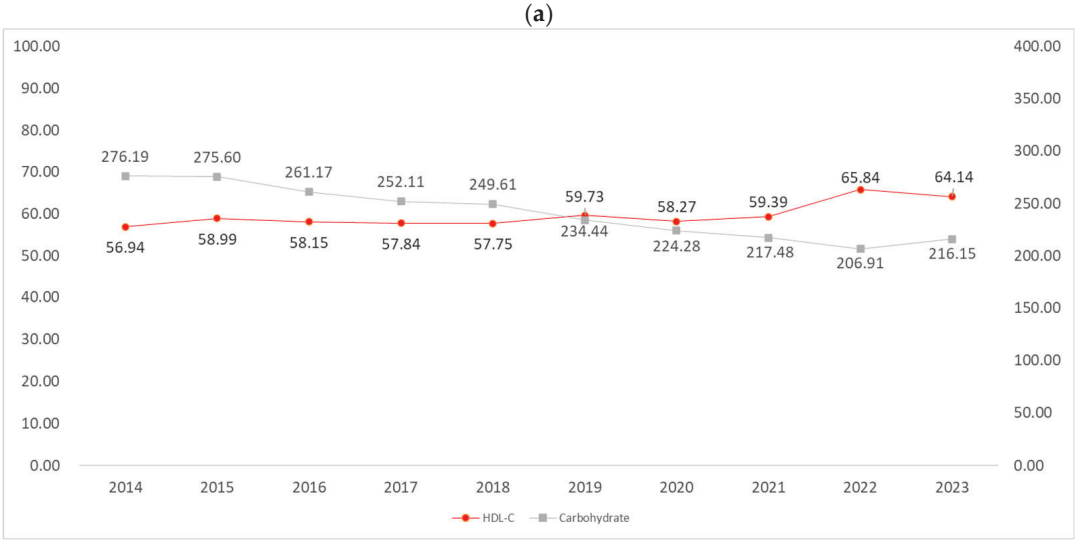
In exact alignment with the declining trajectory of carbohydrate intake, HDL-C levels (red line) displayed a gradual upward trend across all four groups. Post hoc analyses also confirmed that the HDL-C levels in the later half of the study period (2019–2023; f–j) were statistically significantly higher than the average levels in the earlier half (2014–2018; a–e) across all groups. In particular, a more pronounced upward shift was commonly observed starting around 2019 and 2022.

The integrated results of Figure 1 visually and statistically demonstrate that the ‘reduction in carbohydrate intake’ and the ‘elevation of HDL-C’ are synchronized chronologically, forming a perfect cross-over pattern across the entire Korean adult population, rather than being confined to a specific age group (20–30 s vs. 40–50 s) or sex. This strongly corroborates that the cross-sectional association derived from the multivariable regression analysis (Table 3)—identifying carbohydrate intake as an independent risk factor that lowers HDL-C—is in exact alignment with the macroscopic, population-level trend of national dietary transition over the past decade (Figure 1a–d).

https://doi.org/10.3390/nu18071045



(**a**)

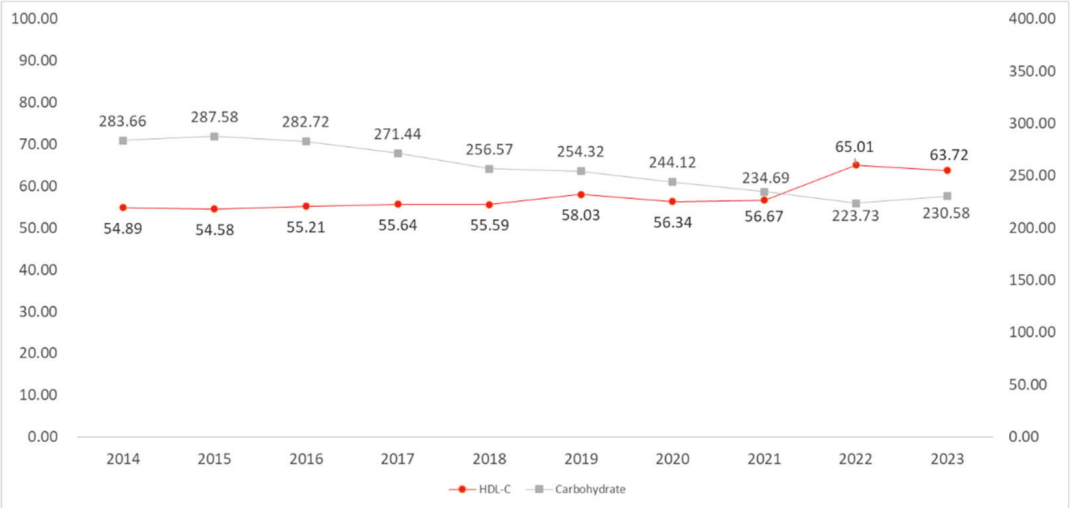


(**b**)



(**c**)

Figure 1. *Cont.*



(**d**)

Figure 1. (**a**). Trends in the relationship between HDL-C and Carbohydrate Intake—Male 20–39; Post hoc group: a = 2014, b = 2015, c = 2016, d = 2017, e = 2018, f = 2019, g = 2020, h = 2021, i = 2022, j = 2023; Carbohydrate Intake Trends: Post hoc: Bonferroni = a, b > c, d, e, f > g, h, i, j; HDL-C Trends Post hoc: Bonferroni = a, b, c, d, e < f, g, h, i, j; (**b**). Trends in the relationship between HDL-C and Carbohydrate Intake—Female 20–39; Post hoc group: a = 2014, b = 2015, c = 2016, d = 2017, e = 2018, f = 2019, g = 2020, h = 2021, i = 2022, j = 2023; Carbohydrate Intake Trends: Post hoc: Bonferroni = a, b, c > d, e > f, g, h, i, j; HDL-C Trends Post hoc: Bonferroni = a, b, c, d, e < f, g, h < i, j; (**c**). Trends in the relationship between HDL-C and Carbohydrate Intake—Male 40–59; Post hoc group: a = 2014, b = 2015, c = 2016, d = 2017, e = 2018, f = 2019, g = 2020, h = 2021, i = 2022, j = 2023; Carbohydrate Intake Trends: Post hoc: Bonferroni = a, b, c, d, e > f > g, h, i, j; HDL-C Trends Post hoc: Bonferroni = a, b, c, d, e < f, g, h < i, j; (**d**). Trends in the relationship between HDL-C and Carbohydrate Intake—Female 40–59; Post hoc group: a = 2014, b = 2015, c = 2016, d = 2017, e = 2018, f = 2019, g = 2020, h = 2021, i = 2022, j = 2023; Carbohydrate Intake Trends: Post hoc: Bonferroni = a, b, c, d > e, f > g, h, i, j; HDL-C Trends Post hoc: Bonferroni = a, b, c, d, e < f, g, h < i, j.

6. Discussion

6.1. Principal Findings and Dietary/Lifestyle Shifts

In this nationwide study of Korean adults, we observed a marked upward trend in HDL cholesterol levels from 2014 to 2023, indicating an overall improvement in population-level metabolic health. Mean HDL-C rose by about 6 mg/dL over the decade, and the prevalence of low HDL-C (a risk factor and diagnostic criterion for metabolic syndrome) decreased dramatically. This favorable trend appears to reflect the net impact of several concurrent public health developments in Korea: most prominently, a significant shift in diet composition and a reduction in smoking. Our analysis suggests that the traditional Korean high-carbohydrate dietary pattern—long implicated in low HDL levels—has attenuated in recent years, with people eating fewer refined carbs and relatively more fats and protein [20]. This nutritional transition is likely a key driver of the improved HDL-C profiles in the population. Meanwhile, sustained tobacco control efforts [19] have lowered smoking rates, removing a source of HDL suppression for many individuals. These positive changes seem to outweigh negative influences such as rising obesity, at least in terms of shaping this specific metabolic biomarker. From a public health perspective, the improvement in HDL-C is encouraging, as low HDL-C has been widespread in Asia [21] and serves as a strong proxy for insulin resistance and elevated cardiometabolic risk. Our findings align with other recent data: a 20-year analysis showed low HDL-C prevalence in Korea nearly halved between 2001 and 2020, especially in women. Interestingly, that study attributed the HDL increase largely to decreased carbohydrate and increased unsaturated fat intake

in the Korean diet—precisely the mechanism our data support [21]. Women historically had particularly low HDL in Korea (despite women usually having higher HDL than men globally), likely due to cultural dietary factors; the dramatic HDL gains in Korean women in the past decade have narrowed this gap [22].

The public health implications of these findings are significant. First, they underscore the impact that population-level dietary changes can have on cardiovascular risk factors. Over the past decade, Korean national health campaigns have targeted reduction in sodium and refined carbohydrate intake (including a "Reduction of White Rice Consumption" initiative and promotion of more diverse staples and healthy oils)—although not originally aimed at HDL, these efforts may have yielded the unintended benefit of improving systemic metabolic profiles, as reflected by rising HDL-C levels. It suggests that encouraging a balanced diet with moderate fat intake (particularly unsaturated fats from fish, nuts, and plant oils) in place of excessive carbohydrates can improve lipid profiles in high-carb-consuming populations. This is consistent with clinical trial evidence that low-carbohydrate or Mediterranean-style diets tend to raise HDL-C more than low-fat, high-carb diets [23,24]. For East Asian countries experiencing a nutrition transition, our results provide evidence in support of dietary guidelines that emphasize reducing refined grains and added sugars while incorporating healthy fats. Of course, any dietary strategy must be culturally acceptable and nutritionally adequate [25–27]. One concern is that the observed drop in whole-grain intake and certain micronutrients (like thiamine and iron) in Korea could pose other health challenges [28,29]. Policymakers should ensure that while carbohydrate quantity is reduced, the quality of diet remains high (e.g., replacing polished rice with vegetables, legumes, and sources of unsaturated fats rather than with processed meats or sugary foods). Additionally, our multivariable analysis highlights the significant impact of alcohol consumption, iron, and certain vitamins on HDL-C levels, which warrants attention. Consistent with previous studies, alcohol drinking was strongly and positively associated with HDL-C across all subgroups. While alcohol is known to increase the transport rates of apolipoproteins A-I and A-II [30], it also increases HDL-C levels by suppressing cholesteryl ester transfer protein (CETP) activity. Importantly, an increase in HDL-C driven by this CETP suppression is generally not expected to confer anti-atherosclerotic benefits [31]. We caution against initiating alcohol consumption solely for this lipid benefit due to its well-documented adverse health effects [32]. Regarding micronutrients, iron intake showed an inverse relationship with HDL-C, particularly in older groups [33]. This may be explained by the pro-oxidant nature of iron; excess iron accumulation can induce oxidative stress and lipid peroxidation [34], potentially impairing HDL functionality and accelerating its clearance [35]. Conversely, certain vitamins (such as vitamins B1 and B2) exhibited significant associations with HDL-C [36]. B-complex vitamins act as essential coenzymes in cellular energy and lipid metabolism [37]. The protective associations observed may reflect not only the direct role of these vitamins in maintaining metabolic homeostasis but also their role as surrogate markers for a generally high-quality, nutrient-dense diet [38].

6.2. The Role of Adiposity and Insulin Resistance

The strong inverse association we found between adiposity and HDL-C reinforces the importance of obesity prevention in cardiometabolic health. The rise in obesity in Korea (though modest compared to Western countries) is an alarming trend that could undermine gains in HDL and other risk factors. Higher BMI and waist circumference each independently predict lower HDL, as well as higher triglycerides and insulin resistance [39]. Therefore, public health policies should intensify focus on healthy weight through interventions like promoting physical activity, improving food environments, and addressing

sedentary lifestyles and caloric over-consumption. Notably, our results show physical activity's direct effect on HDL was quite small after accounting for other factors; however, physical activity is critical for weight control and has many other cardiovascular benefits beyond merely elevating this lipid marker. We interpret this not as physical activity being unimportant, but rather that activity alone (without weight loss or dietary change) has limited capacity to raise HDL by more than a few points. Effective strategies will combine diet, exercise, and weight management to maximize metabolic improvements [40].

The strong inverse association between adiposity indicators (BMI and waist circumference) and HDL-C levels highlights the pivotal role of insulin resistance and glucose tolerance in lipid metabolism. Central obesity, reflected by increased waist circumference, is a primary driver of systemic insulin resistance. When this metabolically compromised state is coupled with a high-carbohydrate diet, it exacerbates postprandial glucose spikes and hyperinsulinemia. From a mechanistic standpoint, insulin resistance decreases the activity of lipoprotein lipase (LPL) and increases hepatic lipase activity, a combination that accelerates the catabolism and renal clearance of HDL particles, thereby lowering circulating HDL-C mass [41,42]. In this pathophysiological context, the importance of physical activity must be emphasized alongside dietary modifications. Although our multivariable model showed a relatively modest direct effect of exercise duration on HDL-C mass, regular physical activity profoundly improves skeletal muscle glucose uptake and systemic insulin sensitivity [43]. Therefore, mitigating insulin resistance through a synergistic combination—reducing refined carbohydrate intake, managing central adiposity, and maintaining regular exercise habits—is essential. This integrated lifestyle approach not only optimizes metabolic biomarkers like HDL-C but also provides comprehensive protection against the broader spectrum of cardiometabolic diseases [44].

6.3. Impact of the COVID-19 Pandemic and Clinical Implications

Some intriguing aspects merit further discussion. The surge in mean HDL-C in 2022 was larger than anticipated. While we have attributed it to ongoing trends, one cannot exclude the influence of the COVID-19 pandemic context. It is possible that lifestyle changes during the pandemic, such as reduced dining out, led to healthier home cooking and a reduction in sugary drinks or carb-heavy convenience foods, which in turn improved HDL. Additionally, a proportion of the population may have become more health-conscious in response to COVID-19, potentially affecting lipid levels [45]. Conversely, other reports noted weight gain and worse dietary habits in some groups during lockdowns, which might have temporarily offset HDL gains in 2020 [46]. By 2022, those effects might have normalized, and the underlying positive trend reasserted itself strongly. The large jump in 2022 HDL could also be partly due to random variation or sampling; although KNHANES is carefully designed, the specific sample in 2022 might, by chance, have had slightly more health-aware individuals. We advise interpreting the 2022 value with caution until additional years (2024 onward) confirm whether HDL has truly "reset" at a higher plateau or if it oscillates. Regardless, the consistent upward direction over the decade is clear.

From a global perspective, the Korean experience provides an interesting contrast to Western countries. In the US and Europe, mean HDL-C has been relatively stable or even declining slightly in some cohorts, partly due to obesity and physical inactivity increases, though low-HDL prevalence is generally lower than it was in Asia. Korea's improvement in HDL-C despite rising obesity highlights the power of dietary composition changes and smoking reduction. Crucially, the population-level rise in HDL observed here should be interpreted primarily as a reflection of broader lifestyle improvements that confer cardiovascular benefits (for example, eating more unsaturated fats and less refined carbs not only raises HDL but also lowers triglycerides and improves insulin sensitivity). Thus, rising

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HDL can be viewed as a marker of positive health behavior change, rather than a direct goal. We caution that a high HDL-C level per se is not automatically protective—recent Mendelian randomization studies and pharmacologic trials have demonstrated that simply elevating HDL by drug therapy does not necessarily translate into fewer heart attacks [3,47]. The quality and function of HDL particles, and the context of other risk factors, matter. In Korea's case, the HDL increase has come alongside dropping smoking rates. The net impact on cardiovascular disease outcomes will depend on all these factors. Encouragingly, national data show a decline in ischemic heart disease mortality in Korea in the 2010s, which might be linked in part to these risk factor trends [48].

6.4. Limitations

First, the data are observational and cross-sectional in nature (even though we looked at trends across time, each year's data is a cross-section), so causal inference must be made cautiously.

Second, dietary intake was assessed by a single 24 h recall, which is subject to measurement error and day-to-day variability. While the large sample size should even out random error, some systematic bias might exist. The trend of decreasing carbohydrate intake is supported by multiple years of data and external studies, but the absolute values might be underestimated. However, this methodology is used as a standardized and validated tool within the KNHANES protocol and is therefore useful for national epidemiological surveys.

Third, we did not extensively stratify our analysis by sex or age, as our goal was to examine the overall national trends.

Fourth, as real-world data, various variables potentially related to HDL-C may exist, but due to the nature of secondary data provision, we could not reflect all of them. However, since we utilized publicly available national healthcare big data, we can be confident in the reliability of the measured variables.

Our findings are robust in illustrating the broad shifts in HDL-C and what likely drove them. The public health message is that population-wide lifestyle modifications—even modest ones—can substantially improve lipid profiles. Korea's case demonstrates that lowering national carbohydrate intake and smoking prevalence, even as BMI rises slightly, was associated with higher HDL-C levels in the populace. For policymakers and health professionals, this emphasizes a multipronged approach: continue to advocate for balanced diets (less sugar and refined grain, more healthy fats and fiber), smoke-free living, and also tackle obesity with urgency. Each of these factors influences different metabolic pathways; together they shape cardiovascular risk.

7. Conclusions

In conclusion, HDL cholesterol levels among Korean adults have significantly increased over the last ten years, reflecting a positive development in cardiovascular risk factor status. This improvement correlates with lifestyle changes, notably dietary transition and smoking reduction, amid relatively stable or mildly worsening other factors. While high HDL-C should not be viewed as an end in itself, it serves as an indicator that Korean public health efforts to improve diet and reduce tobacco use are having measurable success. Going forward, sustaining these trends and addressing remaining challenges will be crucial. These findings contribute to a growing body of evidence that favorable changes in population diet and behavior can ameliorate components of metabolic syndrome. Other countries undergoing similar nutrition transitions may draw lessons from the Korean experience—namely, that reducing excessive carbohydrate intake and curbing smoking can lead to meaningful improvements in HDL-C and presumably cardiovascular health. Continuous monitoring of risk factor trends and outcomes will be important to evaluate

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long-term impacts, especially as Korea and other nations grapple with the dual burden of an aging population and lifestyle-related chronic diseases. Public health strategies that promote a heart-healthy lifestyle at the population level remain a cornerstone of cardiovascular disease prevention in the 21st century.

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Data Availability Statement: Publicly Accessible Repository. The original data presented in the study are openly available in the Korea National Health and Nutrition Examination Survey (KNHANES), Korea Disease Control and Prevention at https://knhanes.kdca.go.kr/knhanes/eng/main.do (accessed on 5 December 2025) [13].

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

References

1. Gordon, D.J.; Probstfield, J.L.; Garrison, R.J.; Neaton, J.D.; Castelli, W.P.; Knoke, J.D.; Jacobs, D.R., Jr.; Bangdiwala, S.; Tyroler, H.A. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* **1989**, *79*, 8–15. [CrossRef] [PubMed]
2. Dankner, R.; Chetrit, A.; Green, M.S. Change in HDL cholesterol as an independent predictor of coronary heart disease mortality in men. *Eur. Heart J.* **2005**, *26*, 888–893.
3. Voight, B.F.; Peloso, G.M.; Orho-Melander, M.; Frikke-Schmidt, R.; Barbalic, M.; Jensen, M.K.; Hindy, G.; Hólm, H.; Ding, E.L.; Johnson, T.; et al. Plasma HDL cholesterol and risk of myocardial infarction: A mendelian randomisation study. *Lancet* **2012**, *380*, 572–580. [CrossRef]
4. Rohatgi, A.; Khera, A.; Berry, J.D.; Givens, E.G.; Ayers, C.R.; Wedin, K.E.; Neeland, I.J.; Yuhanna, I.S.; Rader, D.R.; de Lemos, J.A. HDL cholesterol efflux capacity and incident cardiovascular events. *N. Engl. J. Med.* **2014**, *371*, 2383–2393. [CrossRef]
5. Park, D.; Shin, M.J.; Després, J.P.; Eckel, R.H.; Tuomilehto, J.; Lim, S. 20-year trends in metabolic syndrome among Korean adults from 2001 to 2020: Impact of lifestyle changes on HDL cholesterol. *JACC Asia* **2023**, *3*, 491–502. [CrossRef]
6. Choi, S.; Kim, Y.; Park, S.; Lee, J.; Oh, K. Trends in cigarette smoking among adolescents and adults in South Korea. *Epidemiol. Health* **2014**, *36*, e2014023. [CrossRef]
7. Kweon, S.; Park, J.Y.; Park, M.; Kim, Y.; Yeon, S.Y.; Yoon, L.; Yun, S.; Park, S.; Yang, J.E.; Kim, Y.; et al. Trends in food and nutrient intake over 20 years: Findings from the 1998–2018 Korea National Health and Nutrition Examination Survey. *Epidemiol. Health* **2021**, *43*, e2021027. [CrossRef] [PubMed]
8. Sung, H.; Kim, G.; Ma, X.; Choe, H.; Han, Y.; Yoon, J.; Ryu, Y.; Kim, Y.S. Physical activity trends in Korean adults from Korea National health and nutritional examination survey from 2014 to 2019. *Int. J. Environ. Res. Public Health* **2022**, *19*, 5213. [CrossRef]
9. Chun, O.K.; Lee, H.; Song, W.O. Secular trends in dietary energy, carbohydrate, fat, and protein intake among Korean adults: Analysis of KNHANES data, 2010–2020. *Public Health Nutr.* **2024**, *27*, 1234–1245.
10. Moon, J.; Seo, Y.; Lee, H.-L.; Lee, H.; Kaneko, F.; Shin, S.; Kim, E.; Yum, K.S.; Kim, Y.D.; Baek, J.-H.; et al. Incidence and case fatality of stroke in Korea, 2011–2020. *Epidemiol. Health* **2024**, *46*, e2024001. [CrossRef]
11. Kim, S.; Kwon, Y.; Lee, J.Y. Trends of food supply and nutrient intake in South Korea over the past 30 years. *Food Nutr. J.* **2018**, *7*, 1–10. [CrossRef]
12. Mensink, R.P.; Zock, P.L.; Kester, A.D.; Katan, M.B. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. *Am. J. Clin. Nutr.* **2003**, *77*, 1146–1155. [CrossRef]

13. Lee, H.A.; An, H. The effect of high carbohydrate-to-fat intake ratios on hypo-HDL-cholesterolemia risk and HDL-cholesterol levels over a 12-year follow-up. *Sci. Rep.* **2020**, *10*, 913. [CrossRef] [PubMed]
14. Thompson, P.D.; Buchner, D.; Pina, I.L.; Balady, G.J.; Williams, M.A.; Marcus, B.H. Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease: A Statement From the Council on Clinical Cardiology (Subcommittee on Exercise, Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity). *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, e42–e49.
15. Korea Disease Control and Prevention Agency. *The Korea National Health and Nutrition Examination Survey (KNHANES), 2014–2023*; Korea Disease Control and Prevention Agency: Cheongju-si, Republic of Korea, 2024.
16. Kweon, S.; Kim, Y.; Jang, M.J.; Kim, Y.; Kim, K.; Choi, S.; Chun, C.; Khang, Y.-H.; Oh, K. Data resource profile: The Korea National Health and Nutrition Examination Survey (KNHANES). *Int. J. Epidemiol.* **2014**, *43*, 69–77. [CrossRef]
17. Kim, S.; Kwon, Y.; Lee, J.Y. Trends in KNHANES sampling strategy and implications for statistical analysis. *Epidemiol. Health* **2022**, *44*, e2022021.
18. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2024. Available online: https://www.R-project.org/ (accessed on 14 June 2024).
19. National Center for Health Statistics. *NCHS Data Presentation Standards for Proportions*; Vital and Health Statistics Series 2, No 185; National Center for Health Statistics: Hyattsville, MD, USA, 2023.
20. Ha, K.; Song, Y.J. Low-Carbohydrate Diets in Korea: Why Does It Matter, and What Is Next? *J. Obes. Metab. Syndr.* **2021**, *30*, 222–232. [CrossRef]
21. Park, J.J.; Kim, Y.H. National Smoking Cessation Services (NSCS) enrollment and their effect on long-term tobacco cessation in Korea: Results from a 1-year prospective follow-up of NSCS participants. *Tob. Induc. Dis.* **2024**, *22*, 45. [CrossRef]
22. Shah, C.; Reddy, D. Cardiovascular Disease Prevalence in Asians Versus Americans: A Review of Genetics, Diet, and the Call for Enhanced Prevention and Screening. *J. Community Health* **2024**, *49*, 673–682.
23. Shai, I.; Schwarzfuchs, D.; Henkin, Y.; Shahar, D.R.; Witkow, S.; Greenberg, I.; Golan, R.; Fraser, D.; Bolotin, A.; Vardi, H.; et al. Weight loss with a low-carbohydrate, Mediterranean, or low-fat diet. *N. Engl. J. Med.* **2008**, *359*, 229–241. [CrossRef] [PubMed]
24. Mansoor, N.; Vinknes, K.J.; Veierød, M.B.; Retterstøl, K. Effects of low-carbohydrate diets v. low-fat diets on body weight and cardiovascular risk factors: A meta-analysis of randomised controlled trials. *Br. J. Nutr.* **2016**, *115*, 466–479. [CrossRef]
25. Hu, F.B. Globalization of diabetes: The role of diet, lifestyle, and genes. *Diabetes Care* **2011**, *34*, 1249–1257. [CrossRef]
26. Mozaffarian, D. Dietary and policy priorities for cardiovascular disease, diabetes, and obesity: A comprehensive review. *Circulation* **2016**, *133*, 187–225. [CrossRef] [PubMed]
27. Popkin, B.M.; Adair, L.S.; Ng, S.W. Global nutrition transition and the pandemic of obesity in developing countries. *Nutr. Rev.* **2012**, *70*, 3–21. [CrossRef]
28. Ha, K.; Song, Y.J. Fact sheet: Nationwide trends in dietary intakes among Korean adults, 2013–2022. *Korean J. Intern. Med.* **2025**, *40*, 428–435.
29. Kim, Y.N.; Cho, Y.O. Prevalent low thiamine status among adults living in Seoul metropolitan area, South Korea. *Nutr. Res. Pract.* **2019**, *13*, 407–414. [CrossRef]
30. De Oliveira e Silva, E.R.; Foster, D.; Harper, M.M.; Seidman, C.E.; Smith, J.D.; Breslow, J.L.; Brinton, E.A. Alcohol consumption raises HDL cholesterol levels by increasing the transport rate of apolipoproteins A-I and A-II. *Circulation* **2000**, *102*, 2347–2352. [CrossRef] [PubMed]
31. Hannuksela, M.L.; Liisanantti, M.K.; Savolainen, M.J. Effect of alcohol on lipids and lipoproteins in relation to atherosclerosis. *Crit. Rev. Clin. Lab. Sci.* **2002**, *39*, 225–283. [CrossRef]
32. GBD 2016 Alcohol Collaborators. Alcohol use and burden for 195 countries and territories, 1990–2016: A systematic analysis for the Global Burden of Disease Study 2016. *Lancet* **2018**, *392*, 1015–1035. [CrossRef] [PubMed]
33. Ahluwalia, N.; Genoux, A.; Ferrières, J.; Perret, B.; Carrié, D.; Campagne, F.; Simon, M.; Tack, I. Iron status is inversely associated with high-density lipoprotein cholesterol in apparently healthy French men. *J. Nutr.* **2010**, *140*, 1923–1928.
34. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.T.D.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84. [CrossRef]
35. Kontush, A.; Chapman, M.J. Functionally defective high-density lipoprotein: A new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol. Rev.* **2006**, *58*, 342–374. [CrossRef]
36. Huskisson, E.; Maggini, S.; Ruf, M. The role of vitamins and minerals in energy metabolism and well-being. *J. Int. Med. Res.* **2007**, *35*, 277–289. [CrossRef] [PubMed]
37. Zhuang, P.; Jiao, J.; Dong, J.; Cai, Y.; Wang, J.; Wu, F.; Zhang, Y. Association of B vitamins and their combining effects with polyunsaturated fatty acids on lipid profiles. *Nutrients* **2018**, *10*, 431.
38. Drewnowski, A. Concept of a nutritious food: Toward a nutrient density score. *Am. J. Clin. Nutr.* **2005**, *82*, 721–732. [CrossRef]
39. Kim, K.; Park, Y.; Chae, H.; Kim, G.; Kang, T. 20-Year Trends in Metabolic Syndrome Among Korean Adults from 2001 to 2020. *J. Clin. Med.* **2023**, *12*, 4426.

40. Després, J.P.; Lemieux, I. Abdominal obesity and metabolic syndrome. *Nature* **2006**, *444*, 881–887. [CrossRef]
41. Vergès, B. Pathophysiology of diabetic dyslipidaemia: Where are we? *Diabetologia* **2015**, *58*, 886–899. [CrossRef]
42. Mooradian, A.D. Dyslipidemia in type 2 diabetes mellitus. *Nat. Clin. Pract. Endocrinol. Metab.* **2009**, *5*, 150–159. [CrossRef] [PubMed]
43. Sylow, L.; Kleinert, M.; Richter, E.A.; Jensen, T.E. Exercise-stimulated glucose uptake—Regulation and implications for glycaemic control. *Nat. Rev. Endocrinol.* **2017**, *13*, 133–148. [CrossRef] [PubMed]
44. Myers, J.; Kokkinos, P.; Nyelin, E. Physical activity, cardiorespiratory fitness, and the metabolic syndrome. *Nutrients* **2019**, *11*, 1652. [CrossRef] [PubMed]
45. Di Renzo, L.; Gualtieri, P.; Pivari, F.; Soldati, L.; Attinà, A.; Cinelli, G.; Leggeri, C.; Caparello, G.; Barrea, L.; Scerbo, F.; et al. Eating habits and lifestyle changes during COVID-19 epidemic in Italy. *J. Transl. Med.* **2020**, *18*, 229. [CrossRef] [PubMed]
46. Bhutani, S.; vanDellen, M.R.; Cooper, J.A. Longitudinal weight gain and related risk behaviors during the COVID-19 pandemic in adults in the US. *Nutrients* **2021**, *13*, 671. [CrossRef] [PubMed]
47. Keene, D.; Price, C.; Shun-Shin, M.J.; Francis, D.P. Effect on cardiovascular risk of high density lipoprotein targeted drug treatments niacin, fibrates, and CETP inhibitors: Meta-analysis of randomised controlled trials including 117,411 patients. *BMJ* **2014**, *349*, g4379. [CrossRef]
48. Kim, H.C. Epidemiology of cardiovascular disease and its risk factors in Korea. *Glob. Health Med.* **2021**, *3*, 134–141. [CrossRef]

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Assessing Orthorexic Tendencies and Dietary Patterns: A Cross-Sectional Study of Orthorexia Nervosa and Dietary Patterns in Lithuania

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Abstract

Background/Objectives: Orthorexia nervosa (ON) is an emerging condition marked by a preoccupation with healthy eating that is linked to diminished well-being and social functioning. While research on ON extends across countries, no studies about ON have been found in Lithuania. This study aimed to investigate dietary patterns, socio-demographic correlates, and the prevalence of orthorexic tendencies in a Lithuanian adult sample. **Methods:** A cross-sectional online survey was conducted using the ORTO-R 6-item scale, a Food Frequency Questionnaire (FFQ), and socio-demographic and dietary behavior measures. Principal component analysis (PCA) was applied to the FFQ to identify dietary patterns, and stepwise multiple linear regression was used to examine predictors of orthorexic tendencies. **Results:** Approximately 15% of the Lithuanian adult sample exhibited elevated orthorexic tendencies, while three dietary factors were extracted, including Balanced-Traditional, Processed-Dense and Protein-Rich patterns. Both Balanced-Traditional and Protein-Rich dietary patterns were positively associated with orthorexic tendencies, although only the Balanced-Traditional pattern remained a significant predictor in the fully adjusted regression model, which explained 16.2% of the variance in ORTO-R scores ($F(7,468) = 12.97$, $p < 0.001$). Higher orthorexic tendencies were associated with following a dietary plan, adherence to the Healthy-Traditional pattern, being female, younger age, higher meal frequency, employment status, and being married. **Conclusions:** Orthorexic behaviors were more prevalent among younger women, individuals following structured diets, and those adhering to health-oriented eating patterns. These findings highlight the interplay between demographic and dietary factors in shaping orthorexic tendencies in the Lithuanian population.

Keywords: eating behavior; orthorexia nervosa; food frequency; dietary pattern; eating disorder

1. Introduction

Orthorexia nervosa (ON) is a condition which was initially coined in European research in Italy [1]. The condition appears to impair social functioning as it is often linked to poorer mental health outcomes [2]. Across Europe and Asia while using different ON instruments, researchers have consistently confirmed its unequivocal prevalence and presence [3,4]. Within the European context, Eastern European countries seem to have assimilated Western ideals of beauty and eating behavior [5]. However, research in the Baltic countries including Latvia, Lithuania, and Estonia is scarce. Particularly for Lithuania, research on eating behaviors relates to pediatric and adolescent populations, with some studies revealing

emotional eating patterns among students with higher stress levels [6–9]. Therefore, it is important to understand eating behaviors and styles across the Lithuanian population above 18 years old. Furthermore, it is important to understand the emergence of orthorexic tendencies in Lithuania to add to the body of literature on ON.

1.1. Orthorexia Nervosa

From its initial conception, ON or correct eating has been regarded as a maladaptive behavior borrowed from Western values and consumerism ideology [10]. Prior to ON, healthism was the unofficial term used non-scientifically [11–13]. Since then, ON emerged—scientifically and empirically—following the need for a label related to the Diagnostic and Statistical Manual (DSM-V) [14]. Trends on research related to ON portray a linear emergence of the condition in health, nutrition, and mental health research across Europe, with spikes in the early 2000s, 2010s and 2020s following COVID-19 [1,15].

Scoping further, European researchers have collaborated on consensus to ensure that ON becomes an official annex and be labeled as a disorder within the DSM. The symptoms outlined suggest that ON is a Feeding and Eating Disorder whereby individuals possess a preoccupation with rigid eating patterns which affect psychological, social, and economic functioning [2]. Furthermore, the diagnosis consensus argues ON's distinction from Anorexia Nervosa (AN) and obsessive–compulsive disorder (OCD) owing to ON-specific properties such as compulsions being related to nutrition only, and the preoccupation with nutritious correct eating irrelated to body image [2]. Moreover, a series of factors have been linked to ON across countries, including time spent on social media and social media addictive behaviors, excessive physical exercise and gym culture, and emotion dysregulation and educational/professional background [2].

Difficulties in identifying, recognizing and regulation emotions has been found in several samples, including Lebanon, Germany, Turkey and digitally [16–19]. Individuals with ON tendencies employ dysfunctional metacognitive processes wherein they have difficulties reflecting and monitoring their own thinking processes mediated by adaptive or maladaptive emotional regulation abilities [19]. Such a finding is in line with studies on perfectionism and eating disorders wherein individuals diagnosed with an eating disorder (ED) scored higher on perfectionism and dysfunctional metacognitive beliefs, worry and rumination [20]. To that end, profiles of high-ON-scoring individuals include high scores for alexithymia, emotion regulation difficulties, body dissatisfaction, and excessive physical exercise [21].

Excessive exercise has also been a predominant risk factor for ON [2]. Exercise addiction has been associated with high self-expectations and higher tendencies for ON [22]. Other studies also have found higher ON prevalence across aerobic and strength-training exercise levels, internal exercise motivation and other measures [22–24]. Rather, the condition has been more tightly linked to muscle dysphoria and excessive exercise in CrossFit populations mediated by social media content online and tendencies for perfectionism [25]. Following that, social media addiction, use and type of content have also been linked to higher ON tendencies [25,26]. Platforms like Reddit, Instagram and TikTok have exacerbated ON symptoms among populations that set high self-expectations, exercise, and tend to compulsively obsess over nutritious content in food [27,28]. Out of these platforms, however, Instagram has become a recovery space for self-diagnosed ON individuals [3].

Nevertheless, the work toward better defining ON and understanding its properties remains a long journey. The lack of an official and APA-approved diagnostic body influences researchers, particularly related to measuring symptomatology [29]. The meta-analytic review evaluating the reliability of various ON measuring instruments, however, has not halted the research process. A majority of researchers have been using the

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ORTO-R, the Dusseldorf Orthorexia Scale (DOS) and its translations, and the Orthorexia Nervosa Inventory (ONI) [25]. Fewer studies have utilized the Eating Habits Questionnaire (EAQ-21) [15,25]. The scarcity of these instruments leads to a lack of comparable data, further extending the issue of receiving an APA-approved diagnostic model for ON.

1.2. Eating Patterns Across Europe

Emerging patterns of ON prevalence and overall eating behavior across European contexts highlights the need for a response from dietitians and mental health practitioners alike [2]. As a result of the World Health Organization's (WHO) and relevant agencies' efforts toward lowering obesity and chronic metabolic illnesses, eating behavior has shifted significantly in the past decade across European countries [30]. For example, a cross-national analysis of 22 European countries found that in European adults, 14 to 44% of their total energy intake comes from ultra-processed foods [31]. To track specific dietary behaviors, Food Frequency Questionnaires (FFQs) have been validated across contexts and languages, including Germany, France, Sweden, Italy and Poland [32–36]. Cross-checking through FFQ-based literature, there are cultural similarities and Westernization effects occurring in terms of dietary patterns. German adults consume diets rich in refined grains, processed meats and added fats [33]. This is similar to the UK, the Netherlands and Belgium wherein a consumption of ultra-processed food (UPF) is present [31]. France shows a more moderate consumption of UPF regardless of its western European position, and adults in the country are more prone to consuming stable meat-heavy diets or present mixed dietary patterns [32]. A significant shift is underlined in southern European countries where adults from Italy, Spain and Portugal consume fewer UPFs and more vegetables, legumes and olive oil [31,35]. Northern European countries, including the Scandinavian region, exhibit unique eating behavior patterns where high caffeine consumption and moderate intake of dairy and sweets is noted [34,37].

These studies also highlight significant differences in demographic predictors of specific dietary patterns, suggesting that those with higher socioeconomic status (SES) tend to consume more fruits, vegetables, and legumes in comparison to their lower SES counterparts [37]. Furthermore, age seems to be a fine predictor as adults in Italy, as well as younger adults, rely on packaged snacks and ready-made meals, which emphasizes the Westernization effect [35].

1.3. The Case of Lithuania

While research on eating behavior patterns of adults in Lithuania is scarce, dietary pattern analyses using FFQs in Lithuania consistently identify a diet characterized by frequent consumption of cereals and cereal products, root vegetables, legumes, fruits, vegetables, and fish. In a nationally representative study of Lithuanian adults, a dominant dietary pattern was labeled the cereals pattern, which loaded positively on bread and cereal products, potatoes, vegetables, fruits, and legumes and was distinct from patterns characterized by meat or energy-dense foods [38]. This pattern reflects staple foods traditionally consumed in Lithuania and aligns with long-standing culinary practices shaped by seasonality, local agriculture, and food storage requirements. Subsequent FFQ-based studies confirm the persistence of this plant-forward dietary component within Lithuanian populations. Longitudinal analyses indicate gradual increases in vegetable consumption and modest shifts toward more diversified plant food intake, while cereals and root vegetables remain core components of daily diets [38,39]. Research among Lithuanian young adults similarly identify dietary patterns marked by higher consumption of vegetables, fruits, grains, and fish, contrasting with protein-dominant and highly processed dietary patterns [9]. In addition, contemporary data from national food consumption studies of Lithuanian

adults show that Lithuanians tend to consume about 1691 kilocalories (kcal) per day with many micronutrient insufficiencies and maintain a consistent calorie deficit [40]. Lithuania presents a landscape where dietary patterns are heavily predicted by demographic factors such as age (young adults adhere less to Mediterranean dietary patterns) and education (higher education levels were strongly associated with healthier food consumption patterns) [9,40,41]. Yet there are no studies measuring the prevalence of ON in Lithuania. One study reported that young adults, women, and individuals actively seeking clean or healthy food were more likely to submit to dietary supplements, making up 78.1% of the entire study's sample [42]. Such trends in different European countries and in Lithuania present the need to measure ON in an effort to develop prevention, intervention, and postvention measures in relation to significant predictors.

Utilizing the Revised ORTO-15 scale (ORTO-R) and an adaptation of the Food Frequency Questionnaire (FFQ) to the Lithuanian context, a representative sample of 1091 adults of various socio-demographic backgrounds were surveyed online. The aim of the study was to assess the prevalence of ON in a Lithuanian sample and to identify profiles of vulnerable groups and their dietary patterns. This study is one of the first of its kind for Lithuania. It adds to the current body of literature and informs relevant stakeholders, including nutrition and mental health practitioners and relevant national and international organizations (the WHO, etc.) related to the current situation and needs.

2. Materials and Methods

2.1. Study Design and Participants

This cross-sectional study was conducted among adults living in Lithuania in an attempt to assess ON tendencies and determine how these tendencies associate with eating behaviors and food consumption patterns. A total of 1091 participants completed the questionnaire and were included in the analysis; the criteria for inclusion ensured the participation of adults older than 18 years old residing in Lithuania. Given the exploratory nature of the study, the sample size was determined pragmatically, aiming to maximize participation and ensure stable multivariate analyses rather than to test a predefined hypothesis. This explains the gender imbalance present in the study, owing to which caution is warranted in making gender-related conclusions. The data were collected through a random sampling technique via a self-administered survey combining different instruments from March to June 2025. A Google Forms questionnaire was administered. The study was conducted in compliance with the 1964 Declaration of Helsinki's ethical standards. The research protocol was reviewed and approved by the Research Ethics Committee of SMK College of Applied Sciences. All participants were informed about the purpose, procedures, and voluntary nature of the research before taking part. Informed consent was obtained from all respondents prior to survey completion. Confidentiality and anonymity of the participants' responses were strictly maintained throughout the research process.

2.2. Measures

2.2.1. Orthorexia Tendencies

The ORTO-R is a self-report questionnaire composed of 6 items evaluated on a 1–5 Likert scale where 1 = never, 2 = rarely, 3 = sometimes, 4 = very often, and 5 = always [43]. The questionnaire was forward translated to Lithuanian and back translated to English by the 4-person research team to ensure internal consistency, and it was piloted on a sample of 30 initial participants based on validation principles of 5 participants per item [44]. Higher total scores on the ORTO-R reflect higher levels of orthorexic tendencies. In the current sample, the scale demonstrated acceptable internal consistency (Cronbach's $\alpha = 0.78$), with corrected item–total correlations ranging from 0.47 to 0.63.

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2.2.2. Food Frequency Questionnaire (FFQ)

Dietary patterns were assessed using a 17-item food frequency questionnaire adapted for Lithuanian adults. Participants indicated how often they consumed major food groups (e.g., cereals, vegetables, fruits, meats, dairy, fats, sweets, soft drinks, alcohol, and coffee) on a Likert-type scale where 0 = never/seldom (less than once a month), 1 = 1–3 times per month, 2 = 1–2 times per week, 3 = 3–4 times per week, and 7 = daily consumption. The FFQ showed good internal consistency in the present study (Cronbach's $\alpha = 0.84$). Corrected item–total correlations ranged from 0.28 to 0.60, and no item substantially decreased scale reliability, supporting the cohesiveness of the measure for evaluating dietary habits.

2.2.3. Socio-Demographic and Lifestyle Variables

In an attempt to identify and compile profiles of ON-vulnerable individuals, several socio-demographic and lifestyle variables were assessed. Socio-demographic variables included age, gender, economic household income, household size (persons residing), height, weight, employment status, and marital status. Furthermore, lifestyles were measured by questions on diet behavior, diet source, type of diet, and reasons for dieting.

2.2.4. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics, Version 21.0 (IBM Corp., Armonk, NY, USA). Prior to analysis, all variables were screened for missing data, outliers, normality, and appropriate coding. Descriptive statistics (means, standard deviations, frequencies, and percentages) were computed for all socio-demographic, dietary, and ORTO-R variables. To identify underlying dietary patterns, a principal component analysis (PCA) with Varimax rotation was applied to the Food Frequency Questionnaire (FFQ). Sampling adequacy was evaluated using the Kaiser–Meyer–Olkin (KMO) statistic and Bartlett's test of sphericity. Components with eigenvalues greater than 1.0 and clear interpretability were retained. Regression-based factor scores for each extracted component were generated and used as continuous variables in subsequent analyses. Bivariate associations between orthorexic tendencies (ORTO-R mean score), dietary patterns, and socio-demographic variables were examined using Pearson's correlation coefficients. Assumptions of linearity and homoscedasticity were evaluated through scatterplots and standardized residual diagnostics. To determine the strongest predictors of orthorexic tendencies, a stepwise multiple linear regression model was conducted with the mean ORTO-R score as the dependent variable. Independent variables included age, gender, marital status, employment status, household size, daily meal frequency, current diet plan status, and the three FFQ-derived dietary factor scores. Entry and removal criteria followed SPSS defaults ($p < 0.05$ for entry; $p > 0.10$ for removal). Model fit was evaluated using R^2, adjusted R^2, and F-statistics. Multicollinearity was assessed with Tolerance and Variance Inflation Factor (VIF) values, with VIF < 2 indicating acceptable levels. The Durbin–Watson statistic was used to verify the independence of residuals. Statistical significance for all analyses was set at $p < 0.05$ (two-tailed).

3. Results

3.1. Participant Characteristics and ON Prevalence

The majority of the participants were women (76.4%), with fewer participants reporting as men (22.9%) or preferring not to say their gender (0.6%), whereas the mean age was 33 years (M = ±33.06). Women had a mean age of 33.29 years (SD = 12.24), while men had a mean age of 32.40 years (SD = 12.52). Body mass index (BMI), calculated as weight in kilograms divided by height in meters squared, had a mean value of 24.91 kg/m^2 (SD = 4.95). As shown in Table 1, the majority of our participants reported living with

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3–4 household members (65.6%). Most participants reported being married (41.8%) or in stable relationships (33.6%), while 17.8% were single and 5.7% were divorced. Participants were also asked to describe how many times they eat full meals per day (excluding snacking), to which almost half of the participants (48%) responded by saying they eat two full meals per day, whereas fewer (32.2%) reported three meals, 15.7% reported one meal, and 4.2% reported more than three meals per day. Their dietary behaviors varied as almost half of the participants (40.6%) reported being on some type of diet.

Table 1. Participant characteristics.

Characteristic	Category	n (%)
Gender	Woman	834 (76.4%)
	Man	250 (22.9%)
	Prefer not to say	7 (0.6%)
BMI (kg/m^2)	Mean ± SD	24.91 ± 4.95
Age (years)	Total/mean ± SD	33.06 ± 12.29
	Man/mean ± SD	33.29 ± 12.24
	Woman/mean ± SD	32.40 ± 12.52
Marital status	Single	187 (17.8%)
	Married	439 (41.8%)
	In a relationship	353 (33.6%)
	Divorced	60 (5.7%)
	Widowed	11 (1.0%)
Employment status	Student	217 (20.3%)
	Employed full-time	594 (55.7%)
	Employed part-time	96 (9.0%)
	Freelance	47 (4.4%)
	Unemployed	102 (9.6%)
	Other (shift work/parental leave)	11 (1.0%)
Household size	Living alone	140 (13.0%)
	2 persons	151 (14.0%)
	3–4 persons	708 (65.6%)
	≥5 persons	81 (7.4%)
Full meals per day	One	169 (15.7%)
	Two	516 (48.0%)
	Three	346 (32.2%)
	More than three	45 (4.2%)
Currently following a diet	Yes	442 (40.6%)
	No	648 (59.4%)

Considering the nature of ORTO-R where 1 = never and 5 = always, the mean calculated for the Lithuanian sample (M = 2.73; SD = 0.83) indicates moderate levels of orthorexic tendencies across our sample (see Table 2). In the absence of validated diagnostic cutoff scores for the ORTO-R, a distribution-based approach was applied. Participants scoring at or above the 85th percentile of the sample distribution (M ≥ 3.67) were descriptively categorized as exhibiting relatively elevated orthorexic tendencies. Based on this criterion, approximately 15% of the sample met this threshold exhibiting elevated orthorexic tendencies. Importantly, this percentage represents an estimation of individuals at higher risk for orthorexic behaviors based on the distribution of ORTO-R scores and should not be interpreted as a clinical diagnosis or a national prevalence estimate.

Table 2. ORTO-R mean.

Variable [1]	M	SD	Min	Max
ORTO-R Total Score	2.73	0.83	1.00	5.00

[1] Note. M = mean; SD = standard deviation. ORTO-R scores range from 1 (never) to 5 (always), with higher scores indicating greater orthorexic tendencies.

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3.2. Dietary Patterns

Principal component analysis of the Food Frequency Questionnaire was conducted to identify underlying dietary patterns. Sampling adequacy was confirmed (KMO = 0.86), and Bartlett's test of sphericity was significant ($\chi^2(136) = 4546.24$, $p < 0.001$). Three dietary components were retained based on eigenvalues greater than 1 and scree plot inspection, accounting for a cumulative value of 49.6% of the total variance. The extracted patterns were labeled Balanced-Traditional Diet, Protein-Rich Diet, and Processed-Dense Diet. Regression-based standardized factor scores were computed and retained for subsequent analyses. The *Balanced-Traditional Diet* was labeled based on high loadings of water, fresh fruits and vegetables, root vegetables, cereals, dairy products, meat, fish, and fats, reflecting a pattern consistent with culturally rooted, minimally processed, and nutritionally balanced eating practices and compliant with Lithuanian culinary practices. The *Protein-Rich Diet* was defined by strong loadings of fish and seafood, nuts and seeds, legumes, and eggs, indicating a dietary pattern centered on protein-dense foods commonly associated with health-oriented and structured eating behaviors. In contrast, the *Processed-Dense Diet* was characterized by high consumption of soft drinks and juices, savory snacks, alcoholic beverages, and sugar-rich foods, representing a pattern dominated by energy-dense, highly processed, and discretionary food items. Importantly, the PCA presented in Table 3 captures patterns of food consumption rather than prescriptive nutritional guidelines. The FFQ was constructed to assess the frequency of broad food groups without a priori classification based on macronutrient or micronutrient composition, allowing dietary patterns to emerge empirically from observed eating behaviors.

Table 3. Principal component analysis (PCA) of the Food Frequency Questionnaire (FFQ) items.

Food Group [2]	Balanced-Traditional	Protein-Rich	Processed-Dense
Pure water	0.79		
Fresh vegetables	0.69		
Fresh fruits	0/64		
Root vegetables	0.58		
Cereals and cereal products	0.49		
Milk and dairy products	0.60		
Meat and meat products	0.64		
Fats (vegetable and animal)	0.46		
Fish and seafood		0.76	
Nuts and seeds		0.68	
Legumes		0.65	
Eggs		0.44	
Soft drinks and juices			0.74
Savory snacks			0.72
Alcoholic beverages			0.65
Sugar and sweets			0.59

[2] Note. Principal component analysis with varimax rotation was conducted. Three components were retained based on eigenvalues > 1 and scree plot inspection, accounting for 49.6% of the total variance.

3.3. Correlations

To examine the correlations occurring between variables, Person's correlation analyses were conducted. As depicted in Table 4, Pearson's correlation analyses indicated a significant positive association between ORTO-R scores and both the Healthy-Traditional dietary pattern (r = 0.216, $p < 0.001$) and the Protein-Rich dietary pattern (r = 0.230, $p < 0.001$). No significant association was observed between ORTO-R scores and the Processed-Dense dietary pattern (r = −0.006, $p = 0.855$).

Table 4. Pearson correlations between ORTO-R scores and dietary patterns.

Variable [1]	1	2	3	4
ORTO-R Total score		0.216 ***	0.230 ***	−0.006
Balanced-Traditional Diet			0.196 ***	0.251 ***
Protein-Rich Diet				0.011
Processed-Dense Diet				

[1] Note. Values represent Pearson correlation coefficients. *** $p < 0.001$. N = 931 for correlations involving dietary patterns.

3.4. Stepwise Regression Analysis

Owing to the large set of exploratory predictors for the current study, a stepwise linear regression analysis was chosen to identify the dietary behavior and socio-demographic variables that were most strongly correlated with orthorexic tendencies. Such an analytic method allows for the identification of combinations that uniquely contribute to orthorexic tendencies in the absence of a theoretical framework [45]. Results are interpreted cautiously given known limitations of stepwise procedures, including sensitivity to variable entry order and potential model instability.

In the current study, the total ORTO-R score was calculated as the dependent variable, while predictors (including dietary behavior and socio-demographic variables) were automatically input stepwise. All assumptions of multiple regression were evaluated, including linearity, homoscedasticity, normality of residuals, multicollinearity and influential cases. Tolerance and VIF values were within acceptable limits, indicating no problematic multicollinearity.

In Model 1, the strongest single predictor was whether people followed a dietary plan (coded 1 = yes; 2 = no). This explained the largest proportion of explained variance. Model 2 introduced Healthy/Traditional dietary pattern scores followed by gender in Model 3 (1 = female; 2 = male). Models 4 and 5 introduced marital status and number of meals per day, and Model 6 added employment status. Once Model 7 introduced age (see Table 5), all variables were shown to significantly improve model fit, as indicated by the change in F values across steps. The final model explained 16.2% of the variance in orthorexic tendencies across the Lithuanian sample (adjusted $R^2 = 0.150$).

Table 5. Stepwise regression coefficients, ΔR^2 by step, and model fit indices.

Model	Predictors Entered	R	R^2	ΔR^2	Adj. R^2	SE	F Change	*p* (F Change)
1	Are you currently on a dietary plan?	0.215	0.046	0.046	0.044	0.8465	22.979	<0.001
2	+ Balanced-Traditional eating pattern (FFQ Factor 1)	0.305	0.093	0.047	0.089	0.8263	24.476	<0.001
3	+ Marital status	0.344	0.119	0.025	0.113	0.8155	13.572	<0.001
4	+ Gender	0.365	0.133	0.014	0.126	0.8096	7.993	0.005
5	+ Full meals per day	0.382	0.146	0.012	0.136	0.8046	6.804	0.009
6	+ Employment Status	0.391	0.153	0.008	0.142	0.8019	4.224	0.040
7	+ Age	0.403	0.162	0.009	0.150	0.7983	5.170	0.023

While the model explains a relatively small percentage of the variance, it still accounts for a meaningful proportion of the overall sample. Given the multifactorial nature of orthorexia and the cross-sectional design, a substantial amount of variance remains unaccounted for, likely reflecting psychological constructs (e.g., perfectionism, anxiety, rigidity, and health beliefs) not measured in this dataset. In the fully adjusted model, several variables were significantly associated with higher ON scores.

Following a dietary plan emerged as the strongest predictor of orthorexic tendencies in the final model (β = −0.211, $p < 0.001$), indicating that individuals actively adhering to a structured diet reported greater orthorexic tendencies; the negative coefficient reflects the reverse coding of this variable. Greater adherence to the Healthy-Traditional dietary pattern also predicted higher orthorexic tendencies (β = 0.167, $p < 0.001$). Gender was significant, with women scoring higher than men (β = −0.135, $p = 0.001$), reflecting the coding of the variable. Marital status was similarly associated with orthorexic tendencies (β = 0.130, $p = 0.001$), with partnered individuals reporting slightly higher scores than single participants. Eating a greater number of full meals per day predicted higher orthorexic tendencies (β = 0.131, $p = 0.009$), as did being employed (β = 0.122, $p = 0.008$). Age showed a negative association (β = −0.103, $p = 0.023$), indicating that younger participants exhibited higher orthorexic tendencies. While several predictors reached statistical significance, effect sizes were in the small-to-moderate range (see Table 6). Given the cross-sectional design, these findings reflect associations rather than causal or temporal relationships.

Table 6. Stepwise linear regression models predicting orthorexic tendencies.

Predictor	B [1]	SE	β	*p*
Are you currently on a dietary plan?	−0.366	0.078	−0.211	<0.044
Balanced-Traditional Diet (FFQ Factor 1)	0.151	0.040	0.167	<0.001
Marital status	0.133	0.044	0.130	0.001
Gender	−0.268	0.086	−0.135	0.001
Full meals per day	0.131	0.050	0.131	0.009
Employment status	0.096	0.036	0.122	0.008
Age	−0.007	0.003	−0.103	0.023

[1] Note. B = unstandardized coefficient; SE = standard error; β = standardized coefficient. ORTO-R = orthorexia nervosa tendencies score. N = 931.

3.5. Summary of Findings

Overall, participants exhibit moderate levels of orthorexic tendencies, and three dietary patterns are identified (Balanced-Traditional, Protein-Rich, and Processed-Dense). These three dietary patterns accounted for over half of the variance within the given sample. In addition, correlates emerge, as those adhering to the Balanced-Traditional and Protein-Rich dietary patterns tend to have higher orthorexic tendencies than those adhering to the Processed-Dense dietary pattern. Following that, the stepwise regression analysis indicates that orthorexic tendencies were associated and predicted by individuals' adherence to a dietary plan close to the Balanced-Traditional dietary pattern. Women, individuals in relationships, employed participants, younger participants and those consuming more meals per day reported higher orthorexic tendencies. While the nature of this study is exploratory, these findings provide key indications regarding dietary behavior and eating patterns that correlate with higher orthorexic tendencies in the general population in Lithuania.

4. Discussion

This study aimed to examine the current dietary landscape and tendencies for ON in a general population sample in Lithuania through a cross-sectional design. Combining tools such as the FFQ and ORTO-R and the analyses conducted within the study make this the first of its kind within the Lithuanian context. In this exploratory study, the use of PCA, Pearson's correlation and stepwise linear regression analyses enabled us to define current dietary patterns in Lithuania, assess ON prevalence and correlates, and identify dietary patterns most significantly associated with ON.

The present findings demonstrate significant associations between dietary behaviors, dietary patterns, and selected socio-demographic characteristics in relation to orthorexic tendencies. This study represents the first population-based investigation in Lithuania to assess both the prevalence of orthorexic tendencies and food frequency-derived dietary patterns in a general adult sample. Behavioral variables reflecting structured eating practices, including adherence to a dietary plan, consumption of a greater number of full meals per day, and employment status, were associated with higher orthorexic tendency scores. In addition, orthorexic tendencies were positively associated with health-oriented dietary patterns, specifically the Balanced-Traditional and Protein-Rich patterns, whereas no association was observed with the Processed-Dense dietary pattern. Altogether, these findings indicate that orthorexic tendencies in this Lithuanian sample were more closely related to structured, normatively valued eating behaviors and health-focused dietary choices rather than to patterns characterized by higher consumption of processed foods. Furthermore, two of the dietary patterns of this study's FFQ, namely Balanced-Traditional and Protein-Rich diets, portray an array of food groups that are socially acceptable and perceived as healthy in comparison to the Processed-Dense food groups. Additionally, these food groups may be preferentially chosen by individuals with higher orthorexic tendencies due to their perceived healthfulness. Still, this study raises the question of whether some dietary patterns signal broader eating psychopathology, while others may be more characteristic of orthorexic tendencies. This ties into existing evidence which shows that ON is closely related to higher health consciousness, dietary control and socially reinforced norms as healthy and "clean" as well as functional and "optimal" eating rather than pathological eating alone [46,47]. Furthermore, previous research also highlights that a vegan diet did not directly result in disordered eating, but the prevalence of ON was higher in vegans and vegetarians in comparison to people consuming meat [48]. Vegetarian or vegan diets could increase the risk of ON and individuals' perception of "healthy" eating promoting thinness and weight loss, with the idea that ON encounters motivation associated with weight and/or body shape [49,50]. A similar study in student populations in Poland combined the FFQ and ORTO-15, revealing that students reluctantly eating meat products were less likely to score high on ON tendencies [51]. While the current study found that Protein-Rich diets among Lithuanians scored higher on the ORTO-R, this indicates that consuming protein-rich foods such as meat may reveal a broader relationship with higher ON tendencies. Protein consumption has recently grown in popularity among the broader population, and specifically among athletes, as media promotes protein as a muscle builder while also promoting muscularity as an ideal bodily trait [52,53]. This finding connects to several studies related to ON which highlight that CrossFit, gym-goers, and athletes generally exhibit higher orthorexic tendencies [22,54,55]. Nevertheless, a deeper investigation of the interaction of gender identity, sex, and type of exercise/physical activity alongside chronotypes/sleep quality and other symptomatology is warranted.

The gender-specific orientation of ON is further confirmed as the current study also revealed a higher prevalence of ON among women, although this may be, in part, due to the imbalance of the sample distribution. Findings of one study highlighted a higher prevalence of ON symptoms among women, and the association between ON and a specific interest toward dietary choices [55]. A multitude of studies on ON across cultural contexts show that women are more likely to develop ON, with only few studies indicating this trend in men [24,25,54,55]. Other studies also argue that ON may show up differently in men than in women, with men prioritizing protein consumption over healthy eating with a link to muscularity ideation [52,55]. However, the current study did not examine exercise behavior, muscularity ideation or men under the ON lens. Furthermore, being younger in this study was associated with higher tendencies for ON. One study even stated that younger participants admitted having increased habits of healthy eating that became

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pathological and resulted in malnutrition [55]. Several other studies revealed a decline in orthorexic tendencies as age increased in their samples, attributing more rigid dietary patterns to younger populations [24,56]. The current study confirms this string of findings for the context of Lithuania. Yet some of the socio-demographics considered in this study remain underexplored in the literature. While our findings indicate that being in a relationship and being employed can be significant correlates and predictors of ON, other studies seldom consider employment and relationship status to be significant [56,57]. Being employed may be connected to higher ON scoring since full-time employment may enforce routines and healthy lifestyles for people [57]. Similarly, being in a relationship may be connected to partners sharing the same meals and, therefore, the same dietary patterns or beliefs about eating clean [24]. However, these variables remain to be explored by further research across cultural contexts and interrelation behaviors.

Importantly, these results reinforce the conceptualization of ON as a spectrum of disordered eating behaviors that overlap with, but remain distinct from, other eating disorders. The theoretical implications of this study focus on the alignment with previous findings that inadequate health-oriented eating may evolve into maladaptive eating patterns causing orthorexic risks. This study strengthens the conceptualization of ON as a part of a broader disordered eating background overlapping with other eating disorders but staying different at the same time. The study reveals that these eating patterns in Lithuania highlight the importance of cultural environment and regional dietary habits in orthorexia nervosa research, focusing on potential cross-cultural aspects of eating behavior and its triggers.

4.1. Implications

The findings of this study carry several practical and public health implications. First, the higher prevalence of orthorexic behavior among younger women and individuals with highly structured diets show the demand of targeted health education promoting balanced, rich and flexible eating without any assessment of food choices. Second, the study findings also presuppose the need for health professional and educator training to recognize the early stages of orthorexia nervosa risks, especially in populations that follow strict healthy diets and fall into the categories of younger age, women, being in a committed relationship, and being employed. Third, the study results demonstrate the reason for creating culturally based prevention diets in Lithuania as there is an increased interest in diet trends and wellness culture popularity. Fourth, the research may also propose various awareness campaigns and mindfulness development events in schools, universities, fitness centers or other environments where the risk of orthorexia nervosa prevalence tends to run high.

4.2. Limitations and Future Research

The research limitations include the methodological background (quantitative research), geographical location (Lithuania), and the research sample (which does not fully represent separate demographic groups such as older adults, teenagers, certain profession representatives, etc.). The practical application of orthorexia nervosa is also limited as this concept is too complex, clinically severe and remains non-clinically standardized. All the aspects discussed above make up a foundation for further research. Apart from application, several methodological limitations must be acknowledged. Participant recruitment via online dissemination resulted in a non-probabilistic sample with a marked overrepresentation of women, which may have influenced the observed associations, particularly given known gender differences in health-focused eating behaviors and orthorexic tendencies. In addition, the questionnaire was only administered digitally, which may have unintentionally excluded potential respondents owing to a lower level of digital literacy. Dietary patterns were derived from a FFQ that assessed consumption frequency but did not capture portion sizes, restricting the ability to

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estimate absolute intake or total dietary load. Additionally, a substantial proportion of participants reported currently following a dietary plan, reflecting contemporary dietary norms; however, the study design does not and cannot draw conclusions regarding whether dieting precedes, results from, or co-occurs with orthorexic tendencies. Consequently, ON is not a clinically standardized diagnosis, and although the ORTO-R shows improved psychometric performance compared to earlier instruments, the findings should be interpreted as reflecting orthorexic tendencies rather than clinical prevalence. Finally, the current study is missing several key socio-demographic factors and psychological symptoms that have been explored in concurrent research, including education level, social media behavior/use, perfectionism, and anxiety and depressive symptoms.

This study revealed the importance of exploring ON developmental trends and its potential transition into other eating disorders in the long term (longitudinal research). Future research would benefit more from some psychosocial correlations being highlighted (such as anxiety, perfectionism, social media influence, etc.). This will help researchers to project a more holistic profile of orthorexia nervosa risks. Additionally, seeking a deeper approach, such as qualitative or mixed-method study design, would help researchers reveal the lived experiences of individuals with orthorexic tendencies and would provide deeper insights into the phenomenon. Cross-cultural (cross-national) studies would contribute to determining whether the eating patterns observed in Lithuania reflect broader global trends (including various additional demographics) and may reveal how clearly orthorexia nervosa can be shaped by sociocultural factors.

5. Conclusions

This study provides population-wide insights into orthorexic tendencies and dietary patterns among Lithuanian adults across socio-demographic variables. Although ON is not formally recognized as a clinical diagnosis, it is increasingly examined in research as a pattern of maladaptive health-oriented eating behaviors. Accordingly, the present study focused on orthorexic tendencies rather than clinical diagnosis, using the ORTO-R as a validated instrument to assess behavioral patterns related to excessive preoccupation with healthy eating. As such, the findings reflect exploratory efforts of understanding dietary patterns and how they may relate to the specific behaviors connected to ON. The authors do not claim ON as a diagnosis, but rather as a pattern of behavior that may become maladaptive and dysfunctional, advising caution regarding Lithuanian dietitians and healthcare workers' practices.

Furthermore, the findings link to a series of pan-European research efforts demonstrating the clinical and statistical prevalence of ON. Current findings indicate that orthorexic tendencies are more prevalent among women, young adults, employed individuals, and partnered individuals that are prone to adhering to dietary plans, such as the Balanced-Traditional and Protein-Rich dietary patterns identified in this study. The lack of an association between the Processed-Dense dietary pattern and orthorexic tendencies further demonstrates how ON fits into broader health-oriented eating behaviors. Overall, the results underscore the importance of examining cultural and contextual factors when examining orthorexic tendencies, as well as conducting further research on profiling individuals prone to ON while initiating campaigns and programs that increase awareness around clean, sustainable and flexible eating.

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Abbreviations

The following abbreviations are used in this manuscript:

ON	Orthorexia Nervosa
ORTO-R	ORTO-15 Revised
FFQ	Food Frequency Questionnaire
AN	Anorexia Nervosa
WHO	World Health Organization
PCA	Principal Component Analysis
ED	Eating Disorders
APA	American Psychiatry Association
DSM	Diagnostic Statistical Manual
UPF	Ultra-Processed Food

References

1. Donini, L.M.; Marsili, D.; Graziani, M.P.; Imbriale, M.; Cannella, C. Orthorexia nervosa: A preliminary study with a proposal for diagnosis and an attempt to measure the dimension of the phenomenon. *Eat. Weight Disord.* **2004**, *9*, 151–157. [CrossRef]
2. Donini, L.M.; Barrada, J.R.; Baenas, I.; Barthels, F.; Dunn, T.; Moroze, R.; Roncero, M.; Sepúlveda, A.R.; Solmi, F.; Tóth-Király, I.; et al. A consensus document on definition and diagnostic criteria for orthorexia nervosa. *Eat. Weight Disord.* **2022**, *27*, 3695–3715. [CrossRef]
3. Lecaj, R. Social Media and Its Impact on Orthorexia Nervosa. A Systematic Literature Review. In *Pervasive Computing Technologies for Healthcare*; Springer: Cham, Switzerland, 2025; pp. 215–219. [CrossRef]
4. López-Gil, J.F.; Ruiz-González, L.; García-Hermoso, A.; Smith, L.; Firth, J.; Trott, M.; Jiménez-López, E.; Fagherazzi, G. Overall proportion of orthorexia nervosa symptoms: A systematic review and meta-analysis including 30,476 individuals from 18 countries. *J. Glob. Health* **2023**, *13*, 04087. [CrossRef]
5. Robinson, P.; Wade, T.; Herpertz-Dahlmann, B.; Fernandez-Aranda, F.; Treasure, J.; Wonderlich, S. (Eds.) *Eating Disorders: An International Comprehensive View*; Springer Nature: Cham, Switzerland, 2024. [CrossRef]
6. Petrauskiene, A.; Daugelaite, S.; Salomskiene, A.; Speckauskiene, V. What Lithuanian first-graders eat: Results of a 15-year semi-longitudinal, cross-sectional surveillance study. *Nutrients* **2024**, *16*, 1970. [CrossRef]
7. Kriaucioniene, V.; Raskiliene, A.; Petrauskas, D.; Petkeviciene, J. Trends in eating habits and body weight status, perception patterns and management practices among first-year students of Kaunas (Lithuania) universities, 2000–2017. *Nutrients* **2021**, *13*, 1599. [CrossRef] [PubMed]
8. Dubosaitė, U. Lithuanian II and III-Year Medical Students' Eating Behaviour and Related Factors: Stress and Parental Nurturance. Master's Thesis, Lithuanian University of Health Sciences, Kaunas, Lithuania, 2024. Available online: https://portalcris.lsmuni.lt/server/api/core/bitstreams/ea43619f-5e2f-49f6-b30b-1035d9169441/content (accessed on 12 January 2026).
9. Mieziene, B.; Burkaite, G.; Emeljanovas, A.; Tilindiene, I.; Novak, D.; Kawachi, I. Adherence to Mediterranean diet among Lithuanian and Croatian students during COVID-19 pandemic and its health behavior correlates. *Front. Public Health* **2022**, *10*, 1000161. [CrossRef]
10. Danyliuk, I.; Kuchyna, V. Ethnocultural studies of orthorexia nervosa. *Sci. J. Pol. Univ.* **2024**, *63*, 129–136. [CrossRef] [PubMed]

11. Bratman, S. Health Food Junkie: Obsession with Dietary Perfection Can Sometimes Do More Harm Than Good, Says One Who Has Been There. *Yoga J.* **1997**, *136*, 42–46.
12. Crawford, R. Healthism and the medicalization of everyday life. *Int. J. Health Serv.* **1980**, *10*, 365–388. [CrossRef] [PubMed]
13. Håman, L.; Barker-Ruchti, N.; Patriksson, G.; Lindgren, E.-C. Orthorexia nervosa: An integrative literature review of a lifestyle syndrome. *Int. J. Qual. Stud. Health Well-Being* **2015**, *10*, 26799. [CrossRef]
14. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*, 5th ed.; American Psychiatric Association: Washington, DC, USA, 2022.
15. Niedzielski, A.; Kaźmierczak-Wojtaś, N. Prevalence of orthorexia nervosa and its diagnostic tools-A literature review. *Int. J. Environ. Res. Public Health* **2021**, *18*, 5488. [CrossRef]
16. Yöyen, E.; Dereli, Ş.; Barış, T.G. Predictors of orthorexia nervosa in women: Eating attitudes, emotional regulation difficulties, anxiety, depression and self-esteem. *BMC Psychol.* **2025**, *13*, 554. [CrossRef]
17. Guidotti, S.; Fiduccia, A.; Sanseverino, R.; Pruneti, C. Multidimensional assessment of orthorexia nervosa: A case-control study comparing eating behavior, adherence to the mediterranean diet, body mass index, psychological symptoms, and autonomic arousal. *Nutrients* **2025**, *17*, 317. [CrossRef]
18. Obeid, S.; Hallit, S.; Akel, M.; Brytek-Matera, A. Orthorexia nervosa and its association with alexithymia, emotion dysregulation and disordered eating attitudes among Lebanese adults. *Eat. Weight Disord.* **2021**, *26*, 2607–2616. [CrossRef] [PubMed]
19. Gerges, S.; Azzi, V.; Bianchi, D.; Laghi, F.; Pompili, S.; Malaeb, D.; Obeid, S.; Soufia, M.; Hallit, S. Exploring the relationship between dysfunctional metacognitive processes and orthorexia nervosa: The moderating role of emotion regulation strategies. *BMC Psychiatry* **2023**, *23*, 674. [CrossRef]
20. Palmieri, S.; Mansueto, G.; Ruggiero, G.M.; Caselli, G.; Sassaroli, S.; Spada, M.M. Metacognitive beliefs across eating disorders and eating behaviours: A systematic review. *Clin. Psychol. Psychother.* **2021**, *28*, 1254–1265. [CrossRef]
21. Zohar, A.H.; Zamir, M.; Lev-Ari, L.; Bachner-Melman, R. Too healthy for their own good: Orthorexia nervosa and compulsive exercise in the community. *Eat. Weight Disord.* **2023**, *28*, 55. [CrossRef] [PubMed]
22. Mavrandrea, P.; Gonidakis, F. Exercise dependence and orthorexia nervosa in Crossfit: Exploring the role of perfectionism. *Curr. Psychol.* **2023**, *42*, 25151–25159. [CrossRef]
23. Rudolph, S. The connection between exercise addiction and orthorexia nervosa in German fitness sports. *Eat. Weight Disord.* **2018**, *23*, 581–586. [CrossRef] [PubMed]
24. Oberle, C.D.; Samaghabadi, R.O.; Hughes, E.M. Orthorexia nervosa: Assessment and correlates with gender, BMI, and personality. *Appetite* **2017**, *108*, 303–310. [CrossRef]
25. Vintró-Alcaraz, C.; Ballero Reque, C.; Paslakis, G.; Testa, G. Idealized body images and fitness lifestyles on social media: A systematic review exploring the link between social media use and symptoms of orthorexia nervosa and muscle dysmorphia. *Eur. Eat. Disord. Rev.* **2025**, *34*, 256–280. [CrossRef]
26. Usta Ulutaş, P.; Okan Bakır, B. The type of social media is a greater influential factor for orthorexia nervosa than the duration: A cross-sectional study. *BMC Public Health* **2025**, *25*, 827. [CrossRef] [PubMed]
27. Noebel, N.A.; Howard, K.J.; Oberle, C.D. Social media, diet and fitness content, and orthorexia nervosa: A pilot investigation of viewership and orthorexic behaviors. *J. Soc. Media Soc.* **2024**, *13*, 90–108. Available online: https://thejsms.org/index.php/JSMS/article/view/1357 (accessed on 14 November 2025).
28. Turner, P.G.; Lefevre, C.E. Instagram use is linked to increased symptoms of orthorexia nervosa. *Eat. Weight Disord.* **2017**, *22*, 277–284. [CrossRef] [PubMed]
29. Opitz, M.-C.; Newman, E.; Alvarado Vázquez Mellado, A.S.; Robertson, M.D.A.; Sharpe, H. The psychometric properties of orthorexia nervosa assessment scales: A systematic review and reliability generalization. *Appetite* **2020**, *155*, 104797. [CrossRef]
30. World Health Organization Regional Office for Europe. *European Food and Nutrition Action Plan 2015–2020*; WHO: Copenhagen, Denmark, 2015.
31. Mertens, E.; Colizzi, C.; Peñalvo, J.L. Ultra-processed food consumption in adults across Europe. *Eur. J. Nutr.* **2022**, *61*, 1521–1539. [CrossRef]
32. Toujgani, H.; Berlivet, J.; Berthy, F.; Allès, B.; Brunin, J.; Fouillet, H.; Touvier, M.; Lairon, D.; Mariotti, F.; Baudry, J.; et al. Dietary pattern trajectories in French adults of the NutriNet-Santé cohort over time (2014–2022): Role of socio-economic factors. *Br. J. Nutr.* **2024**, *132*, 1184–1193. [CrossRef]
33. Thieleking, R.; Schneidewind, L.; Kanyamibwa, A.; Hartmann, H.; Horstmann, A.; Witte, A.V.; Medawar, E. Nutrient scoring for the DEGS1-FFQ—From food intake to nutrient intake. *BMC Nutr.* **2023**, *9*, 12. [CrossRef]
34. Wennberg, M.; Kastenbom, L.; Eriksson, L.; Winkvist, A.; Johansson, I. Validation of a digital food frequency questionnaire for the Northern Sweden Diet Database. *Nutr. J.* **2024**, *23*, 83. [CrossRef]
35. Cardamone, E.; Iacoponi, F.; Fiori, F.; Marinoni, M.; Agrimi, U.; Silano, M.; Parpinel, M. The development of a food frequency questionnaire for the assessment of ultra-processed food consumption in the Italian adult population: Protocol for a validity and reproducibility study. *Nutrients* **2024**, *16*, 3896. [CrossRef]

36. Kowalkowska, J.; Slowinska, M.A.; Slowinski, D.; Dlugosz, A.; Niedzwiedzka, E.; Wadolowska, L. Comparison of a Full Food-Frequency Questionnaire with the Three-Day Unweighted Food Records in Young Polish Adult Women: Implications for Dietary Assessment. *Nutrients* **2013**, *5*, 2747–2776. [CrossRef]
37. Klink, U.; Intemann, T.; Bogl, L.H.; Lissner, L.; Gwozdz, W.; De Henauw, S.; Molnár, D.; Mazur, A.; Moreno, L.A.; Pala, V.; et al. Consumer attitudes towards dietary behaviors: A mediator between socioeconomic status and diet quality in European adults. *Eur. J. Nutr.* **2025**, *64*, 127. [CrossRef]
38. Kriaučionienė, V.; Petkevičienė, J.; Klumbienė, J. Dietary patterns and their association with sociodemographic factors in Lithuanian adult population. *Medicina* **2008**, *44*, 799. [CrossRef]
39. Lemming, E.W.; Pitsi, T. The Nordic Nutrition Recommendations 2022—Food Consumption and Nutrient Intake in the Adult Population of the Nordic and Baltic Countries. *Food Nutr. Res.* **2022**, *66*, 8572. [CrossRef]
40. Bulotaitė, G.; Bartkevičiūtė, R.; Barzda, A.; Stukas, R. Intakes of energy, macronutrients, and micronutrients in adult Lithuanian population: A national study of 2019–2020. *J. Nutr. Sci.* **2024**, *13*, e46. [CrossRef] [PubMed]
41. Petkeviciene, J.; Smalinskiene, A.; Luksiene, D.I.; Jureniene, K.; Ramazauskiene, V.; Klumbiene, J.; Lesauskaite, V. Associations between apolipoprotein e genotype, diet, body mass index, and serum lipids in Lithuanian adult population. *PLoS ONE* **2012**, *7*, e41525. [CrossRef] [PubMed]
42. Arlauskas, R.; Austys, D.; Dobrovolskij, V.; Stukas, R. Consumption of dietary supplements among working-age residents of Lithuania in the period from 2021 to 2023. *Medicina* **2024**, *60*, 669. [CrossRef] [PubMed]
43. Rogoza, R.; Donini, L.M. Introducing ORTO-R: A revision of ORTO-15: Based on the re-assessment of original data. *Eat. Weight Disord.* **2021**, *26*, 887–895. [CrossRef]
44. Lamm, K.W.; Lamm, A.J.; Edgar, D.W. Scale development and validation: Methodology and recommendations. *J. Int. Agric. Ext. Educ.* **2020**, *27*, 24–35. [CrossRef]
45. Tabachnick, B.G.; Fidell, L.S. *Using Multivariate Statistics*, 7th ed.; Pearson: Boston, MA, USA, 2019.
46. Dunn, T.M.; Bratman, S. On orthorexia nervosa: A review of the literature and proposed diagnostic criteria. *Eat. Behav.* **2016**, *21*, 11–17. [CrossRef]
47. Pontillo, M.; Zanna, V.; Demaria, F.; Averna, R.; Di Vincenzo, C.; De Biase, M.; Di Luzio, M.; Foti, B.; Tata, M.C.; Vicari, S. Orthorexia nervosa, eating disorders, and obsessive-compulsive disorder: A selective review of the last seven years. *J. Clin. Med.* **2022**, *11*, 6134. [CrossRef]
48. Brytek-Matera, A.; Czepczor-Bernat, K.; Jurzak, H.; Kornacka, M.; Kołodziejczyk, N. Strict health-oriented eating patterns (orthorexic eating behaviours) and their connection with a vegetarian and vegan diet. *Eat. Weight Disord.* **2019**, *24*, 441–452. [CrossRef]
49. Atchison, A.E.; Zickgraf, H.F. Orthorexia nervosa and eating disorder behaviors: A systematic review of the literature. *Appetite* **2022**, *177*, 106134. [CrossRef]
50. Hafstad, S.M.; Bauer, J.; Harris, A.; Pallesen, S. The Prevalence of Orthorexia in Exercising Populations: A Systematic Review and Meta-Analysis. *J. Eat. Disord.* **2023**, *11*, 15. [CrossRef] [PubMed]
51. Plichta, M.; Jezewska-Zychowicz, M. Orthorexic Tendency and Eating Disorders Symptoms in Polish Students: Examining Differences in Eating Behaviors. *Nutrients* **2020**, *12*, 218. [CrossRef] [PubMed]
52. Nagata, J.M.; Murray, S.B.; Bibbins-Domingo, K.; Garber, A.K.; Mitchison, D.; Griffiths, S. Predictors of muscularity-oriented disordered eating behaviors in U.S. young adults: A prospective cohort study. *Int. J. Eat. Disord.* **2019**, *52*, 1380–1388. [CrossRef]
53. Daas, M.C.; Van 't Veer, P.; Temme, E.H.M.; Kuijsten, A.; Gurinović, M.; Biesbroek, S. Diversity of dietary protein patterns across Europe—Impact on nutritional quality and environmental sustainability. *Curr. Res. Food Sci.* **2025**, *10*, 101019. [CrossRef] [PubMed]
54. Surała, O.; Malczewska-Lenczowska, J.; Sadowska, D.; Grabowska, I.; Białecka-Dębek, A. Traits of Orthorexia Nervosa and the Determinants of These Behaviors in Elite Athletes. *Nutrients* **2020**, *12*, 2683. [CrossRef]
55. Brytek-Matera, A.; Donini, L.M.; Krupa, M.; Poggiogalle, E.; Hay, P. Orthorexia nervosa and self-attitudinal aspects of body image in female and male university students. *J. Eat. Disord.* **2015**, *3*, 2. [CrossRef]
56. Łucka, I.; Mazur, A.; Łucka, A.; Trojniak, J.; Kopańska, M. Orthorexia Nervosa Tendencies in Two Cohorts of Polish Young Adults: A Comparative Analysis of Prevalence, Correlates, and Comorbidity. *Nutrients* **2025**, *17*, 2208. [CrossRef]
57. Dell'Osso, L.; Cremone, I.M.; Chiarantini, I.; Arone, A.; Casagrande, D.; Massimetti, G.; Carmassi, C.; Carpita, B. Investigating orthorexia nervosa with the ORTO-R in a sample of university students with or without subthreshold autism spectrum: Focus on dietary habits and gender differences. *Front. Psychiatry* **2022**, *13*, 900880. [CrossRef] [PubMed]

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Article

Octenyl Succinic Anhydride Starch Alleviates Alcoholic Liver Disease by Modulating Gut Microbiota and Metabolism

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Abstract

Background/Objectives: Alcoholic liver disease (ALD) is intricately linked to gut microbiota dysbiosis and metabolic disturbances along the gut–liver axis. Octenyl succinic anhydride (OSA) starch escapes digestion in the small intestine and ferments in the colon, modulating gut microbiota and metabolism. This study explored the protective effects of OSA starch against ALD and elucidated the underlying gut microbiota–metabolite interactions. **Methods:** A chronic ethanol-fed mouse model was conducted to evaluate the protective effects of OSA starch against ALD, and multi-omics analyses integrating 16S rRNA sequencing, PICRUSt2 functional predictions, and metabolomics were used to reveal potential mechanism. **Results**: OSA starch supplementation in ALD mice significantly reduced liver fat accumulation, lowered the liver index to 4.11%, and restored serum transaminase levels closer to normal. Multi-omics analyses revealed that OSA starch enriched beneficial gut bacteria such as *Faecalibaculum rodentium* and *Bifidobacterium adolescentis*. OSA starch also enhanced microbial metabolic functions, including pyruvate, butanoate, and propanoate metabolism. These shifts were accompanied by regulation of fecal and serum metabolites, including pyruvate, 2-hydroxybutanoic acid, and lactic acid. Structural equation modeling further confirmed that OSA starch ameliorates ALD via coordinated modulation of gut microbiota, microbial functions, metabolites, and serum markers. **Conclusions**: OSA starch protects against alcoholic liver injury by remodeling the gut–liver metabolic network, presenting a promising dietary strategy for ALD.

Keywords: OSA starch; alcoholic liver disease; gut microbiota; host metabolism

1. Introduction

Alcoholic liver disease (ALD) is a significant contributor to chronic liver injury and mortality worldwide, predominantly driven by prolonged excessive alcohol consumption [1]. The progression of ALD involves hepatic steatosis, underpinned by complex interactions between the liver and the gut [2,3]. Growing evidence highlights the gut–liver axis as a central player in ALD pathogenesis, particularly through gut microbiota dysbiosis and increased intestinal permeability [4]. These alterations facilitate the translocation of various microbial metabolites into the portal circulation, promoting hepatic steatosis and metabolic disruption. Moreover, shifts in gut-derived metabolites—such as short-chain fatty acids, amino acid derivatives, and bile acids—profoundly influence hepatic lipid metabolism, oxidative stress, and immune responses, all of which contribute to ALD

progression [5]. ALD is associated with gut bacterial products and their translocation to the liver, although the role of specific microbiota changes remains unclear [6]. Therefore, nutritional interventions targeting the microbiome have emerged as a promising strategy for ALD prevention and treatment [7,8].

Among nutritional interventions, resistant starches have emerged as promising prebiotic agents capable of reshaping gut microbial communities and their metabolic outputs [9,10]. Octenyl succinic anhydride (OSA) starch, as a modified resistant starch, escapes digestion in the small intestine and undergoes fermentation in the colon by commensal bacteria, leading to the production of short-chain fatty acids like butyrate, propionate, and acetate [11,12]. These microbial metabolites play essential roles in enhancing gut barrier integrity and regulating lipid and glucose metabolism. Additionally, resistant starch intake has been linked to favorable shifts in microbial functional pathways, such as those involved in energy metabolism, amino acid synthesis, and bile acid transformation, which collectively support host metabolic health [13,14]. OSA is a commonly used esterification agent that modifies starches, making the derivative amphiphilic and capable of acting as an emulsion stabilizer [15]. Approved as a food additive, OSA-modified starch has undergone rapid global consumption due to its beneficial properties, including reducing fat absorption in the gut by encapsulating fat particles [16]. Given that OSA starch has also been proven to have a highly similar ability to regulate the microbiota and SCFAs, this type of resistant starch may have the potential to prevent ALD. While resistant starch has shown efficacy in improving metabolic diseases like obesity and non-alcoholic fatty liver disease [17,18], OSA starch's potential to mitigate alcohol-induced liver injury through modulation of gut microbiota and microbial metabolism remains underexplored.

In this study, the protective effects of OSA starch against ALD were systematically evaluated using a chronic alcohol-fed mouse model. Comprehensive assessments, including liver histopathology, serum biochemistry, fecal and serum metabolomics, and gut microbiota profiling via 16S rRNA gene sequencing, were conducted. Functional predictions and pathway analyses were further employed to elucidate microbial metabolic functions influenced by OSA starch. Structural equation modeling was applied to clarify the mechanistic pathways linking OSA starch, gut microbiota, microbial metabolites, and liver health. These findings underscore the potential of OSA starch as a dietary strategy for ALD.

2. Materials and Methods

2.1. Establishment of Alcoholic Liver Injury Model and Dietary OSA Starch Intervention

A total of 24 male C57BL/6J mice aged eight weeks were purchased from Gem Pharmatech Co., Ltd. (Nanjing, China) and maintained under specific pathogen-free conditions with controlled environment parameters. All animal procedures received ethical approval from the Animal Ethics Committee of Jiangnan University (JN. No 20240930c0641123), following the ARRIVE guidelines for animal research. Mice were randomly assigned to one of three experimental groups (n = 8 per group) using a random number generator to ensure unbiased group allocation: Control, Model, and OSA starch intervention groups. Both the Control and ALD Model groups were fed a standard chow containing native corn starch as the main carbohydrate source. The OSA starch group was provided with a diet where native starch was fully replaced by OSA-modified starch. The dietary formulations followed established protocols from a previous study [19].

To induce alcoholic liver damage, mice in the Model and OSA starch groups received daily oral gavage of ethanol. For the initial 27 days, 42% (v/v) ethanol was administered, followed by a single dose of 50% (v/v) ethanol on day 28. Ethanol dosing began at 0.1 mL per 10 g body weight in the first week, and was progressively increased to 0.15 mL/10 g

from the third week onward. Control group mice were gavaged with an equivalent volume of sterile water throughout the experiment.

2.2. Serum Biochemical Assays

Following an overnight fast, mice were anesthetized via intraperitoneal injection of 2–3% isoflurane. Blood was drawn, and liver tissues were promptly collected for further analysis. Serum was isolated by centrifugation at 3000× *g* for 10 min at 4 °C. Key biochemical markers, including total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), were quantified using a BS-350E automated biochemical analyzer (Myriad Biomedical, Shenzhen, China).

2.3. Liver Histology via Hematoxylin and Eosin Staining

Liver specimens were fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated through a graded ethanol series, cleared with xylene, and embedded in paraffin. Paraffin blocks were sliced into 4 μm sections using a rotary microtome (Leica, IL, USA). The tissue sections were then stained with hematoxylin and eosin (H&E) and examined microscopically for histopathological evaluation.

2.4. Untargeted Metabolomics Profiling

Metabolic profiling was conducted using a QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA) connected to an ultra-high-performance liquid chromatography (UHPLC) system. Both positive and negative ionization modes of electrospray ionization (ESI) were employed. For extraction, 50 μL/mg of each sample was combined with 400 μL of pre-cooled methanol containing internal standards. Fecal samples were processed with 2–3 stainless steel beads and homogenized using a tissue grinder (60 Hz, 15 s per cycle, 3–4 cycles) under chilled conditions. Samples were then subjected to 15 min of sonication in an ice-water bath and centrifuged at 10,000× *g* for 20 min at 4 °C.

Approximately 300 μL of the supernatant was collected, evaporated to dryness under vacuum at low temperatures, and reconstituted in 100 μL of 20% methanol. The mixture was vortexed, sonicated, and centrifuged again before being transferred to autosampler vials for UHPLC–MS/MS analysis. Chromatographic separation was carried out using a BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters, Milford, MA, USA) with 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B for positive mode. For negative ion mode, an HSS T3 column (1.8 μm, 2.1 × 100 mm, Waters, Milford, MA, USA) was utilized. The column temperature was maintained at 50 °C. Data acquisition and initial analysis were performed using Analyst software 1.7.2 (AB SCIEX, Framingham, MA, USA). The identity of the metabolites was confirmed based on the MS/MS spectra, comparing the observed fragmentation patterns with available reference spectra in the database. This included matching retention times and the specific mass-to-charge values, as well as interpreting the fragmentation pathways for structural confirmation. Additionally, the metabolite analysis was further validated by comparing the results with data available on MetaboAnalyst 5.0, which provides comprehensive information on known metabolites and their fragmentation profiles [20].

2.5. 16S rRNA Gene Sequencing and Microbial Functional Prediction

Genomic DNA from fecal samples was extracted with the TIANamp Stool DNA Kit (DP328, TIANGEN, Beijing, China), following the manufacturer's instructions. The quality and quantity of DNA were assessed via a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis. The V3–V4 regions of the bacterial 16S rRNA gene were amplified using specific primers with Illumina adapters.

Purified PCR products were quantified and pooled in equimolar amounts. Libraries were prepared using the TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego CA, USA), and sequencing was conducted on the Illumina NovaSeq 6000 platform (paired-end, 2 × 250 bp).

Raw sequences underwent quality filtering with Trimmomatic to remove adapters and low-quality reads, and merged using FLASH. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity via USEARCH. Taxonomic classification was performed against the SILVA database (version 138). Microbial functional potential was predicted using PICRUSt2 to infer KEGG Orthologs (KOs) based on 16S rRNA profiles. All sequencing and computational analyses were completed by Wekemo Tech Group Co., Ltd. (Shenzhen, China).

2.6. Statistical and Computational Analyses

Data are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post hoc test. A p-value < 0.05 was considered statistically significant (* $p < 0.05$). Differential microbial taxa were identified using linear discriminant analysis effect size (LEfSe) on the Galaxy platform (https://usegalaxy.org/).

3. Results and Discussion

3.1. OSA Starch Ameliorates Hepatic Injury and Lipid Dysregulation in ALD Mice

To evaluate the protective effects of OSA starch on ALD, a chronic alcohol feeding model in mice, together with a 28-day intervention with OSA starch, was established (Figure 1A). Alcohol exposure significantly elevated the liver index, indicating hepatomegaly as a consequence of fat accumulation ($p < 0.01$, Figure 1B) [21]. OSA starch supplementation markedly reduced the liver index compared to the ALD Model group ($p < 0.05$), suggesting mitigation of alcohol-induced liver enlargement. This reduction corresponds with a significant decrease in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels ($p < 0.01$ and $p < 0.05$, respectively; Figure 1C,D), key biomarkers for liver injury [22]. Consistently, histological examination revealed pronounced steatosis and hepatocellular ballooning in the ALD Model group, whereas OSA starch intervention substantially alleviated these pathological features, maintaining liver architecture closer to that of controls (Figure 1I). These outcomes align with prior studies indicating that dietary fibers can modulate the gut–liver axis, thereby alleviating alcohol-induced hepatic injury [23].

In parallel, alcohol exposure disrupted lipid metabolism, as reflected by elevated total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), alongside decreased high-density lipoprotein cholesterol (HDL-C) (Figure 1E–H) [24,25]. OSA starch supplementation significantly lowered TG levels ($p < 0.05$) and restored HDL-C levels ($p < 0.01$), both of which are critical in mitigating the progression of steatosis and cardiovascular risk. Interestingly, OSA starch did not significantly alter TC or LDL-C levels compared to the ALD Model group. Together, these findings demonstrate that OSA starch exerts benefits on liver morphology, function, and lipid metabolism in ALD.

3.2. OSA Starch Intervention Reshapes Gut Microbiota Composition in ALD Mice

To investigate the regulatory effects of OSA starch on gut microbial dysbiosis induced by alcohol exposure, alpha diversity and microbial community structure in ALD mice were assessed [26]. Shannon indices (Figure 2A) revealed that chronic alcohol consumption increased species richness, reflecting an unstable gut microbial species [27]. Interestingly,

OSA starch supplementation attenuated the ethanol-induced increase in microbial species richness, indicating a selective regulation of the microbial composition. Principal component analysis (PCA) based on Bray–Curtis distance (Figure 2B) demonstrated a clear segregation of microbial profiles, with the OSA starch group distinctly clustering away from the ALD model, indicating a pronounced shift in microbial structure attributable to the dietary intervention.

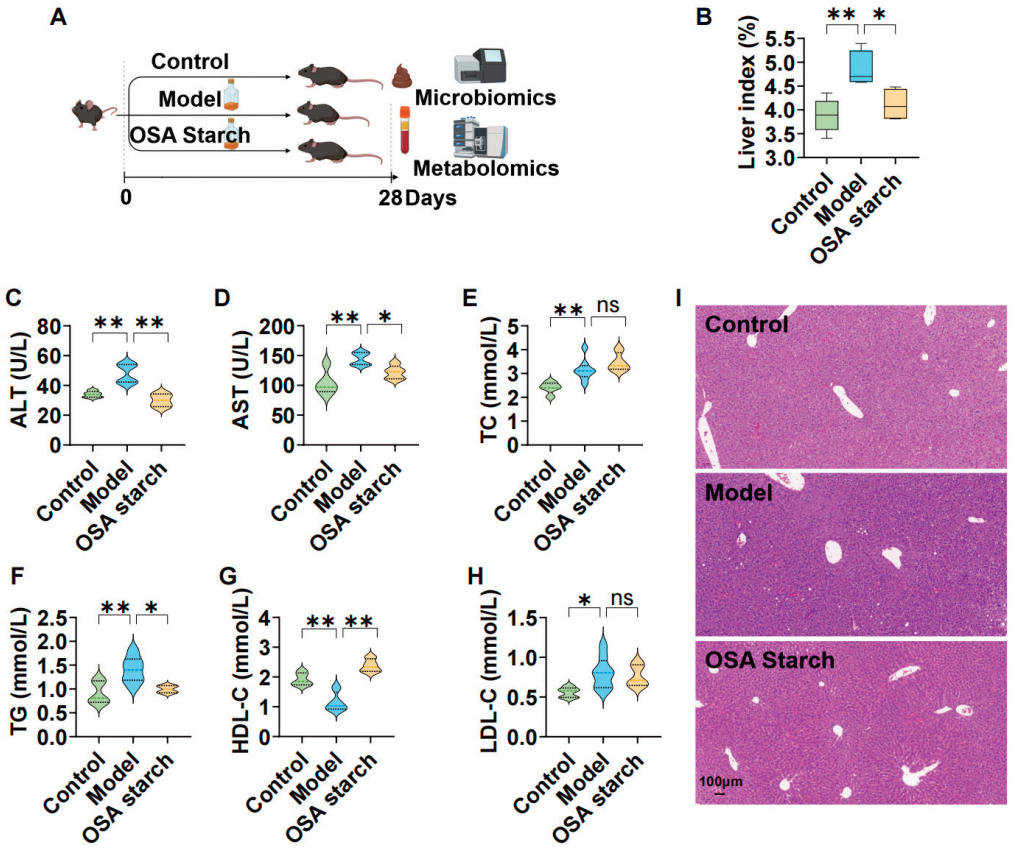


Figure 1. OSA starch ameliorates hepatic injury and lipid dysregulation in AL mice. (**A**) Schematic representation of the experimental protocol. (**B**) Liver index. (**C**,**D**) Serum ALT and AST levels. (**E**,**F**) Serum TC and TG levels. (**G**,**H**) Serum HDL-C and LDL-C levels. (**I**) Representative liver histology with H&E staining. Data are presented as mean ± SD (n = 8). Statistical significance was determined by one-way ANOVA with Dunnett's post hoc test: ns, not significant $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

At the taxonomic level, phylum-level analysis (Figure 2C) showed that alcohol exposure disrupted the balance between Firmicutes and Bacteroidota. OSA starch partially restored this ratio, primarily by reducing the relative abundance of Proteobacteria, a phylum often associated with barrier dysfunction [28]. At the phylum level, alcohol exposure increased the relative abundance of Proteobacteria while decreasing Firmicutes, consistent with dysbiosis patterns commonly observed in liver injury (Figure 2C) [29]. Species-level profiling (Figure 2D) revealed that OSA starch increased beneficial microbes such as *Faecalibaculum rodentium* and *Bifidobacterium adolescentis*, while reducing the abundance of potentially pathogenic taxa like *Muribaculum gordoncarteri*. These compositional changes are consistent with the known prebiotic effects of resistant starches, which modulate the gut microbiota to enhance host metabolic and immune health [9,30]. Collectively, these results suggest that OSA starch ameliorates alcohol-induced gut dysbiosis through microbiome modulation.

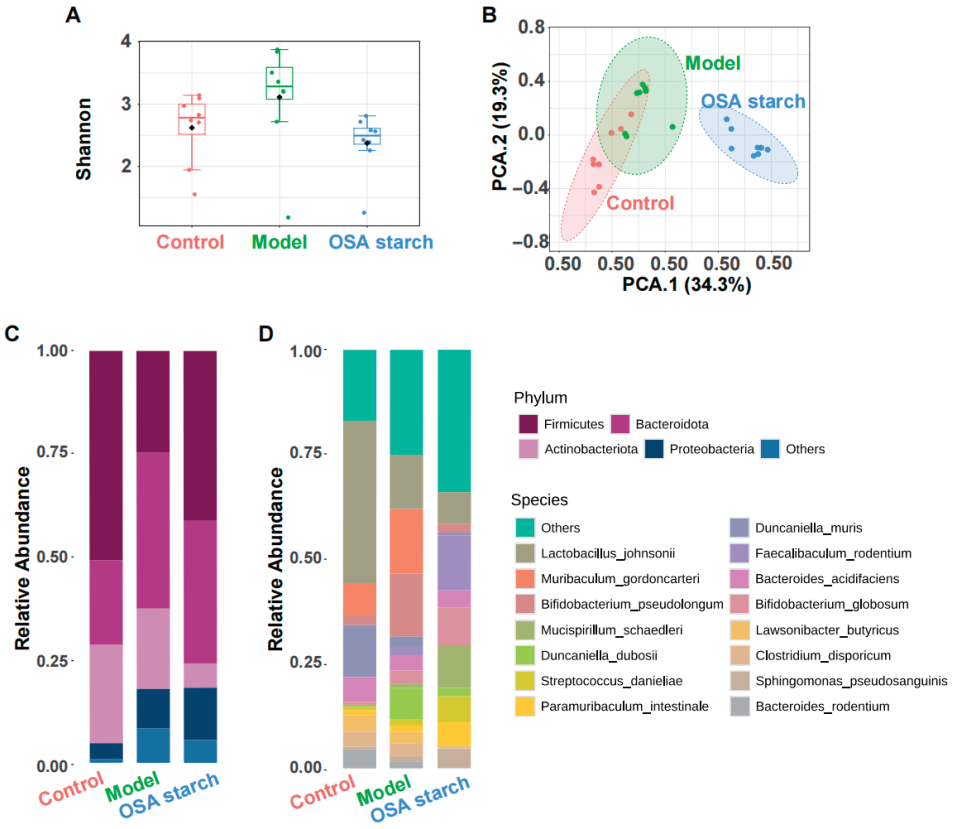


Figure 2. OSA starch intervention reshapes gut microbiota composition in ALD mice. (**A**) Alpha diversity indices—Shannon. (**B**) PCA based on Bray–Curtis dissimilarity reveals distinct clustering. (**C**) Gut microbiota composition at the phylum level. (**D**) Gut microbiota composition at the species level.

3.3. OSA Starch Modulates Key Gut Microbial Taxa and Their Functional Profiles in ALD Mice

Comprehensive analysis of gut microbiota alterations induced by OSA starch intervention in ALD mice revealed significant shifts in bacterial composition and diversity. LEfSe analysis identified distinct microbial biomarkers across experimental groups (Figure 3A). The ALD Model group exhibited an enrichment of potentially pathogenic species such as *Haemophilus parainfluenzae*, *Veillonella atypica*, and *Muribaculum gordoncarteri*, which have been implicated in gut barrier dysfunction and inflammation [31,32]. Conversely, OSA starch supplementation selectively enriched beneficial taxa, including *Faecalibaculum rodentium*, *Bacteroides acidifaciens*, and *Bifidobacterium adolescentis*. These species are recognized as producers of short-chain fatty acids, particularly butyrate, known to reinforce intestinal integrity and exert anti-inflammatory effects, potentially contributing to the alleviation of ALD pathology [23,33,34].

Random forest classification further validated the discriminative microbial features between groups, achieving low classification error rates (Figure 3B). The most influential taxa contributing to group separation, as indicated by the mean decrease in accuracy, included *Faecalibaculum rodentium*, *Haemophilus parainfluenzae*, and *Bifidobacterium adolescentis* (Figure 3C). These key species are involved in metabolic pathways essential for maintaining host lipid homeostasis and modulating immune responses [35,36]. Co-occurrence network analysis (Figure 3D) demonstrated that *Bifidobacterium adolescentis* and *Faecalibaculum rodentium* formed central nodes within the microbial ecosystem of OSA starch-treated mice, suggesting that OSA starch fosters a more interconnected microbial community structure.

Functional predictions based on PICRUSt2 and KEGG pathway analysis revealed that OSA starch intervention notably influenced microbial metabolic potential (Figure 3E).

Specifically, pathways associated with propanoate metabolism, glycolysis/gluconeogenesis, and galactose metabolism were reduced in the OSA starch group, which linked to metabolic disturbances in ALD [37]. In contrast, pathways such as glyoxylate and dicarboxylate metabolism were relatively enhanced, indicating a metabolic shift towards improved energy efficiency and substrate utilization. These microbial functional adaptations align with the biochemical improvements observed in liver function and lipid profiles, supporting the role of OSA starch in mitigating ALD progression through targeted modulation of the gut microbiome and its metabolism.

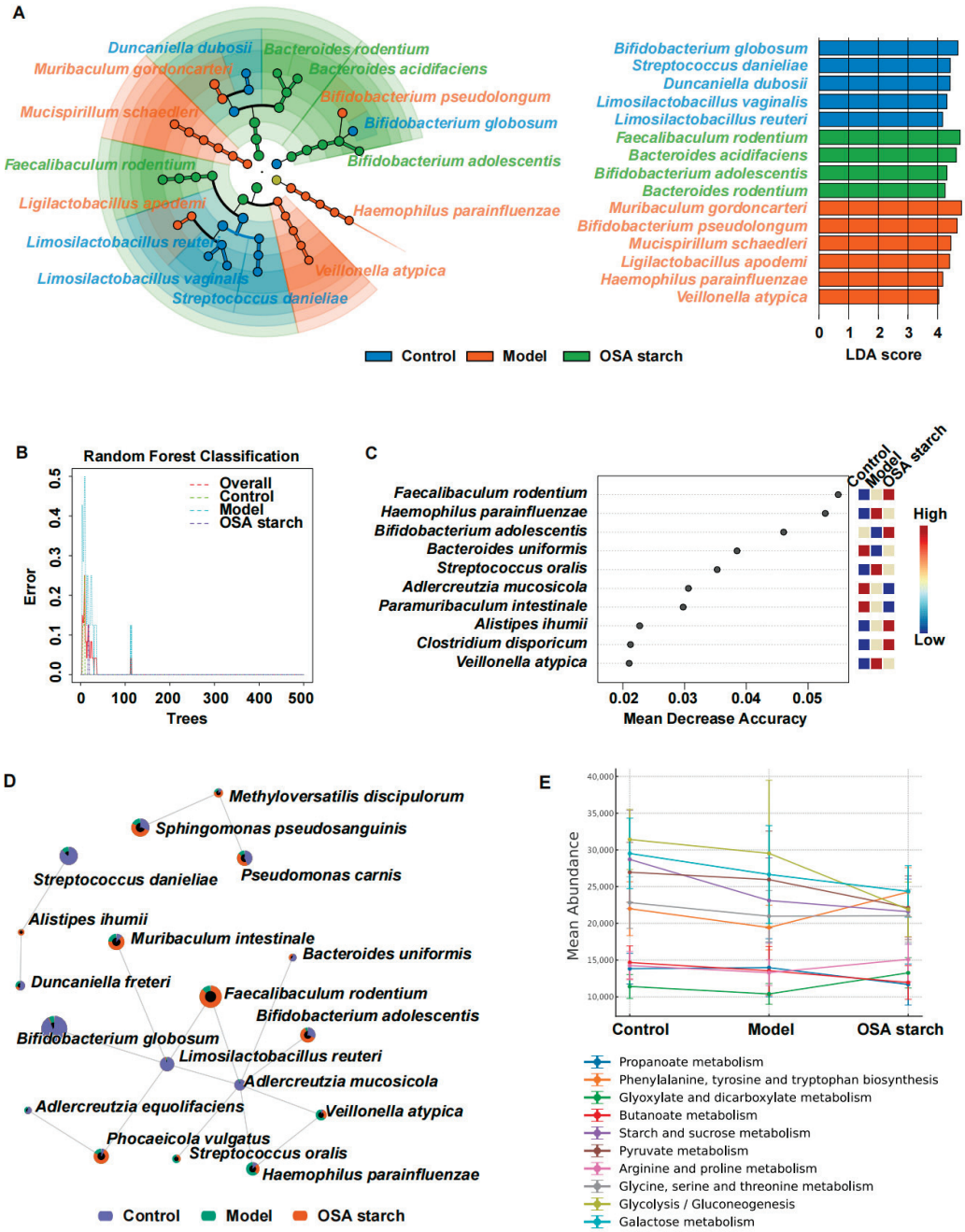


Figure 3. OSA starch modulates key gut microbial taxa and their functional profiles in ALD mice. (**A**) LEfSe cladogram and LDA score of differential species. (**B**) Random forest classification error rates for overall and individual groups. (**C**) Top microbial features ranked by mean decrease accuracy contributing to group discrimination. (**D**) Co-occurrence network of differential gut microbes colored by group prevalence. (**E**) Predicted KEGG metabolic pathways (Level 3) of gut microbiota across groups.

3.4. Key Gut Microbial Metabolic Pathways Modulated by OSA Starch in ALD Mice

Functional pathway analysis based on PICRUSt2 prediction revealed that OSA starch notably regulated gut microbial metabolic functions centered on carbon metabolism, including the pyruvate, glyoxylate, and dicarboxylate, butanoate, and propanoate metabolism pathways (Supplementary Figure S1). In the pyruvate metabolism pathway, several critical enzymes responsible for converting pyruvate into acetyl-CoA, lactate, and other intermediates were significantly altered (Supplementary Figure S1A). Hepatic pyruvate metabolism is critical and complementary for maintenance of antioxidant capacity [38]. Additionally, modulation of glyoxylate and dicarboxylate metabolism suggests enhanced microbial capacity for the glyoxylate cycle (Supplementary Figure S1B), which is pivotal in maintaining energy efficiency under metabolic stress conditions commonly associated with liver diseases [39].

Moreover, the butanoate and propanoate metabolism pathways were distinctly influenced, as evidenced by the differential regulation of enzymes involved in the synthesis of key short-chain fatty acids, such as butyrate and propionate (Supplementary Figure S1C,D). Short-chain fatty acids are critical mediators of gut–liver axis health, with butyrate known to strengthen intestinal barrier integrity and propionate contributing to lipid metabolism regulation [40,41]. The attenuation of propanoate metabolism alongside the remodeling of butanoate pathways may help restore short-chain fatty acid homeostasis disrupted in ALD. This metabolic reconfiguration in the gut microbiota highlights a potential mechanism by which OSA starch exerts its protective effects against hepatic injury via microbial metabolic modulation.

3.5. OSA Starch Reshapes Fecal Metabolome and Modulates Key Metabolic Pathways in ALD Mice

Untargeted fecal metabolomics revealed a distinct metabolic reprogramming following OSA starch intervention in ALD mice. PCA demonstrated clear separation between the Control, Model, and OSA starch groups, reflecting the overall impact of OSA starch on metabolic profiles (Figure 4A). The volcano plots identified pyruvate accumulation as a hallmark of ALD progression, with the Model group showing significant decreases compared to Control (Figure 4B). Conversely, OSA starch supplementation not only sustained elevated pyruvate and 2-hydroxy-butanoic acid levels but also substantially reduced the abundance of lactic acid, L-glutamic acid, beta-leucine, and creatine—metabolites linked to disrupted amino acid and energy metabolism (Figure 4C). These metabolic shifts suggest that OSA starch may redirect pyruvate flux away from lactate production and abnormal amino acid turnover, promoting more efficient energy homeostasis. Further analysis using Upset plots identified metabolites converging on four pivotal metabolic pathways: pyruvate metabolism, propanoate metabolism, butanoate metabolism, and glyoxylate and dicarboxylate metabolism (Figure 4D). These pathways are central to maintaining carbon flow between glycolysis, the TCA cycle, and short-chain fatty acid production [42]. The heatmap clustering emphasized the normalized metabolic landscape in the OSA starch group relative to the Model (Figure 4E).

A detailed metabolite–pathway interaction network (Figure 5A) mapped the relationships between these four key pathways and their representative metabolites. Pyruvate metabolism emerged as a central hub, directly connected to citrulline, ornithine, creatine, lactic acid, and beta-leucine, among others. This centrality underscores pyruvate's critical role as a metabolic crossroad linking amino acid biosynthesis and energy production [43]. Additionally, propanoate, butanoate, and glyoxylate metabolism were interconnected through shared metabolites such as glutamine, glyceric acid, and malic acid, highlighting the intricate metabolic crosstalk restored by OSA starch intervention. KEGG enrichment analysis further revealed significant regulation of pathways involved in arginine and pro-

line metabolism, pyruvate metabolism, histidine metabolism, and glyoxylate pathways (Figure 5B), which are also relevant to hepatic and intestinal health [44]. Complementarily, SMPDB enrichment confirmed involvement of beta-alanine, purine, and arachidonic acid metabolism (Figure 5C), suggesting broader impacts on amino acid turnover and inflammatory responses. Collectively, these findings suggest that OSA starch exerts hepatoprotective effects by orchestrating the gut-derived metabolic networks.

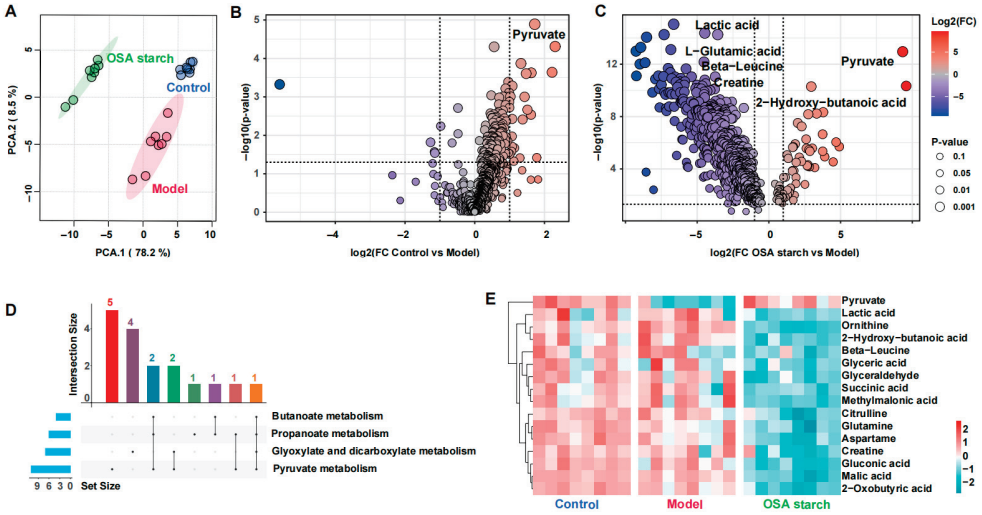


Figure 4. OSA starch reshapes the fecal metabolome in ALD mice. (**A**) PCA score plot of fecal metabolome showing group separation. (**B**) Volcano plot of differential metabolites between Control and Model groups. (**C**) Volcano plot of differential metabolites between OSA starch and Model groups. (**D**) Upset plot indicating metabolite overlap among four key pathways (pyruvate, propanoate, butanoate, glyoxylate/dicarboxylate metabolism). (**E**) Heatmap of representative differential metabolites across groups.

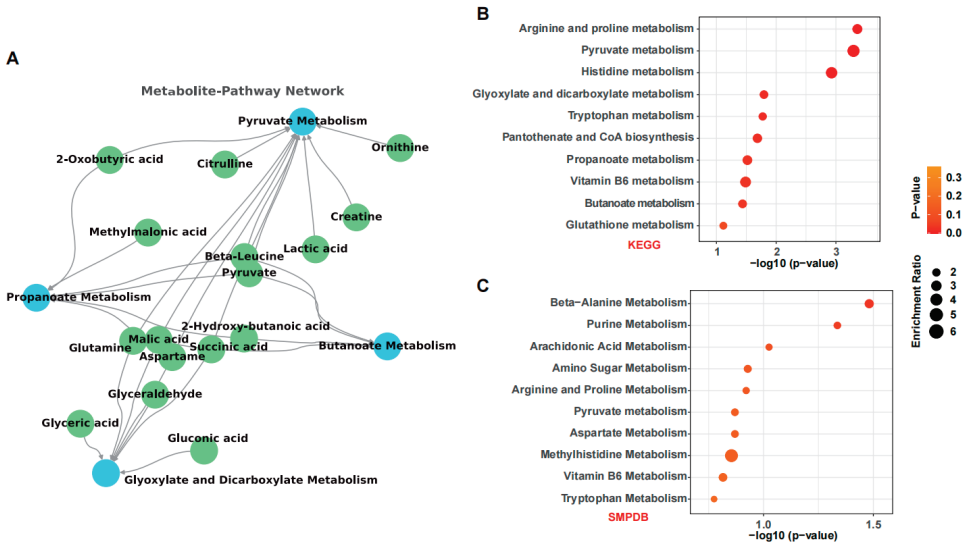


Figure 5. OSA starch modulates key metabolic pathways in ALD mice. (**A**) Metabolite–Pathway network connecting key metabolites to their respective metabolic pathways. Blue represents metabolic pathways, and green represents metabolic metabolites. (**B**) KEGG pathway enrichment analysis of altered metabolites ($p < 0.01$). (**C**) SMPDB pathway enrichment analysis of altered metabolites ($p < 0.01$). Red represents altered metabolic pathways in KEGG Database and SMPDB database, respectively.

3.6. OSA Starch Improves Serum Bile Acid Profiles in ALD Mice

Untargeted serum metabolomics revealed that OSA starch supplementation significantly remodeled the serum metabolic landscape in ALD mice. PCA illustrated distinct

clustering of the Control, Model, and OSA starch groups, indicating that OSA starch markedly shifted the serum metabolome towards a healthier profile (Figure 6A). Differential analysis between the OSA starch and Model groups identified several metabolites that were significantly altered, particularly those associated with amino acid metabolism and energy balance (Figure 6B). Key metabolites such as lactic acid, L-glutamic acid, beta-leucine, creatine, and propionyl-L-carnitine showed substantial reductions following OSA starch intervention, suggesting improved mitochondrial function and reduced metabolic stress [45].

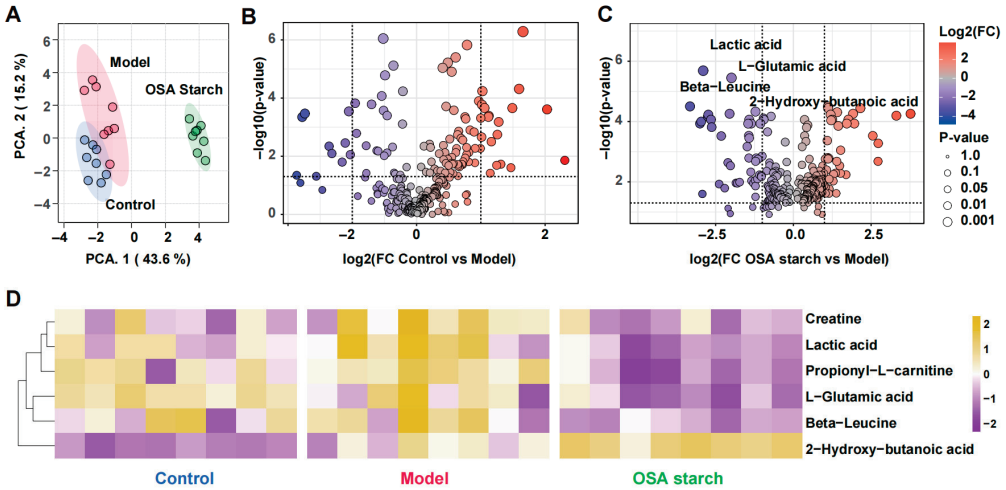


Figure 6. OSA starch improves serum metabolism profiles in ALD mice. (**A**) PCA based on untargeted serum metabolomics. (**B**) Volcano plot of differentially expressed serum metabolites between the Control and Model groups. (**C**) Volcano plot of differentially expressed serum metabolites between OSA starch and Model groups. (**D**) Heatmap showing the relative abundance of significantly altered metabolites.

Heatmap visualization further highlighted that OSA starch restored the serum levels of these representative metabolites (Figure 6D). The reduction in lactic acid and glutamic acid implies mitigation of anaerobic glycolysis and excitotoxicity, respectively, both of which are aggravated in liver disease [46,47]. Additionally, the decrease in beta-leucine and propionyl-L-carnitine may reflect a normalization of branched-chain amino acid metabolism and fatty acid oxidation, respectively, further indicating metabolic reprogramming favoring hepatic health [48]. These findings are consistent with previous reports where targeting amino acid and lipid metabolism alleviated liver injury and improved metabolic resilience [18]. Overall, the serum metabolomic data support the hypothesis that OSA starch exerts protective effects against ALD by rebalancing energy production.

3.7. OSA Starch Modulates Gut Microbiota–Metabolite–Host Axis to Alleviate ALD

To further elucidate the mechanism underlying the beneficial effects of OSA starch on ALD, a comprehensive multi-layer network was constructed integrating gut microbiota, microbial functions, fecal and serum metabolites, and clinical serum indicators. The interaction network (Figure 7A) revealed *Faecalibaculum rodentium* and *Bifidobacterium adolescentis* as central bacterial nodes connected to key metabolic pathways, including butanoate and propanoate metabolism, and to signature fecal metabolites such as pyruvate, lactic acid, and 2-hydroxy-butanoic acid. These metabolites exhibited strong links with serum markers like ALT, AST, and TG, suggesting that gut microbial modulation impacts host metabolic health. Additionally, microbial functional pathways including pyruvate and propanoate metabolism were identified as pivotal nodes linking gut microbiota alterations to metabolic outputs, reinforcing the microbiota–metabolite–host axis hypothesis.

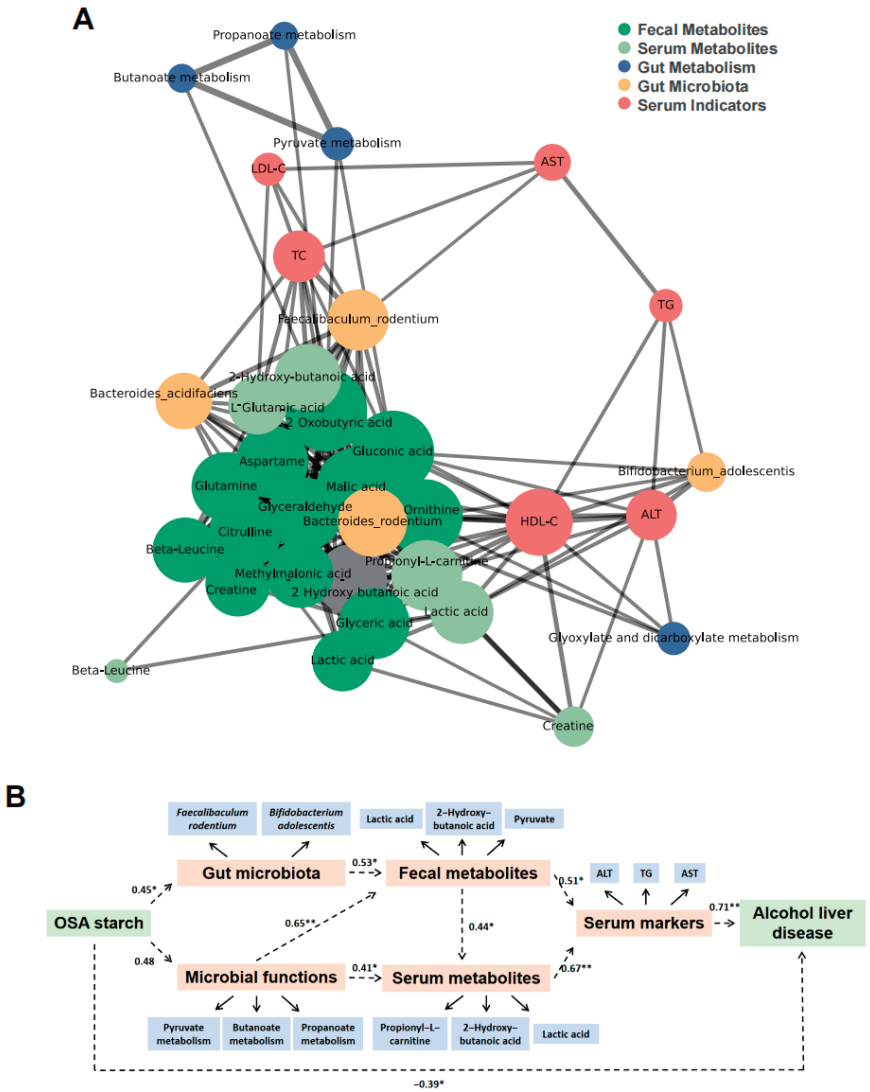


Figure 7. OSA starch modulates gut microbiota–metabolite–host axis to alleviate ALD. (**A**) Interaction network depicting relationships among gut microbiota, microbial metabolism, fecal metabolites, serum metabolites, and serum indicators. (**B**) SEM illustrating the pathways from OSA starch intervention through gut microbiota, microbial functions, fecal and serum metabolites, and serum markers to alcoholic liver disease outcome. * $p < 0.05$, ** $p < 0.01$.

A structural equation model (SEM) was constructed using AMOS software version 28 (Armonk, NY, USA) to quantify the direct and indirect pathways involved (Figure 7B). The final model exhibited good fit to the data, with fit indices of RMSEA = 0.065 and CFI = 0.975, indicating an acceptable model fit. OSA starch positively regulated gut microbiota (path coefficient = 0.45, $p < 0.01$) and microbial functions (0.48, $p < 0.01$), which sequentially affected fecal metabolites (0.53, $p < 0.01$) and serum metabolites (0.41, $p < 0.05$). Both fecal and serum metabolites significantly influenced serum markers (0.51, $p < 0.01$ and 0.44, $p < 0.05$, respectively). OSA starch exhibited a strong negative correlation with ALD progression (−0.39, $p < 0.05$). These findings suggest that the gut microbiota–metabolite axis serves as a critical mediator of OSA starch's therapeutic benefits in ALD.

Network and SEM analyses strongly support the hypothesis that OSA starch alleviates ALD by modulating the gut microbiota–metabolite–host axis. This central role of the gut microbiota aligns with the protective mechanisms attributed to other prebiotics and dietary fibers in ALD research, such as polysaccharides [49]. The unique structure and physical and chemical properties of OSA starch may affect the way this regulatory mechanism is realized,

thereby influencing the succession of microorganisms and the availability of ecological niches. This indicates that although the core protective axis (gut–liver communication) is the common goal, different prebiotic fibers participate in it through subtle mechanisms.

While these findings show the beneficial effects of OSA starch on the gut–liver axis in ALD mice, several limitations exist. First, murine models differ from humans in alcohol metabolism and disease progression, limiting translation to human ALD. Second, the 28-day intervention may not reflect long-term OSA starch effects or chronic ALD progression, warranting longer studies. Third, replacing native starch entirely with OSA starch does not mimic typical human diets, suggesting that future studies should examine OSA starch in more complex dietary contexts.

4. Conclusions

In conclusion, this study suggests that dietary OSA starch may ameliorate alcoholic liver disease by orchestrating a gut microbiota–metabolite–host axis. OSA starch selectively enriched beneficial gut microbes such as *Faecalibaculum rodentium* and *Bifidobacterium adolescentis*, which appeared associated with enhanced microbial metabolic functions, including pyruvate, butanoate, and propanoate metabolism. Consistent with this, favorable changes in fecal and serum metabolites (e.g., elevated pyruvate and reduced lactic acid), along with improvements in serum lipid profiles and hepatic injury markers, were observed. Structural equation modeling indicates that OSA starch exerts its protective effects through a multi-level regulatory cascade spanning the gut microbiota, microbial functions, metabolic intermediates, and serum indicators. These findings highlight the potential of OSA starch as a dietary intervention for alcoholic fatty liver disease by modulating the gut–liver metabolic axis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu17172779/s1, Figure S1: Key Gut Microbial Metabolic Pathways Modulated by OSA Starch in ALD mice.

Author Contributions: C.L. and X.P. designed and performed the experiments, analyzed the data, interpreted the results, and wrote the article. T.L., R.M. and Y.T. designed the experiments, interpreted the results. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal procedures received ethical approval from the Animal Ethics Committee of Jiangnan University (JN. No. 20240930c0641123 (17 July 2025)), following the ARRIVE guidelines for animal research.

Informed Consent Statement: Not applicable.

Data Availability Statement: The Dataset available on request from the authors.

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

Abbreviations

The following abbreviations are used in this manuscript:

ALD	Alcoholic liver disease
OSA	Octenyl succinic anhydride

References

1. Yan, M.Y.; Man, S.L.; Ma, L.; Guo, L.P.; Huang, L.Q.; Gao, W.Y. Immunological mechanisms in steatotic liver diseases an overview and clinical perspectives. *Clin. Mol. Hepatol.* **2024**, *30*, 620–648. [CrossRef]
2. Jia, W.; Xie, G.X.; Jia, W.P. Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. *Nat. Rev. Gastroenterol. Hepatol.* **2018**, *15*, 111–128. [CrossRef]
3. Bajaj, J.S. Alcohol, liver disease and the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* **2019**, *16*, 235–246. [CrossRef]
4. Xu, Q.; Zhang, R.S.; Mu, Y.; Song, Y.; Hao, N.; Wei, Y.B.; Wang, Q.B.; Mackay, C.R. Propionate Ameliorates Alcohol-Induced Liver Injury in Mice via the Gut-Liver Axis: Focus on the Improvement of Intestinal Permeability. *J. Agric. Food Chem.* **2022**, *70*, 6084–6096. [CrossRef] [PubMed]
5. Yang, Y.; Ren, Q.X.; Zhou, Z.L.; Li, X.; Ren, D.L.; Ji, Z.W.; Mao, J. Structural elucidation of a highly branched α-D-glucan from Huangjiu and its hepatoprotective activity via gut microbiome regulation and intestinal barrier repairment. *Carbohydr. Polym.* **2024**, *324*, 121423. [CrossRef]
6. Li, F.; McClain, C.; Feng, W. Microbiome dysbiosis and alcoholic liver disease. *Liver Res.* **2019**, *3*, 218–226. [CrossRef]
7. Bluemel, S.; Wang, L.R.; Kuelbs, C.; Moncera, K.; Torralba, M.; Singh, H.; Fouts, D.E.; Schnabl, B. Intestinal and hepatic microbiota changes associated with chronic ethanol administration in mice. *Gut Microbes* **2020**, *11*, 265–275. [CrossRef]
8. Wu, H.Y.; Chen, J.J.; Guo, S.Y.; Deng, J.H.; Zhou, Z.M.; Zhang, X.; Qi, T.T.; Yu, F.; Yang, Q. Advances in the acting mechanism and treatment of gut microbiota in metabolic dysfunction-associated steatotic liver disease. *Gut Microbes* **2025**, *17*, 2500099. [CrossRef]
9. Chang, R.R.; Jin, Z.Y.; Tian, Y.Q. Insights into the structural, morphological, and thermal property changes in simulated digestion and fermentability of four resistant starches from high amylose maize starch. *Food Hydrocoll.* **2023**, *142*, 108770. [CrossRef]
10. Chang, R.R.; Wang, F.; Huang, J.T.; Jin, Z.Y.; Tian, Y.Q. Recrystallized Resistant Starch: Structural Changes in the Stomach, Duodenum, and Ileum and the Impact on Blood Glucose and Intestinal Microbiome in Mice. *J. Agric. Food Chem.* **2023**, *71*, 12080–12093. [CrossRef]
11. Wang, N.; Zhang, C.; Li, H.X.E.; Wu, J.H.; Zhang, D.C.; Li, Y.; Yang, L.; Zhang, N.; Wang, X.Y. Structure properties of Canna edulis RS3 (double enzyme hydrolysis) and RS4 (OS-starch and cross-linked starch): Influence on fermentation products and human gut microbiota. *Int. J. Biol. Macromol.* **2024**, *265*, 130700. [CrossRef]
12. Liu, C.; Li, X.M.; Li, J.Y.; Yang, F.W.; Guo, Y.H.; Yao, W.R.; Qian, H.; Cheng, Y.L. Role of the gut microbiota in dietary patterns rich in torularhodin OSA colon-targeted delivery. *Food Funct.* **2022**, *13*, 11034–11048. [CrossRef]
13. Devarakonda, S.L.S.; Superdock, D.K.; Ren, J.N.F.; Johnson, L.M.; Loinard-González, A.P.; Poole, A.C. Gut microbial features and dietary fiber intake predict gut microbiota response to resistant starch supplementation. *Gut Microbes* **2024**, *16*, 2367301. [CrossRef]
14. Huicui, L.; Min, Z.; Qingyu, M.; Baoming, T.; Chenxi, N.; Zhifei, C.; Juxiu, L. Health beneficial effects of resistant starch on diabetes and obesity via regulation of gut microbiota: A review. *Food Funct.* **2020**, *11*, 5749–5767. [CrossRef]
15. Sun, Z.H.; Chen, Z.W.; Xu, B.; Shi, Y.C. Distribution of octenylsuccinate substituents within a single granule of modified waxy maize starch determined by Raman microspectroscopy. *Carbohydr. Polym.* **2019**, *216*, 282–286. [CrossRef]
16. Chen, J.P.; Maierheba, K.; Zhang, Y.; Cheng, H.; Lin, B.B.; Yue, P.; Wang, L.H.; Liu, F.Z.; Shi, J.W.; Wan, Z.X.; et al. Octenyl Succinic Anhydride-Modified Starch Attenuates Body Weight Gain and Changes Intestinal Environment of High-Fat Diet-Fed Mice. *Foods* **2022**, *11*, 2980. [CrossRef] [PubMed]
17. Li, H.T.; Zhang, L.; Li, J.; Wu, Q.; Qian, L.L.; He, J.S.; Ni, Y.Q.; Kovatcheva-Datchary, P.; Yuan, R.; Liu, S.B.; et al. Resistant starch intake facilitates weight loss in humans by reshaping the gut microbiota. *Nat. Metab.* **2024**, *6*, 578–597. [CrossRef]
18. Ni, Y.; Qian, L.; Siliceo, S.L.; Long, X.; Nychas, E.; Liu, Y.; Ismaiah, M.J.; Leung, H.; Zhang, L.; Gao, Q.; et al. Resistant starch decreases intrahepatic triglycerides in patients with NAFLD via gut microbiome alterations. *Cell Metab.* **2023**, *35*, 1530–1547. [CrossRef] [PubMed]
19. Wang, T.; Liu, C.; Ma, R.R.; Pan, X.H.; Shen, W.Y.; Tian, Y.Q. Esterified starches enhance short-term satiety in mice structural and physicochemical alterations. *Food Funct.* **2024**, *15*, 12058–12068. [CrossRef]
20. Pang, Z.; Zhou, G.; Ewald, J.; Chang, L.; Hacariz, O.; Basu, N.; Xia, J. Using MetaboAnalyst 5.0 for LC-HRMS spectra processing, multi-omics integration and covariate adjustment of global metabolomics data. *Nat. Protoc.* **2022**, *17*, 1735–1761. [CrossRef] [PubMed]
21. Song, K.; Kwon, Y.-J.; Lee, E.; Lee, H.S.; Youn, Y.H.; Baik, S.J.; Shin, H.J.; Chae, H.W. Optimal cutoffs of fatty liver index and hepatic steatosis index in diagnosing pediatric MASLD. *Clin. Gastroenterol. Hepatol.* **2025**, *25*, 00530. [CrossRef]
22. Abeysekera, K.W.A.; Fernandes, G.S.; Hammerton, G.; Portal, A.J.; Gordon, F.H.; Heron, J.; Hickman, M. Prevalence of steatosis and fibrosis in young adults in the UK: A population-based study. *Lancet Gastroenterol.* **2020**, *5*, 295–305. [CrossRef] [PubMed]
23. Shen, H.Y.; Zhou, L.L.; Zhang, H.; Yang, Y.R.; Jiang, L.; Wu, D.Q.; Shu, H.; Zhang, H.J.; Xie, L.X.; Zhou, K.C.; et al. Dietary fiber alleviates alcoholic liver injury via Bacteroides acidifaciens and subsequent ammonia detoxification. *Cell Host Microbe* **2024**, *32*, 1331–1346. [CrossRef] [PubMed]

24. Zhang, J.H.; Liu, X.J.; Jin, X.; Mao, X.D.; Xu, X.L.; Zhang, X.; Shang, K.; Xu, Y.; Zhang, Y.H.; Meng, G.F.; et al. Liver-specific inactivation of Cideb improves metabolic profiles and ameliorates steatohepatitis and fibrosis in animal models for MASH. *Pharmacol. Res.* **2025**, *214*, 107664. [CrossRef]
25. Hersberger, M.; von Eckardstein, A. Low high-density lipoprotein cholesterol—Physiological background, clinical importance and drug treatment. *Drugs* **2003**, *63*, 1907–1945. [CrossRef]
26. Tsiavos, A.; Antza, C.; Trakatelli, C.; Kotsis, V. The Microbial Perspective: A Systematic Literature Review on Hypertension and Gut Microbiota. *Nutrients* **2024**, *16*, 3698. [CrossRef]
27. Wang, R.; Tang, R.Q.; Li, B.; Ma, X.; Schnabl, B.; Tilg, H. Gut microbiome, liver immunology, and liver diseases. *Cell. Mol. Immunol.* **2021**, *18*, 4–17. [CrossRef]
28. Shi, Y.J.; Zou, Y.X.; Xiong, Y.H.; Zhang, S.Y.; Song, M.M.; An, X.F.; Liu, C.; Zhang, W.X.; Chen, S.Y. Host Gasdermin D restrains systemic endotoxemia by capturing Proteobacteria in the colon of high-fat diet-feeding mice. *Gut Microbes* **2021**, *13*, 1946369. [CrossRef]
29. DeMinicis, S.; Rychlicki, C.; Agostinelli, L.; Saccomanno, S.; Candelaresi, C.; Trozzi, L.; Mingarelli, E.; Facinelli, B.; Magi, G.; Palmieri, C.; et al. Dysbiosis Contributes to Fibrogenesis in the Course of Chronic Liver Injury in Mice. *Hepatology* **2014**, *59*, 1738–1749. [CrossRef]
30. Dobranowski, P.A.; Stintzi, A. Resistant starch, microbiome, and precision modulation. *Gut Microbes* **2021**, *13*, 1926842. [CrossRef]
31. Olagunju, A.; Martinez, J.; Kenny, D.; Gideon, P.; Mookadam, F.; Unzek, S. Virulent endocarditis due to Haemophilus parainfluenzae: A systematic review of the literature. *World J. Cardiol.* **2022**, *14*, 546–556. [CrossRef]
32. Leibovitzh, H.; Nayeri, S.; Borowski, K.; Hernandez-Rocha, C.; Lee, S.H.; Turpin, W.; Stempak, J.M.; Sandhu, I.; Milgrom, R.; Smith, M.; et al. Inflammatory Bowel Disease Associated with Primary Sclerosing Cholangitis is Associated with an Altered Gut Microbiome and Bile Acid Profile. *J. Crohn's Colitis* **2024**, *18*, 1957–1966. [CrossRef]
33. Rios-Covian, D.; Gueimonde, M.; Duncan, S.H.; Flint, H.J.; de los Reyes-Gavilan, C.G. Enhanced butyrate formation by cross-feeding between Faecalibacterium prausnitzii and Bifidobacterium adolescentis. *FEMS Microbiol. Lett.* **2015**, *362*, 176. [CrossRef]
34. Zagato, E.; Pozzi, C.; Bertocchi, A.; Schioppa, T.; Saccheri, F.; Guglietta, S.; Fosso, B.; Melocchi, L.; Nizzoli, G.; Troisi, J.; et al. Endogenous murine microbiota member Faecalibaculum rodentium and its human homologue protect from intestinal tumour growth. *Nat. Microbiol.* **2020**, *5*, 511–523. [CrossRef]
35. Kawano, Y.; Edwards, M.; Huang, Y.M.; Bilate, A.M.; Araujo, L.P.; Tanoue, T.; Atarashi, K.; Ladinsky, M.S.; Reiner, S.L.; Wang, H.H.; et al. Microbiota imbalance induced by dietary sugar disrupts immune-mediated protection from metabolic syndrome. *Cell* **2022**, *185*, 3501–3519. [CrossRef]
36. Ogura, Y.; Suzuki, S.; Shirakawa, T.; Masuda, M.; Nakamura, H.; Iijima, K.; Yoshikawa, N. antigen and antibody in children with IgA nephropathy and Henoch-Schonlein nephritis. *Am. J. Kidney Dis.* **2000**, *36*, 47–52. [CrossRef]
37. Geng, X.; Zhuang, M.M.; Tian, W.N.; Shang, H.Y.; Gong, Z.Y.; Lv, Y.F.; Li, J.R. Green Radish Polysaccharide Prevents Alcoholic Liver Injury by Interfering with Intestinal Bacteria and Short-Chain Fatty Acids in Mice. *Foods* **2024**, *13*, 3733. [CrossRef] [PubMed]
38. Yiew, N.K.H.; Vazquez, J.H.; Martino, M.R.; Kennon-McGill, S.; Price, J.R.; Allard, F.D.; Yee, E.U.; Layman, A.J.; James, L.P.; Mccommis, K.S.; et al. Hepatic pyruvate and alanine metabolism are critical and complementary for maintenance of antioxidant capacity and resistance to oxidative insult. *Mol. Metab.* **2023**, *77*, 101808. [CrossRef] [PubMed]
39. Dean, J.T.; Tran, L.; Beaven, S.; Tontonoz, P.; Reue, K.; Dipple, K.M.; Liao, J.C. Resistance to Diet-Induced Obesity in Mice with Synthetic Glyoxylate Shunt. *Cell Metab.* **2009**, *9*, 525–536. [CrossRef] [PubMed]
40. Recharla, N.; Geesala, R.; Shi, X.Z. Gut Microbial Metabolite Butyrate and Its Therapeutic Role in Inflammatory Bowel Disease: A Literature Review. *Nutrients* **2023**, *15*, 2275. [CrossRef]
41. Cani, P.D. Is colonic propionate delivery a novel solution to improve metabolism and inflammation in overweight or obese subjects? *Gut* **2019**, *68*, 1352–1353. [CrossRef] [PubMed]
42. Zhang, Y.T.; Deng, Y.K.; Zou, Y.F.; Han, B.L.; Pu, J.Z.; Rao, J.Q.; Huang, D.; Luo, H.B. Linking Microbial Functional Gene Abundance and Daqu Extracellular Enzyme Activity: Implications for Carbon Metabolism during Fermentation. *Foods* **2022**, *11*, 3623. [CrossRef] [PubMed]
43. Deja, S.; Fletcher, J.A.; Kim, C.W.; Kucejova, B.; Fu, X.R.; Mizerska, M.; Villegas, M.; Pudelko-Malik, N.; Browder, N.; Inigo-Vollmer, M.; et al. Hepatic malonyl-CoA synthesis restrains gluconeogenesis by suppressing fat oxidation, pyruvate carboxylation, and amino acid availability. *Cell Metab.* **2024**, *36*, 1088–1104. [CrossRef] [PubMed]
44. Xue, C.; Li, G.L.; Zheng, Q.X.; Gu, X.Y.; Shi, Q.M.; Su, Y.S.; Chu, Q.F.; Yuan, X.; Bao, Z.Y.; Lu, J.; et al. Tryptophan metabolism in health and disease. *Cell Metab.* **2023**, *35*, 1304–1326. [CrossRef]
45. Harris, P.S.; McGinnis, C.D.; Michel, C.R.; Marentette, J.O.; Reisdorph, R.; Roede, J.R.; Fritz, K.S. Click chemistry-based thiol redox proteomics reveals significant cysteine reduction induced by chronic ethanol consumption. *Redox Biol.* **2023**, *64*, 102792. [CrossRef]

46. Puhakka, E.; Ahmed, H.; Haikonen, R.; Leclercq, S.; Hanhineva, K.; Maccioni, L.; Amadieu, C.; Lehtonen, M.; Maennistoe, V.; Rysae, J.; et al. Serum Metabolite Profile in Progressive Versus Nonprogressive Alcohol-Related Liver Disease: A Cross-Sectional Metabolomics Study. *Liver Int.* **2025**, *45*, 70128. [CrossRef]
47. Zhu, X.P.; Huang, Q.X.; Ma, S.; Chen, L.Y.; Wu, Q.; Wu, L.; Ma, H.; Li, X.M.; Li, Q.; Aleteng, Q.; et al. Presence of sarcopenia identifies a special group of lean NAFLD in middle-aged and older people. *Hepatol. Int.* **2023**, *17*, 313–325. [CrossRef]
48. Tamai, Y.; Chen, Z.; Wu, Y.; Okabe, J.; Kobayashi, Y.; Chiba, H.; Hui, S.P.; Eguchi, A.; Iwasa, M.; Ito, M.; et al. Branched-chain amino acids and l-carnitine attenuate lipotoxic hepatocellular damage in rat cirrhotic liver. *Biomed. Pharmacother.* **2021**, *135*, 111181. [CrossRef]
49. Song, X.; Cui, W.; Meng, F.; Xia, Q.; Li, X.; Hou, M.; Jia, L.; Zhang, J. Glucopyranose from Pleurotus geesteranus prevent alcoholic liver diseases by regulating Nrf2/HO-1-TLR4/NF-κB signalling pathways and gut microbiota. *Food Funct.* **2022**, *13*, 2441–2455. [CrossRef]

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Dietary Intake of a Milk Sphingolipid-Rich MFGM/EV Concentrate Ameliorates Age-Related Metabolic Dysfunction

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Abstract

Background/Objectives: Nutraceuticals containing milk fat globule membranes (MFGMs) and extracellular vesicles (EVs) are purported to abate age-related metabolic dysfunction due to their richness in milk sphingolipids. As such, nutraceuticals offer a compelling strategy to improve metabolic health through dietary means, especially for elderly persons who are unable to adhere to common therapeutic interventions. To address this, we examined the effects of supplementing aged sedentary rats with an MFGM/EV-rich concentrate. **Methods/Results:** In a 25-week study, 89-week-old male rats received either a milk sphingolipid-rich MFGM/EV concentrate or a control supplement. Analysis of metabolic health using a battery of tests, including MS^{ALL} lipidomics of plasma, liver, and other peripheral tissues, revealed that MFGM/EV supplementation promotes accretion of unique sphingolipid signatures, ameliorates ceramide biomarkers predictive of cardiovascular death, and has a general lipid-lowering effect. At the functional level, we find that these health-promoting effects are linked to increased lipoprotein particle turnover, showcased by reduced levels of triglyceride-rich particles, as well as a metabolically healthier liver, assessed using whole-body lipidomic flux analysis. **Conclusions:** Altogether, our work unveils that MFGM/EV-containing food holds a potential for ameliorating age-related metabolic dysfunction in elderly individuals.

Keywords: dietary supplementation; milk fat globule membranes; extracellular vesicles; lipidomics; ceramide biomarkers; hyperlipidemia; lipoprotein particles; lipidomic flux analysis

1. Introduction

The global population is projected to exceed 9 billion by 2050, largely due to rising life expectancy [1]. This demographic shift is leading to a significant rise in the elderly population, with individuals aged 60 and older expected to double to over 2 billion over the next 25 years. As the population ages, the prevalence of age-related metabolic dysfunction [2,3] and its sequelae of cardiometabolic disorders (e.g., obesity, steatohepatitis and cirrhosis, type 2 diabetes, and coronary artery disease) is also rising [4]. This inevitably

places an increasing socioeconomic burden on healthcare systems worldwide. While effective pharmacological treatments and lifestyle interventions, such as physical exercise, reduce morbidity and mortality related to cardiometabolic diseases [5,6], these regimens can be difficult to implement in elderly individuals with high medication burdens, undesirable side effects of drugs, and mobility limitations. In this context, nutraceuticals, unique food constituents that have health benefits beyond their nutritional value, can serve as a compelling complementary approach to support cardiometabolic health.

As nutraceuticals, milk sphingolipids are associated with the amelioration of age-related metabolic dysfunction and improved cardiometabolic health [7–9]. The mechanisms underlying these effects remain largely unknown but are plausibly linked to the essential roles that these lipids play as structural components of biological membranes and signaling molecules [10,11]. Several epidemiological studies suggest that dairy foods can alleviate cardiometabolic risk factors in elderly individuals [12]. Furthermore, clinical trials have shown that intake of food rich in milk sphingolipids can lower risk factors in elderly persons predisposed to cardiovascular events [12,13]. Notably, a recent study of overweight postmenopausal women demonstrated that consumption of a milk sphingolipid-containing cream cheese reduces risk markers [14,15], including lowered plasma levels of cholesterol lipids, triglycerides, and ceramide (Cer) 18:1;2/24:1 is a clinical risk marker for cardiovascular death [16,17] (An overview of lipid class abbreviations used for lipid molecules is provided in Table S1). These health-promoting effects were linked to a reduced intestine-derived chylomicron (CM) load following dietary intake, which in itself is a risk marker of cardiovascular disease (CVD) [18,19]. Additionally, milk sphingolipids can serve as a dietary source for the protective ceramide biomarker Cer 18:1;2/24:0, which helps reduce the risk of fatal cardiovascular outcomes [16], as well as its close structural analogs, Cer 18;1;2/23:0 and Cer 17:1;2/24:0 [20–23], which have not yet been tested for their abilities to reduce CVD risk. Despite these emerging links between the intake of milk sphingolipids and their health-promoting effects in elderly individuals, the molecular underpinnings of these benefits remain poorly understood.

A major source of milk sphingolipids is whey-based concentrates rich in milk fat globule membranes (MFGMs) and extracellular vesicles (EVs) [8,24,25]. MFGM/EVs are colloidal particles that are produced by mammary epithelial cells and contain a high proportion of lipid molecules, especially very-long-chain-containing sphingomyelins and lactosylceramides [26–28]. Following their digestion and uptake, these sphingolipids can serve as structural building blocks for the biosynthesis of lipids involved in a variety of physiological processes, including energy production, membrane formation, and signal transduction events [11]. This is corroborated by several intervention studies in animals and humans showing that milk sphingolipids from MFGM/EV-containing concentrates, or the structural components of these lipids, are taken up and incorporated into tissues at the whole-body level [20–23]. Moreover, we recently demonstrated that adolescent mice fed an MFGM/EV-rich diet had a metabolically healthier liver with a greater flux through metabolic pathways for the production of phosphatidylcholine lipids [20], which, in hepatocytes, are functionally coupled to the production of very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) particles [29–31]. Collectively, these findings suggest that MFGM/EV supplementation may provide health benefits in the context of age-related metabolic dysfunction and related diseases in sedentary elderly individuals.

Here, we address this issue by investigating whether long-term dietary intervention with a milk sphingolipid-rich MFGM/EV concentrate improves the metabolic health of aged sedentary male rats (i.e., quiescent rats housed under regular conditions without exercise regimes). We uncovered that MFGM/EV supplementation promotes accretion of unique sphingolipid signatures at the whole-body level and that this translates to the ame-

lioration of numerous cardiometabolic risk factors. Altogether, our findings suggest that nutraceuticals based on milk sphingolipid-rich MFGM/EV concentrates hold a therapeutic potential for ameliorating age-related metabolic dysfunction in elderly individuals.

2. Materials and Methods

2.1. Chemicals

Cow milk-derived MFGM/EV-rich whey concentrate (derived from skimmed milk) [28], Arla® MIPRODAN 40 calcium caseinate, and Lacprodan® SP-9224 whey protein isolate were procured from Arla Food Ingredients (Viby J, Denmark). Methanol, 2-propanol, and water were purchased from Biosolve BV (Valkenswaard, The Netherlands). Chloroform was acquired from Rathburn Chemicals (Walkerburn, UK). Ammonium formate, $^{2}H_{9}$-choline chloride, $^{13}C_{3}$-choline chloride, and $^{13}C_{6}$-glucose were obtained from Sigma-Aldrich (Buchs, Switzerland). All solvents and chemicals were of the highest analytical grade. Synthetic lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Preparation of Supplements

Nutritional drinks with supplements were prepared at room temperature in an airflow bench. The MFGM/EV drink was prepared as a 10% (*w*/*v*) suspension by dissolving the MFGM/EV-rich ingredient in tap water. To have an equal total protein level, the control drink was prepared as an 8.26% (*w*/*v*) suspension by dissolving equal amounts (1:1, *w*/*w*) of the whey protein isolate and the caseinate in tap water. These nutritional drinks were made on the days of utilization and poured into sterile water drinking bottles. The energy contents of the MFGM/EV-rich concentrate and whey protein–caseinate blend were 1742 and 1574 kJ/100 g, respectively (Table S2).

2.3. In Vivo Study in Rats

The in vivo study was conducted in accordance with European standards and the Danish Law on Animal Experiments, approved by the Danish Animal Experiment Inspectorate (protocol 2020-15-0201-00728; date of issue: 21 December 2020), and carried out at the laboratory animal facility of the Copenhagen University Hospital (Rigshospitalet). Male Wistar rats were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and allowed to acclimatize for one week with ad libitum access to water and regular chow. Moreover, the rats were maintained in pairs of two per cage and at a constant temperature (23 ± 1 °C) and humidity (40 ± 5%) under a 12 h light/12 h dark cycle. The rationale for using only male rats was due to budgetary constraints, as we minimized long-term housing costs by using commercially available aged rats, which were only offered as males.

A 25-week intervention study was carried out with elderly rats (62–63 weeks old at arrival). These rats were allocated into two dietary groups, each with 22 animals housed in 11 cages. During the first eight weeks, each pair of rats received 300 mL of the MFGM/EV drink or the control drink three times per week by giving access to an additional drinking bottle in the cage. For the remainder of this study, the pairs of rats received 200 mL of the nutritional drinks three times per week. This down-scaling was performed to avoid exhausting the limited amount of available MFGM/EV ingredient prior to the end of the intervention study. The bottles with nutritional drinks were generally emptied within 48 h. Across the study period, the MFGM/EV-supplemented and control rats received a daily dose of approximately 365 mg and 8 mg of milk phospholipids, respectively, per kg of body weight (Table S2). The body weights of rats were measured weekly starting six weeks after onset and through to the end of this study (25 weeks). After 24 weeks, the rats were subjected to the Morris Water Maze test [32]. Nineteen (19) and eighteen (18) rats from

the MFGM/EV-fed group and control group, respectively, survived throughout the study period. In the 25th and final week, 5 rats per dietary group were randomly chosen for an oral choline tracer test (OCTT) (see details below). Moreover, another 10 rats from each group were randomly chosen for the sampling of blood plasma and tissues for the analysis of steady-state lipid and metabolite levels as well as lipoprotein particles.

2.4. Sampling for Steady-State Lipidomics and Metabolomics

For the analyses of steady-state levels, the rats were fasted overnight (~18 h) and then euthanized. Blood plasma was collected via cardiac puncture, followed by whole-body perfusion using ~200 mL of 4 °C phosphate-buffered saline. Biopsies were then taken from the liver, duodenum, lungs, and kidneys. These samples were stored in 2 mL Eppendorf tubes, snap-frozen in dry ice, and kept at −80 °C until further processing.

2.5. Sampling of Blood Plasma for OCTT

For the OCTT, the rats were fasted overnight, baseline blood plasma samples were collected, and subsequently, the animals were dosed orally with 53 mg/kg $^{2}H_{9}$-choline chloride, formulated in a suspension with 5% (*w*/*v*) MFGM/EV ingredient in sterile tap water. Afterward, the rats were provided access to food, and serial sampling of blood plasma via the jugular vein was performed after 1, 2, 5, 30, 51, and 75 h, as well as after 96 h, at the terminal timepoint by cardiac puncture. Notably, the rats were fasted for ~4.5 h prior to the plasma sampling at the 30, 51, and 75 h timepoints, and overnight until the 96 h timepoint. Whole blood was collected in K_3EDTA-coated microvettes, and the plasma was prepared by centrifugation (3000× *g*; 15 min; 4 °C). After centrifugation, the plasma samples were collected in 2 mL Eppendorf tubes, which were snap-frozen in dry ice and stored at −80 °C until further processing.

2.6. Sample Preparation for Steady-State Lipidomic and Metabolomics

The blood plasma and tissue samples were subjected to two-step lipid extraction and MS^{ALL}-based lipidomic analysis, as previously described [31,33,34]. The sample preparation was carried out in a cold room at 4 °C using cold solvents and consumables. The plasma samples (5 µL) were mixed with 195 µL of 155 mM ammonium formate buffer and spiked with 30 µL of an internal lipid standard mixture as well as 10 µL of an internal metabolite standard mixture with $^{13}C_3$-choline and $^{13}C_6$-glucose. The tissue biopsies were homogenized at 80 °C in 155 mM ammonium formate using an ULTRA-TURRAX® (IKA, Staufen, Germany), as previously described [31]. Aliquots (70 µL) of the homogenates corresponding to 0.1 mg of liver, 0.1 mg of kidney, 0.25 mg of lung, and 0.35 mg of duodenum (22 µg of total protein) were mixed with 130 µL of 155 mM ammonium formate buffer and spiked with 30 µL of an internal lipid standard mixture. All samples were extracted by adding 990 µL of chloroform/methanol (15:1, *v*/*v*), followed by vigorous shaking (1400 rpm; 2 h; 4 °C) and centrifugation (1000× *g*; 2 min; 4 °C), to promote phase separation. The lower organic phase, denoted as the apolar lipid extract, was collected in a new vial. The remaining aqueous phase was reextracted with 990 µL of chloroform/methanol (2:1, *v*/*v*) for 1.5 h, followed by centrifugation to promote phase separation. The lower organic phase, denoted as the polar lipid extract, was collected in a new vial. An aliquot of the remaining aqueous phase (350 µL) was also collected in a new vial for the extracts of plasma samples. All extracts were vacuum-evaporated and stored at −20 °C until analysis.

2.7. Steady-State Lipidomics

The lipid extracts were dissolved in 100 µL of chloroform/methanol (1:2, *v*/*v*), gently shaken (700 rpm; 3 min), and centrifuged (13,000 rpm; 3 min). The apolar lipid extracts

(10 μL) were loaded into 96-well plates and mixed with 12.9 μL of 13.3 mM ammonium formate in 2-propanol for analysis in positive-ion mode and 12.9 μL of 1.33 mM ammonium formate in 2-propanol for analysis in negative-ion mode. The polar lipid extracts (10 μL) were loaded into 96-well plates and mixed with 0.01% methylamine in methanol for analysis in negative-ion mode. The lipid extracts were analyzed by MS^{ALL} analysis in positive- and negative-ion mode using an Orbitrap Fusion Tribrid (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion Biosciences, Ithaca, NY, USA). The apolar extracts were infused using a back pressure of 1.25 psi and an ionization voltage of ±0.95 kV. The polar extracts were infused using a back pressure of 0.6 psi and an ionization voltage of −0.96 kV. High-resolution survey Fourier-transform MS ($FTMS^1$) spectra were recorded across the range of *m/z* 280 to 1400 using a max. injection time of 100 ms, automated gain control at 1×10^5, three microscans, and a target resolution of 500,000. Consecutive $FTMS^2$ spectra were acquired for all precursors in the range of *m/z* 398.3 to 1000.8 in steps of 1.0008 Da, recorded across a range starting from *m/z* 150 until the precursor *m/z* value of + 10 Da, using a max. injection time of 100 ms, automated gain control at 5×10^4, one microscan, a target resolution of 30,000, HCD fragmentation, and a quadrupole ion isolation width of 1.0 Da. Targeted monitoring of ammoniated cholesterol (*m/z* 404.38869) and its internal standard cholesterol+$^2H_7$ (*m/z* 411.43265) was performed by MSX analysis using a max. injection time of 600 ms, automated gain control at 5×10^4, five microscans, a target resolution of 120,000, HCD fragmentation at 8%, and a quadrupole ion isolation width of 1.5 Da for each precursor [34].

2.8. Steady-State Metabolomics

The metabolite extracts were dissolved in 100 μL of methanol/water (5:1, *v*/*v*) and gently shaken (700 rpm; 5 min) and centrifuged (20,000 rpm; 3 min). The extracts (10 μL) were loaded into 96-well plates and analyzed directly by MS^{ALL} analysis in positive-ion mode using an Orbitrap Fusion Tribrid (Thermo Fisher Scientific) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion Biosciences). The extracts were infused using a back pressure of 0.75 psi and an ionization voltage of 1.0 kV. High-resolution survey Fourier-transform MS ($FTMS^1$) spectra were recorded across the range of *m/z* 50 to 450 using a max. injection time of 100 ms, automated gain control at 1×10^5, three microscans, and a target resolution of 500,000. Consecutive $FTMS^2$ spectra were acquired for all precursors in the range of *m/z* 60 to 424 in 1 Da steps, using a max. injection time of 100 ms, automated gain control at 5×10^4, one microscan, a target resolution of 120,000, HCD fragmentation, and a quadrupole ion isolation width of ±0.5 Da.

2.9. Lipidomic Flux Analysis of OCTT Samples

Plasma samples (5 μL) from the OCTT were extracted and analyzed essentially as described above. Notably, a higher target resolution of 240,000 was used for the $FTMS^2$ analysis. Otherwise, instrument settings were similar to those specified in the previous section.

2.10. Profiling of Lipoprotein Particles

The plasma samples were analyzed by gel permeation chromatography coupled to online monitoring of total levels of glyceride and sterol levels at Immuno-Biological Laboratories (Gunma, Japan), as previously described [35,36].

2.11. Lipid Quantification

The identification and quantification of lipid molecules was performed using the ALEX123 software and a data-processing pipeline in SAS 9.4 (SAS Institute, Cary, NC, USA) [33,37–39]. Briefly, lipid molecules detected by full-scan FTMS were identified using

a maximum *m/z* tolerance of ±0.0040 amu, corrected for potential ^{13}C isotope interference, and reported at the "species level". Lipid molecules were confirmed through molecular lipid species-specific fragments and lipid class-specific fragments detected by $FTMS^2$ analysis using a maximum *m/z* tolerance of ±0.0065 amu [38,39]. For the analysis of OCTT data, a maximum *m/z* tolerance of ±0.0015 amu was used for the identification of stable isotope-labeled phosphocholine fragments derived from labeled choline-containing lipid precursor ions (i.e., *m/z* 193.1298 for lipids labeled with $^2H_9$, *m/z* 190.1110 for lipids labeled with $^2H_6$, *m/z* 187.0922 for lipids labeled with $^2H_3$, and *m/z* 184.0733 for unlabeled lipids). Identified lipid molecules were quantified by normalizing their intensities to those of the respective internal lipid standards, subsequent multiplication by the amounts of the respective lipid standards, and normalization to the extracted sample amount (i.e., μL of plasma, mg of tissue, or μg of protein).

2.12. Metabolite Quantification

The quantitative profiling of metabolites was carried out using the ALEX123 software with a manually curated metabolite database and a data-processing pipeline in SAS 9.4 (SAS Institute). Sodiated glucose and the internal $^{13}C_6$-glucose standard were monitored by high-resolution $FTMS^1$ analysis. Choline, protonated betaine, protonated dimethylglycine, and a $^{13}C_3$-labeled choline internal standard were monitored by high-resolution $FTMS^2$ analysis. The absolute level of glucose was quantified by normalizing its intensity to that of the internal $^{13}C_6$-glucose standard, multiplication by the molar spike amount (10 nmol), and, finally, normalization to the extracted amount of plasma (5 μL). The absolute levels of the other metabolites were estimated by normalizing the sum of their fragment intensities to the sum of the fragment intensity of the internal $^{13}C_3$-choline standard, multiplication by the molar spike amount (100 pmol), and normalization to the extracted amount of plasma.

2.13. Proteomics

Untargeted analysis of the plasma proteome and a targeted quantitative analysis of ApoB48 and ApoB100 were performed using plasma samples. For targeted analysis, a mixture of synthetic unlabeled peptides (ApoB48: LSQLETYAI; ApoB100: ASEAVYDYVK (Thermo Fisher Scientific)) was prepared and serially diluted to generate seven standard solutions ranging from 0.5 μmol/L to 10 μmol/L. The isotopically labeled peptides LSQLETYA-[$^{13}C_6{}^{15}N$]I and ASEAVYDYV-[$^{13}C_6{}^{15}N_2$]K were used as internal standards and added to the digestion buffer prior to the sample preparation. The standard solutions and plasma samples were processed using the ProteinWorks™ eXpress kit (Waters Corporation, Milford, MA, USA), as described previously [40,41]. Briefly, 20 μL of each sample was reduced, alkylated, and digested overnight with trypsin. The resulting peptides were purified in 30 mg Oasis HLB cartridges (Waters Corporation), dried under nitrogen, and reconstituted in 100 μL of 5% acetonitrile containing 0.1% formic acid.

Targeted analyses were performed using an Xevo TQ Absolute triple quadrupole mass spectrometer (Waters Corporation) equipped with an electrospray ionization interface and coupled to an Acquity Premier UPLC system (Waters Corporation). Peptide separation was achieved in an Acquity Premier BEH C_{18} column (2.1 × 100 mm, 1.7 μm, VanGuard™ FIT; Waters Corporation), maintained at 60 °C, using a linear gradient of mobile phase B (100% acetonitrile with 0.1% formic acid) in mobile phase A (5% acetonitrile with 0.1% formic acid), as previously described. Peptides were detected in positive-ion mode under the following conditions: a capillary voltage of 3 kV; a desolvation gas (N_2) flow rate of 650 L/h; a desolvation temperature of 450 °C; and a source temperature of 120 °C. The multiple reaction monitoring mode was used for detection (Table S3). Data acquisition and analysis were performed using the MassLynx and TargetLynx software (version 4.1; Waters Corpo-

ration). Quantification was based on the chromatographic peak area ratios of unlabeled to labeled peptides, and calibration curves were established from the standard solutions.

Untargeted proteomic analyses were carried out on the same plasma digests using label-free quantification by nano-LC-MS/MS [41]. The samples were analyzed with an Exploris 480 mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source and coupled to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). Peptides were separated in a 75 μm × 500 mm C_{18} column (EASY-Spray PepMap; Thermo Fisher Scientific). Data acquisition was performed using the Xcalibur software (version 2.9; Thermo Fisher Scientific). Protein identification was realized using Proteome Discoverer (version 2.5; Thermo Fisher Scientific) through a database search against the rat proteome. Trypsin/P was selected as the proteolytic enzyme, allowing up to two missed cleavages and a peptide length of greater than six amino acids. The precursor and fragment mass tolerances were set to ±10 ppm and ±20 ppm, respectively. The following peptide modifications were considered during the search: carbamidomethylation of cysteine (+57.0214 Da, fixed), *N*-terminal acetylation (+42.0105 Da, variable), and methionine oxidation (+15.9949 Da, variable).

2.14. Statistical Analysis

Statistical analyses were carried out using SAS 9.4 (SAS Institute). This included ANOVA with post hoc testing using multiple hypothesis correction (PROC GLM), nonparametric testing (PROC NPAR1WAY), and mixed-effects modeling (PROC MIXED). A *p*- or q-value of less than 0.05 was considered statistically significant. Outliers were identified and removed using the interquartile range test prior to statistical testing. Visualization of the data was realized using Tableau Desktop 2024 (Tableau Software) or SAS 9.4.

3. Results and Discussion

3.1. Study Design

To explore the metabolic effects of supplementing aged rats with the milk sphingolipid-rich MFGM/EV-based concentrate, we carried out a 25-week intervention study (Figure 1A). Here, elderly male rats, 63–64 weeks (~14.6 months) old, at the beginning of this study, were allocated into a treatment group or a control group. Rats in the treatment group were supplemented three times per week with a nutritional drink containing the MFGM/EV concentrate. Rats in the control group were supplemented three times per week with a drink containing a milk lipid-deficient whey protein–caseinate blend. Throughout this study, the sedentary rats (i.e., regularly housed rats not subjected to exercise regimens) were also fed a base diet ad libitum composed of regular chow. This intervention study included longitudinal monitoring of body weight (Figure 1A). At the end of this study, we carried out an oral choline tracer test (OCTT), together with lipidomic flux analysis, to assess the metabolic fitness of the rats. We also performed cognitive tests, involving physical activity, the results of which are reported separately [32]. Following euthanasia, samples of multiple tissues were collected for lipidomic analysis, as well as blood plasma for profiling metabolites and lipoprotein particles.

Over the course of this study, body weight demonstrated no significant difference between the two dietary regimes (Figure 1B). However, physical activity from the cognitive testing resulted in significant weight loss for both groups, which was marginally greater for the MFGM/EV-fed rats compared with the control rats (Figure 1C). Testing the fasting blood glucose level, a proxy for glucose intolerance, showed no difference (Figure 1D). Since the MFGM/EV concentrate is a source of choline [28], we also assessed the plasma levels of choline and its metabolites, betaine and dimethylglycine. The levels of these were also not different between the two groups (Figure 1D). Altogether, these data show that

the MFGM/EV supplementation was well-tolerated by the aged rats and suggest that the concentrate might have health-promoting effects, given the slightly greater weight loss in response to physical activity in the treatment group.

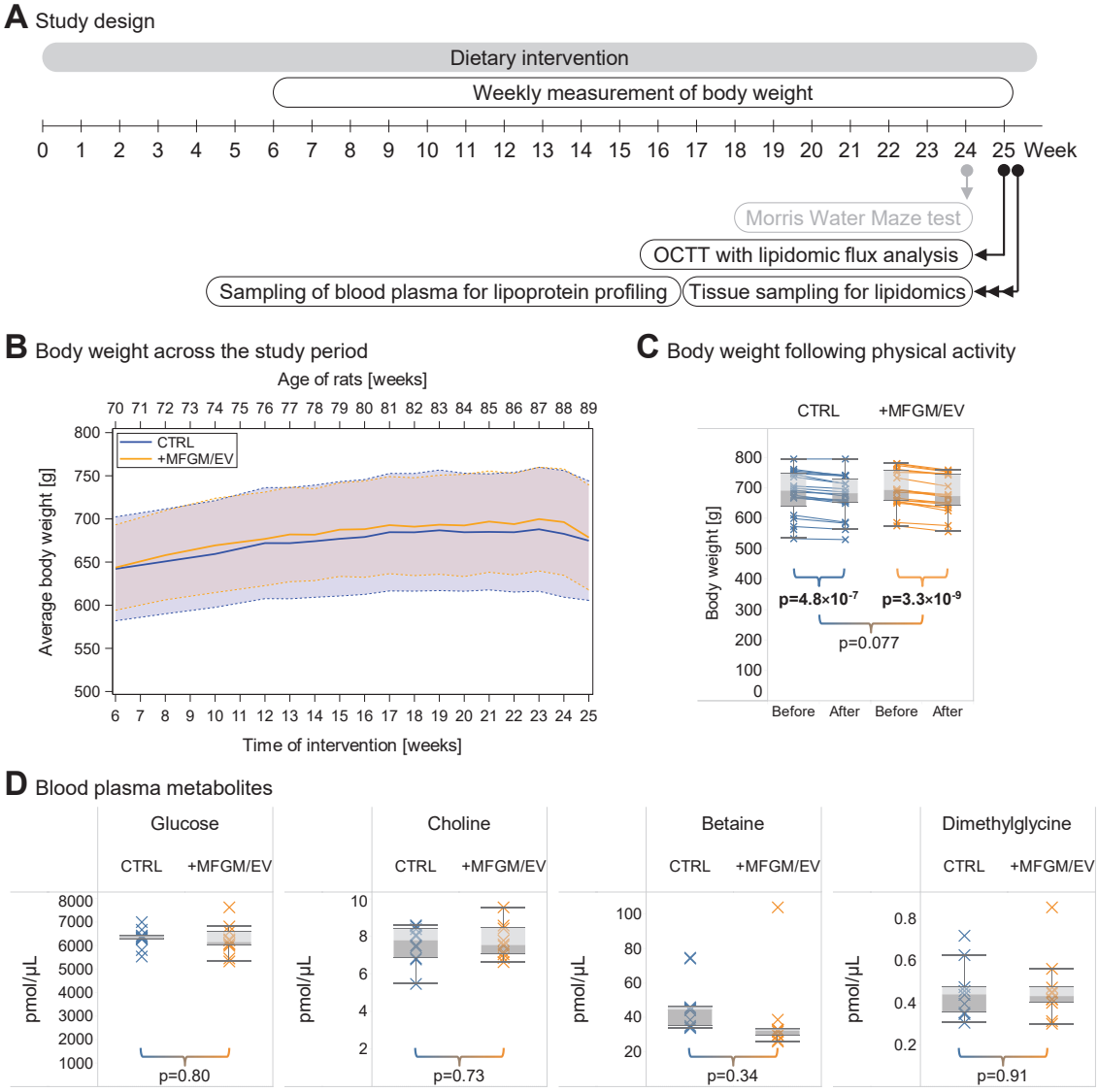


Figure 1. The study design and basic endpoints. (**A**) The design of the intervention study with highlights of timepoints for longitudinal and endpoint measurements. A Morris Water Maze test was carried out after 24 weeks to assess the cognitive performance of the animals. The results are reported [32]. OCTT, oral choline tracer test. (**B**) Body weight as a function of intervention time (lower *x*-axis) and rat age (upper *x*-axis). Each line plot represents the mean ± SD indicated by the band of the same color (n = 19–21 rats/group). A repeated-measures ANOVA F-test shows no significant differences in body weight for the two groups of rats (p = 0.8). (**C**) Changes in body weight following physical activity. The data are represented as box plots with medians, and crosses show individual values (n = 19–20 rats/group). Statistical analysis was carried out by Student's t-test. Significant differences are highlighted in bold. (**D**) Levels of metabolites in plasma. The data are represented as box plots with medians, and crosses show individual values (n = 10 rats/group). Statistical analysis was performed by Student's t-test.

3.2. MFGM/EV Supplementation Promotes Accretion of Milk-Related Sphingolipids in Blood Plasma

Next, we examined whether the dietary intervention elevated levels of very-long odd-chain sphingolipids, a change we previously observed in adolescent mice [20]. To this

end, we carried out in-depth MS^{ALL} lipidomic analysis [33] of blood plasma collected after 25 weeks of supplementation. This analysis quantified (i.e., pmol/μL plasma) 673 lipid molecules, encompassing 21 lipid classes (Table S4). A total of 417 lipid molecules were monitored at the molecular species level, with assignments of individual hydrocarbon chains (e.g., PC 16:0–18:2), and 256 lipids were monitored at the species level with assignment of the total number of C atoms, double bonds, and hydroxyl groups in all hydrocarbon chains (e.g., PE 38:4).

To survey overall effects, we carried out an analysis of variance (ANOVA) with multiple hypothesis correction. Using a stringent statistical threshold with a q-value of <0.01 (i.e., a multiple hypothesis-corrected *p*-value), we shortlisted a total of 96 lipids with significantly altered abundance (Figure S1A). Using these lipid concentrations for unsupervised principal component analysis demonstrated that the plasma lipid profiles of the MFGM/EV-supplemented rats were clustered together and separately from the control rats (Figure 2A).

To examine the metabolic differences in further detail, we generated a volcano plot, which revealed that the lipid profiles of the MFGM/EV-fed rats were unique in having primarily elevated levels of sphingolipids and glycerophospholipids, whereas the lipids elevated in the aged control rats belonged to all lipid categories (Figures 2B and S1B). More specifically, for the MFGM/EV-fed rats, we found that the elevated lipids were primarily sphingomyelins, ceramides, and phosphatidylcholines, with odd-numbered sphingoid and fatty acyl chains (e.g., Cer 17:1;2/24:1, SM 18:1;2/23:0, and PC 15:0–18:2), which was similar to our observation for the MFGM/EV-fed mice. In contrast, the control rats had higher levels of triglycerides, cholesteryl esters, phosphatidylcholines, sphingomyelins, and ceramides, with even-numbered fatty acyl and sphingoid chains (e.g., TAG 62:11, CE 22:4, PC 16:0–20:4, SM 18:1;2/24:1, and Cer 18:1;2/24:1) (Figure S1B).

To explore the lipid metabolic effects in further detail, we inspected the overlap between elevated lipids in the aged rats and adolescent mice fed the MFGM/EV-rich concentrate [20]. Notably, 37 of 86 lipids consistently elevated in mouse plasma were also increased in the plasma of the aged MFGM/EV-fed rats (Figure 2C). Among these were 12 sphingolipids (e.g., 5 ceramides and 6 sphingomyelins), 18 glycerophospholipids (e.g., 11 phosphatidylcholines), and 5 cholesteryl esters (i.e., sterol lipids) (Figure 2D). Notably, these lipid molecules featured primarily odd-numbered fatty acyl and sphingoid chains (Figure 2E).

Strikingly, among the common lipids, we again found several very-long-odd-chain sphingolipids, including Cer 18:1;2/23:0 and its isomer Cer 17:1;2/24:0, as well as the downstream metabolic products SM 18:1;2/23:0 and HexCer 18:1;2/23:0 (Figure 2E). Furthermore, we also found Cer 18:1;2/23:1 and its isomer Cer 17:1;2/24:1, as well as the metabolic product SM 18:1;2/23:1. The concentrations of these sphingolipids were generally low in the control rats, ranging from ~0.5 pmol/μL for the ceramides to ~6 pmol/μL for the sphingomyelins (Figure 2F). The intake of the MFGM/EV concentrate increased these sphingolipids by 2.4- to 8.7-fold, which is similar to what we observed in mice. Overall, these data show that the MFGM/EV supplementation increased the plasma levels of sphingolipids with molecular structures identical to those of prominent sphingolipids in the MFGM/EV concentrate (e.g., 18:1;2/23:0 and 17:1;2/24:0) [28]. Additionally, the similarities between the aged rats and adolescent mice demonstrate that the effects of the MFGM/EV supplementation are generalizable.

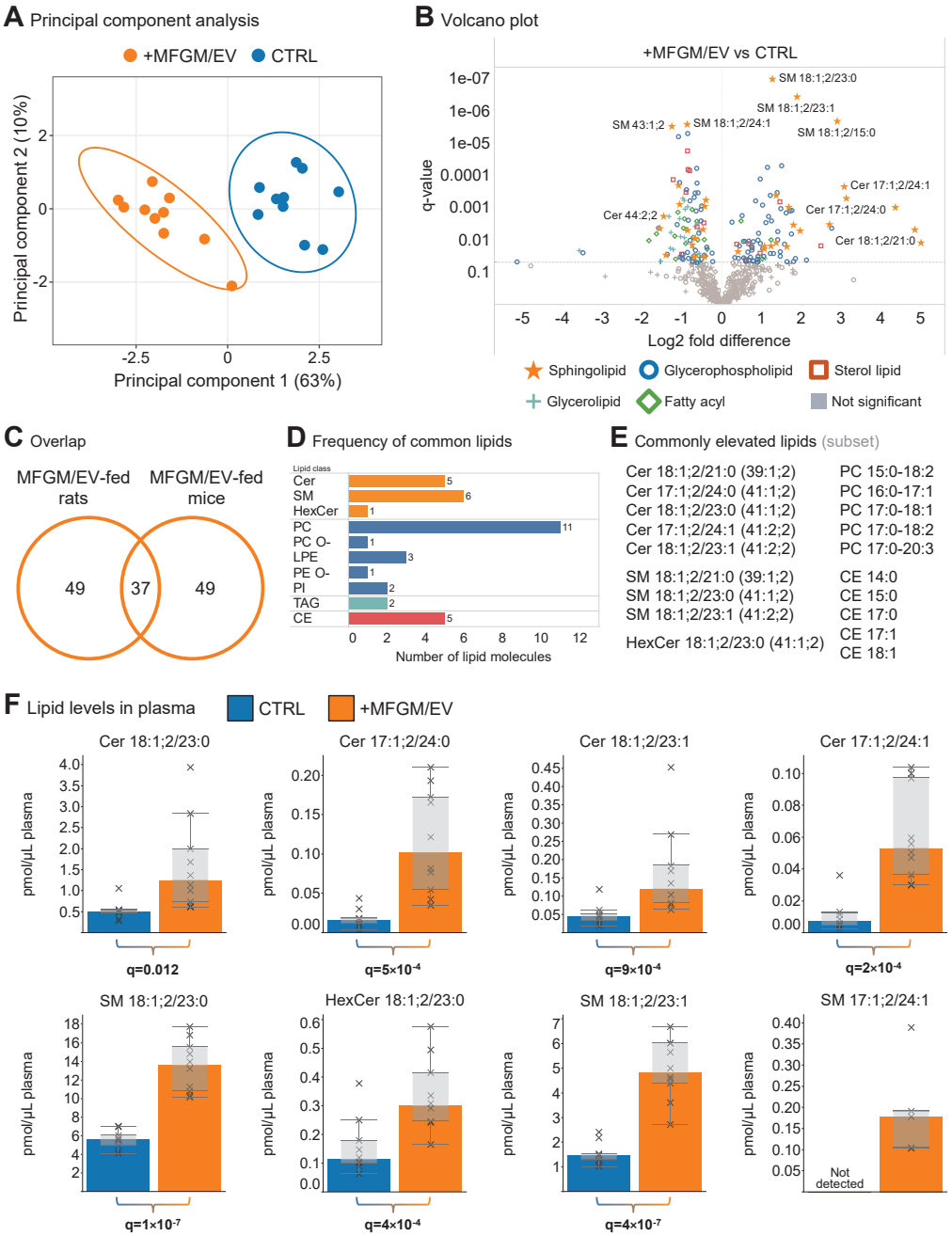


Figure 2. MFGM/EV supplementation elevates plasma levels of very-long-odd-chain sphingolipids. (**A**) Principal component analysis of significantly different plasma lipids (n = 10 rats/group). Ellipses indicate 95% confidence intervals. (**B**) Volcano plot showing lipids with significantly elevated and lowered plasma levels. The horizontal line indicates the significance level at q-value = 0.05. Statistical analysis was carried out using ANOVA with multiple hypothesis correction. (**C**) Venn diagram showing numbers of lipid molecules that are significantly elevated in plasma of MFGM/EV-fed rats and mice. The shortlist of lipids in mice is taken from [20]. The shortlists are based on q-value < 0.05 and fold change > 1. (**D**) Distribution of significantly elevated lipids that are common for MFGM/EV-fed rats and mice. (**E**) Examples of lipid molecules that are consistently elevated in both MFGM/EV-fed rats and mice. Sphingolipid molecules are denoted at both the molecular species level and species level (in parentheses) to emphasize isomeric and metabolic relationships between the ceramide (Cer), sphingomyelin (SM), and hexosylceramide (HexCer) species. (**F**) Concentrations of representative sphingolipids that are significantly and consistently elevated in plasma of rats and mice fed the MFGM/EV ingredient. Data are represented by medians, box plots, and crosses show individual values (n = 5–10 rats/group). Statistical analysis was performed by ANOVA with multiple hypothesis correction. Significant differences are highlighted in bold.

3.3. Accretion of Unique Sphingolipid Signatures in Tissues

Our study with mice also revealed that an MFGM/EV-rich diet prompts a variety of lipid metabolic effects in tissues with different metabolic and physiological functions. To examine whether this is also the case for the aged rats, we analyzed the lipidomes of the duodenum, liver, lungs, and kidneys. On average, the lipidomic analyses quantified (e.g., pmol/mg of tissue) 661 lipid molecules covering 26 lipid classes (Table S5). To pinpoint lipidomic differences, we carried out ANOVA with multiple hypothesis correction. A stringent statistical threshold with a q-value of <0.01 shortlisted 126, 129, 90, and 79 significantly altered lipid abundances in the duodenum, liver, lungs, and kidneys, respectively (Figure 3A). Altogether, this shows that the MFGM/EV supplementation affected the lipid metabolic activities of primarily the duodenum and liver and, to a lesser degree, those of the lungs and kidneys (Figure 3A–E).

To better understand the metabolic effects of the MFGM/EV concentrate at the systemic level, we examined the overlap between lipid molecules that increased concurrently in blood plasma and the four tissues. To do so, we selected lipids that were significantly elevated in the MFGM/EV treatment group (q-value < 0.05 and fold change > 1) and shortlisted lipids that were elevated in four or five of the biopsies. This list featured a total of 27 lipid molecules, whereof 26 were increased in the duodenum, 16 were increased in the plasma, 23 were increased in the liver, 25 were increased in the lungs, and 24 were increased in the kidneys (Figures 3F and S2). Among the common lipids, 10 were sphingolipids (e.g., 4 ceramides and 5 sphingomyelins) and 16 were glycerophospholipids (e.g., 10 phosphatidylcholines). Remarkably, and similar to our finding for mice, these molecules featured primarily odd-numbered fatty acyl and sphingoid chains (e.g., Cer 17:1;2/24:0 and PE 17:0–20:4).

Upon closer inspection of the lipid molecules, we again observed a striking molecular relationship between the subset of odd-chain sphingolipids. Across all tissues, we consistently identified Cer 18:1;2/23:0 and its downstream metabolic products SM 18:1;2/23:0 and HexCer 18:1;2/23:0 (Figure 3F). Similarly, Cer 18:1;2/23:1 and its metabolic product SM 18:1;2/23:1 were also consistently elevated in MFGM/EV-fed rats. The concentrations of these sphingolipids were generally low in the tissues of the aged control rats (Figure 4). The intake of the MFGM/EV supplement consistently increased these sphingolipids by 1.7- to 5.9-fold. Notably, several other odd-chain ceramide and sphingomyelin species were also elevated across all the tissues (Figure 3F; Tables S4 and S5). Taken together, these observations mirror our previous finding in mice and underpin a causal relationship between prominent very-long-odd-chain sphingolipid constituents in the ingested MFGM/EV-rich concentrate [28] and the specific accretion of endogenous sphingolipids having identical backbone structures (e.g., 18:1;2/23:0 and 17:1;2/24:0).

3.4. High-Risk CVD Biomarkers Are Reduced by MFGM/EV Supplementation

Certain plasma ceramides are high-risk biomarkers and purported mediators of cardiometabolic morbidity and mortality in humans (Figure 5A) [16,17,42]. Given that MFGM/EV supplementation influences levels of ceramides at the whole-body level, we assessed whether the dietary intervention also modulates plasma ceramide biomarkers of CVD in aged rats. Notably, the molar ratio between the even-numbered long-chain Cer 18:1;2/16:0 species and the very-long-chain Cer 18:1;2/24:0 species is a high-risk marker predictive of a fatal cardiovascular outcome. Likewise, the molar ratio between Cer 18:1;2/24:1 and Cer 18:1;2/24:0 is also a high-risk marker. Assessing these high-risk markers demonstrated that the MFGM/EV-fed rats had a significant 1.4-fold (−29%) lower Cer 18:1;2/16:0-based risk score ($p = 0.038$) as well as a significant 1.8-fold (−44%) lower Cer 18:1;2/24:1-based risk score ($p = 0.00033$) (Figure 5B). Underpinning this is the lowering

of plasma concentrations for the risk markers Cer 18:1;2/16:0 and Cer 18:1;2/24:1, together with a constant level of the protective marker Cer 18:1;2/24:0 (Figure S3A).

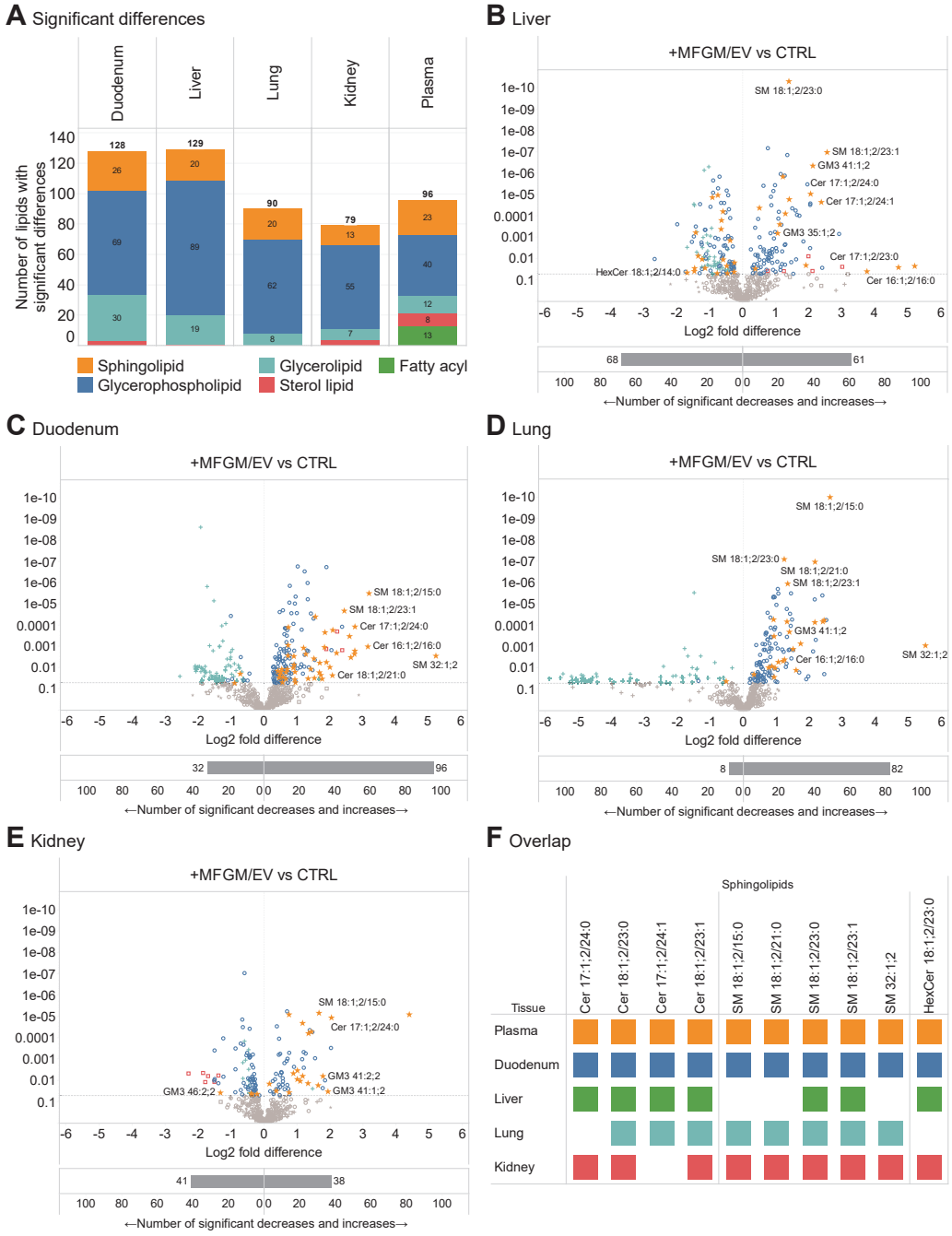


Figure 3. The MFGM/EV concentrate remodels the sphingolipidome of tissues. (**A**) Number of lipid molecules with a significant difference between the two dietary groups. Statistical analysis was performed using ANOVA followed by post hoc testing with multiple hypothesis correction. Lipids with q-value < 0.01 are counted as significantly different. (**B**–**E**) Volcano plots comparing lipids with significant difference between the groups of rats in the liver, duodenum, lungs, and kidneys, respectively. The horizontal line indicates the significance level at q-value = 0.05. Color-symbol scheme is the same as in Figure 2B. Underlying bar graphs show the number of significantly reduced and increased lipid abundances. Statistical analysis was performed as specified for panel A. (**F**) Overlap between significantly and consistently elevated sphingolipids in plasma and tissues of aged rats fed the MFGM/EV concentrate. Sphingolipids with q-value < 0.05 and fold difference > 1 are considered significantly different.

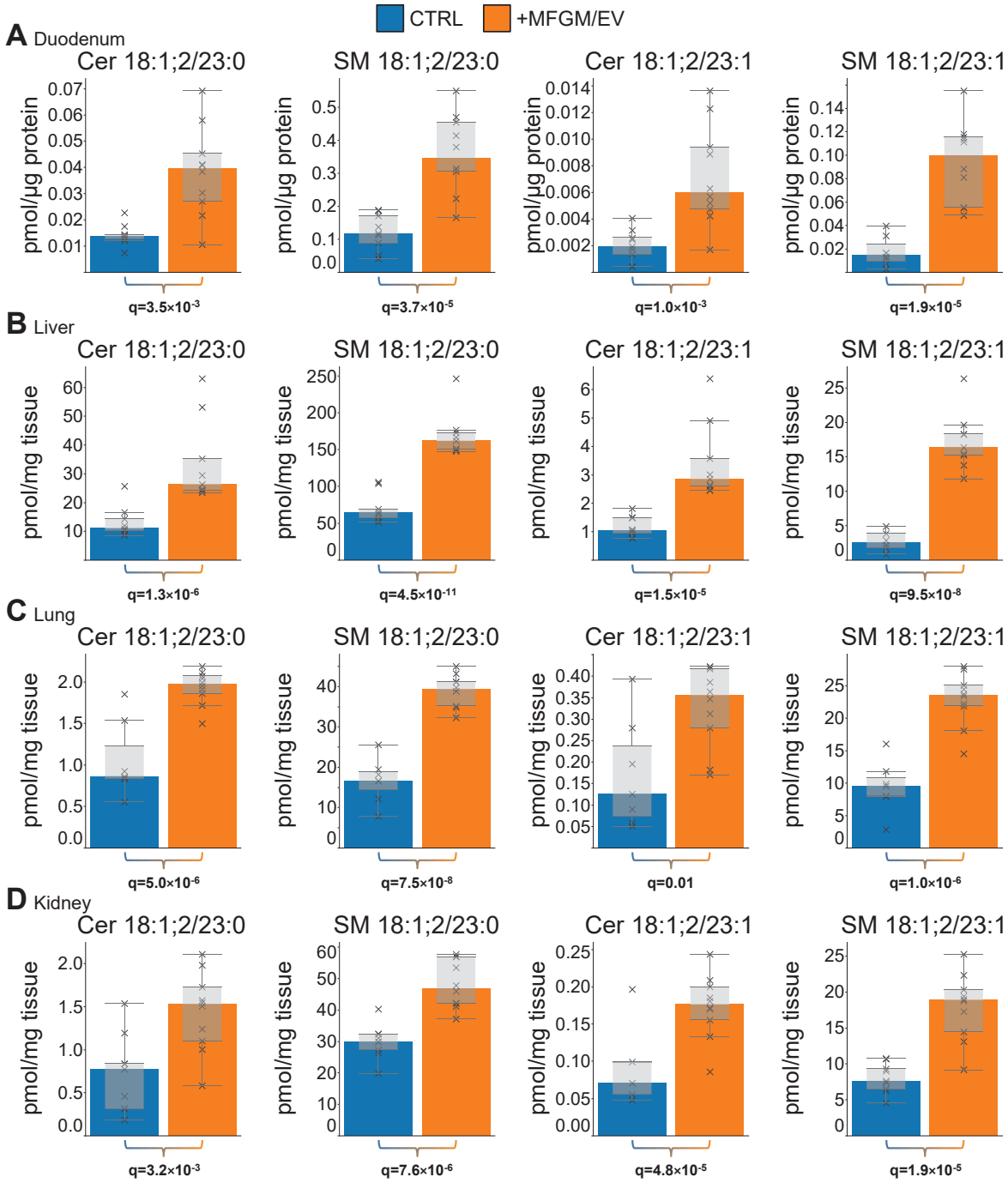


Figure 4. MFGM/EV supplementation promotes accretion of very-long-odd-chain sphingolipids at the whole-body level. (**A**–**D**) Levels of 23:0- and 23:1-containing sphingolipids that are significantly and consistently elevated in the indicated tissues of aged rats fed the MFGM/EV ingredient in the duodenum, liver, lungs, and kidneys, respectively. Data represent box plots, with bars indicating medians and crosses showing individual values. Statistical analysis was performed by ANOVA with multiple hypothesis correction. Significant differences are highlighted in bold.

Importantly, MFGM/EV supplementation specifically increases levels of ceramides that are close structural analogs of the even-chain risk markers. Specifically, Cer 18:1;2/23:0 and Cer 17:1;2/24:0 are potential protective markers as they closely mimic Cer 18:1;2/24:0 by a single methylene unit in their hydrocarbon chains (Figure 5C). Similarly, Cer 18:1;2/15:0 is a potential risk marker as it resembles Cer 18:1;2/16:0, and Cer 18:1;2/23:1 and Cer 18:1;2/24:1 are potential risk markers as they mimic Cer 18:1;2/24:1.

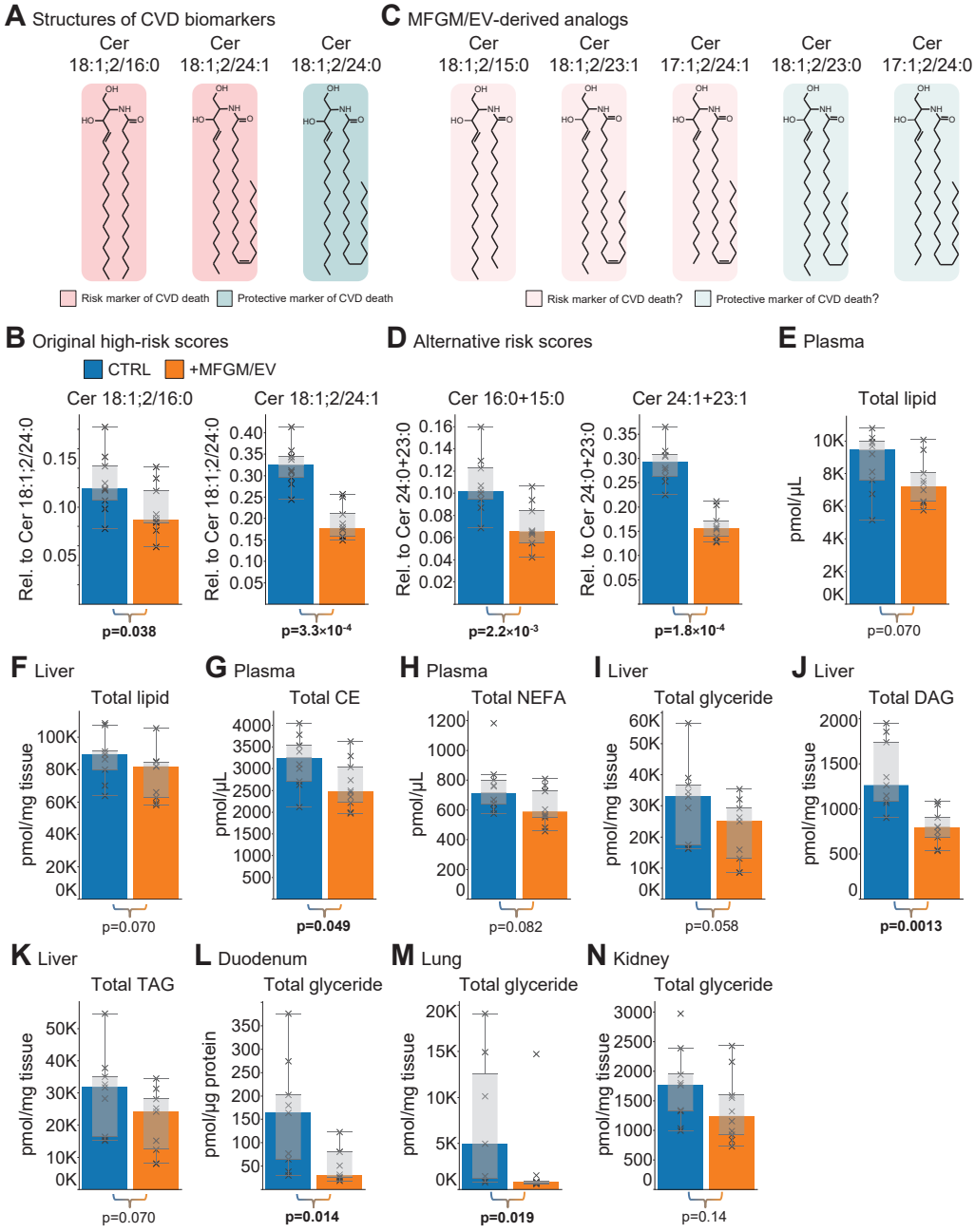


Figure 5. The MFGM/EV concentrate reduces lipid biomarkers associated with cardiometabolic disease. (**A**) Molecular structures of plasma ceramide biomarkers predictive of CVD mortality. (**B**) Original ceramide high-risk scores predictive of CVD death. (**C**) Molecular structures of ceramide biomarker analogs identified in aged MFGM/EV-fed rats. (**D**) Alternative ceramide risk scores that consider levels of original ceramide biomarkers and their structural analogs. (**E**–**N**) Total levels of indicated lipid categories and classes in specified biopsies. Data are represented by medians, box plots, and crosses show individual values (n = 7–10 rats/group). Statistical analysis was performed by a non-parametric unpaired two-sample Wilcoxon test. Significant differences ($p < 0.05$) are highlighted in bold.

To determine whether the reduction in the original CVD risk scores (Figure 5B) is biased by the accretion of odd-chain ceramides from the MFGM/EV concentrate, we recalculated the high-risk scores by factoring in the plasma concentrations of the different odd-chain analogs. Notably, this revealed a more pronounced and significant 1.6-fold (−38%) lowering ($p = 0.0022$) of the long-chain risk score that considers 16:0- and 15:0-ceramides relative to 24:0- and 23:0-ceramides (Figure 5D). Concordantly, the very-long-chain risk score,

considering 24:1- and 23:1-ceramides relative to 24:0- and 23:0-ceramides, also showed a more pronounced and significant 1.9-fold (−47%) lowering (p = 0.00018) (Figure 5D). This lowering of the high-risk scores is primarily driven by the increase in the plasma concentration of the protective analog Cer 18:1;2/23:0 (Figure S3B). Altogether, these data show that the intake of the MFGM/EV-rich concentrate ameliorates high-risk markers of CVD death in aged sedentary rats. Furthermore, these data indicate that the diagnostic utility of the high-risk markers, which currently considers only even-chain ceramides [42], should be extended to also monitor levels of the odd-chain ceramide analogs, to allow better stratification of individuals who frequently consume dairy products [43].

3.5. MFGM/EV Supplementation Improves Biomarkers of Cardiometabolic Health

To assess whether the lowering of the ceramide biomarkers translates to improvement of other, more conventional lipid biomarkers, we examined whether the dietary supplementation also modulates the concentration of all lipids as well as the total levels of glycerides (i.e., diglycerides and triglycerides), non-esterified fatty acids, and cholesterol lipids in plasma and each of the tissues. Overall, this revealed that the MFGM/EV concentrate had a general lipid-lowering effect (Figure S4). For example, the supplementation led to a 1.3-fold (p = 0.070) and 1.1-fold (p = 0.070) lower total lipid concentration in the plasma and liver, respectively (Figure 5E,F). In the plasma, this effect was primarily caused by a significant reduction in the cholesteryl ester level (p = 0.049) (Figure 5G) and a marginal lowering of the non-esterified fatty acid level (p = 0.082) (Figure 5H). In the liver, the effect was primarily due to a subtle lowering of the total glyceride level (p = 0.058) (Figure 5I), which was compounded by a significant reduction in the diglyceride level (p = 0.0013) (Figure 5J) and a marginal lowering of the triglyceride level (p = 0.070) (Figure 5K). Further supporting the notion of a lipid-lowering effect, we also found lower lipid levels in the other tissues, including a significant reduction in the total glyceride level in the duodenum (5.5-fold; p = 0.014) and lungs (6.3-fold; p = 0.019) as well as a marginal reduction in the kidneys (1.4-fold; p = 0.14) in the MFGM/EV-supplemented rats (Figures 5L–N and S4).

Given that lipid markers of cardiovascular disease risk often reflect changes in lipoprotein metabolism, we examined the profile of lipoprotein particles in the two groups of aged rats. To this end, plasma samples were analyzed by gel permeation chromatography with dual monitoring of total levels of glycerides as well as cholesterol lipids (i.e., the sum of free cholesterol and cholesteryl esters) (Figure 6A) [35]. Contrary to traditional lipoprotein-selective assay kits that yield a single concentration value (e.g., LDL-C), the chromatographic approach provides a much broader overview of the full continuum of lipoprotein particles, ranging from large, intestinal-derived CMs to hepatocyte-derived VLDL particles and CM remnants, followed by smaller low-density lipoprotein (LDL) and HDL particles. The analysis demonstrated significantly different profiles for the two groups of rats. Specifically, both the glyceride- and cholesterol-based profiles were elevated in the control rats (Figure 6A), corroborating our observation that the MFGM/EV supplement has a lipid-lowering effect. More specifically, the MFGM/EV-fed rats had significantly lower glyceride levels across most groups of lipoprotein particles as well as a significantly lower level of sterol-containing HDL particles (Figure 6B).

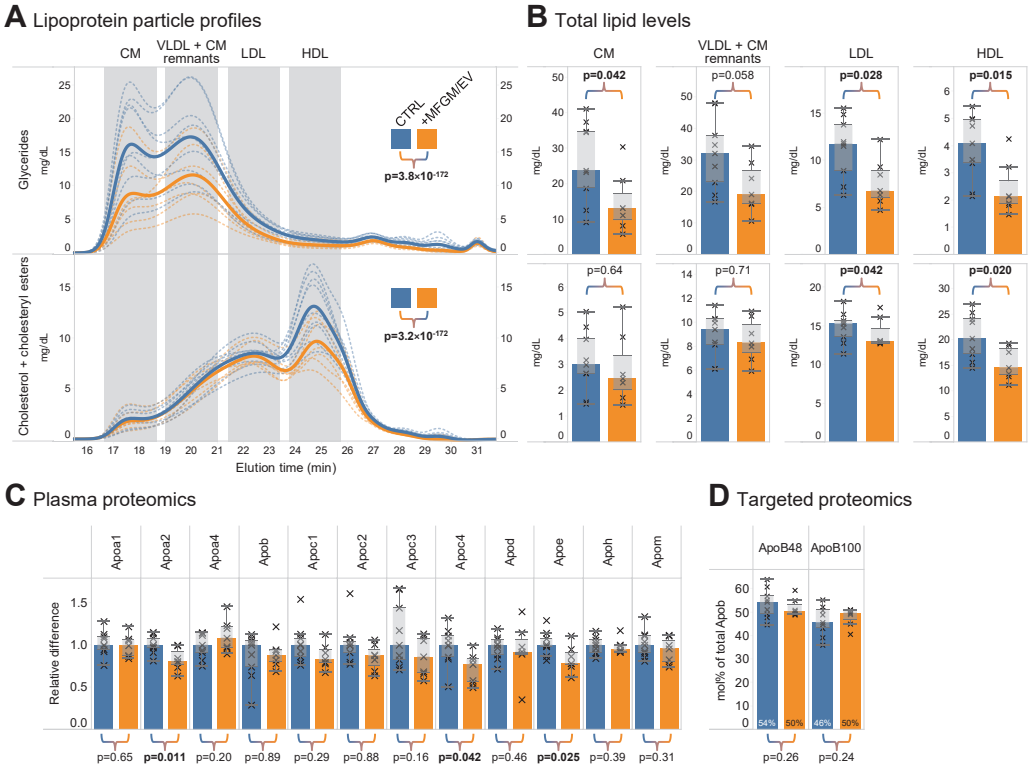


Figure 6. MFGM/EV supplementation modulates the profile of lipoprotein particles. (**A**) Profiles of lipoprotein particles determined by gel permeation chromatography with dual monitoring of total glycerides as well as cholesterol lipid levels. Dashed lines show individual elution profiles, and thick lines show their median profile per intervention group. Statistical analysis was performed by repeated-measures analysis using a linear mixed-effect model. Significant differences are highlighted in bold (n = 7–9 rats). (**B**) Total level of glycerides (upper panel) and cholesterol lipids (lower panel) in major classes of lipoprotein particles. (**C**) Profile of apolipoproteins in plasma. (**D**) Molar fractions of ApoB isoforms. The data are represented by box plots, with bars indicating medians and crosses showing individual measurements (n = 7–9 rats). Statistical analysis was performed by Student's t-test. Significant differences ($p < 0.05$) are highlighted in bold.

Given the functional relationship between lipoprotein particles and the proteins that decorate their surfaces, we explored whether the observed differences in lipoprotein profiles were reflected in the apolipoprotein composition of plasma. Untargeted proteomic analysis identified twelve apolipoproteins, among which three were significantly reduced in the MFGM/EV-fed rats (Figure 6C). These proteins comprise ApoE and ApoC4, which modulate the production and clearance of triglyceride-rich lipoprotein particles (e.g., chylomicrons and VLDL) [44,45], and ApoA2, which is involved in cholesterol efflux from cells to HDL particles [46].

Surprised to find that the level of ApoB, a hallmark of chylomicrons and VLDL particles, did not differ between the two groups of rats, we carried out a targeted proteomic analysis to quantify the molar abundances of the two isoforms ApoB48 and ApoB100, which, in humans, are markers of chylomicrons and VLDL particles, respectively. This analysis showed that the molar ratio of ApoB48 to ApoB100 was 54:46 in aged control rats and 50:50 in MFGM/EV-fed rats (Figure 6D). However, this slight shift in the ApoB48-to-ApoB100 ratio is not statistically significant. Notably, in contrast with humans, the *APOB* transcript in rats is also edited in the liver, resulting in the secretion of both ApoB100- and ApoB48-containing VLDL particles [47]. Accordingly, the lack of a significant difference in the isoform ratio, intended here as a proxy for intestine- versus liver-derived triglyceride-rich lipoproteins, may be masked by the species-specific mRNA editing.

Taken together, these results suggest that supplementing aged rats with food rich in MFGM/EVs primarily lowers lipids and improves a wide range of lipid and lipoprotein biomarkers associated with cardiometabolic health, while the effects on the apolipoprotein profile are comparatively modest.

3.6. Intake of MFGM/EV Concentrate Increases Lipid Metabolic Turnover

Lipoprotein metabolism is tightly coupled to the dynamics of membrane lipid synthesis, especially of choline-containing lipids that decorate the surface of lipoprotein particles. Genetic ablation of the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway reduces hepatic VLDL secretion and renders mice susceptible to steatosis and liver failure [29,48]. Based on this pathophysiological association, we assessed whether intake of the MFGM/EV supplement modulates lipid metabolic turnover at the whole-body level. To do so, we subjected the aged rats to an OCTT (oral choline tolerance test) at the end of the 25 weeks of intervention. In combination with high-resolution lipidomic flux analysis, this test determines the kinetics of $^{2}H_{9}$-choline tracer incorporation into phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin molecules, which is a proxy for the flux through the CDP-choline pathway (Figure 7A). In addition, it also determines the kinetics of $^{2}H_{3}$- and $^{2}H_{6}$-choline incorporation, which is a proxy for the flux through the hepatic PEMT pathway [49]. Altogether, the analysis quantified 186 labeled lipids (Table S6).

The total level of tracer incorporation showed kinetic timelines with apices in the proportion of $^{2}H_{9}$- and $^{2}H_{6}$-labeled lipids after 5 h, and in the proportion of $^{2}H_{3}$-labeled lipids after 24 h (Figure 7B). Comparing the timelines revealed that the MFGM/EV-fed rats had higher production and turnover rates of all labeled lipids (Figure 7B), which indicates that intake of the MFGM/EV concentrate increases lipid metabolic flux through both the CDP-choline pathway as well as the hepatic PEMT pathway.

Next, we examined how the differences in metabolic activity affect the timelines of individual lipids. Statistical analysis showed that the timelines of 43 labeled lipids were significantly different between the two groups of aged rats (Figure 7C). The majority of these lipids were phosphatidylcholines (18), followed by sphingomyelins (14) and lysophosphatidylcholines (11). These timelines were predominantly related to lipids labeled with $^{2}H_{3}$, which result from PEMT activity (Figure 7D). Interestingly, we found that timelines of several phosphatidylcholines with polyunsaturated acyl chains, including the archetypal PEMT product PC(+$^{2}H_{3}$) 18:0–22:6 exhibited large differences (Figure 7E). Concordantly, the timelines for the other archetypal PEMT products, PC(+$^{2}H_{3}$) 16:0–22:6 and LPC(+$^{2}H_{3}$) 22:6, were marginally elevated (Figure S5A). Interestingly, the timelines of analogous PC molecules labeled with $^{2}H_{9}$, and thus produced by the CDP-choline pathway and fatty acyl chain remodeling, were also significantly increased for the MFGM/EV-supplemented rats (Figure S5A). Taken together, these findings show that intake of the MFGM/EV concentrate increases the metabolic turnover of phosphatidylcholine molecules with a 22:6 chain through both the PEMT pathway as well as the CDP-choline pathway.

Sphingomyelin molecules, such as $^{2}H_{3}$- and $^{2}H_{9}$-labeled analogs of the odd-numbered very-long-chain species SM 41:1;2 and SM 41:2;2, also exhibited significant differences (Figures 7F and S5B). This finding is consistent with the observation that MFGM/EV-fed rats have higher steady-state levels of the two 41:1;2 isomers SM 18:1;2/23:0 and SM 17:1;2/24:0, as well as the two 41:2;2 isomers SM 18:1;2/23:1 and SM 17:1;2/24:1 (Figure 2F and Table S4). Furthermore, the timelines for $^{2}H_{3}$- and $^{2}H_{9}$-labeled SM 42:1;2, SM 42:2;2 and SM 34:1;2, corresponding to analogs with 18:1;2/24:0, 18:1;2/24:1, and 18:1;2/16:0 backbones, respectively, were all greater in MFGM/EV-supplemented rats compared with the control rats ((Figures 7G and S5C). Of note, the lipidomic flux analysis was not able to identify the sphingoid and fatty acyl chains of the labeled sphingomyelin species due

to their low abundance. Thus, the proportions of the labeled sphingomyelins reflect the contribution from the pool of the underlying isomers. The elevated metabolic turnover of sphingomyelins with odd- and even-numbered chains likely reflects the backbone structure of sphingolipids present in the MFGM/EV-rich supplement [28].

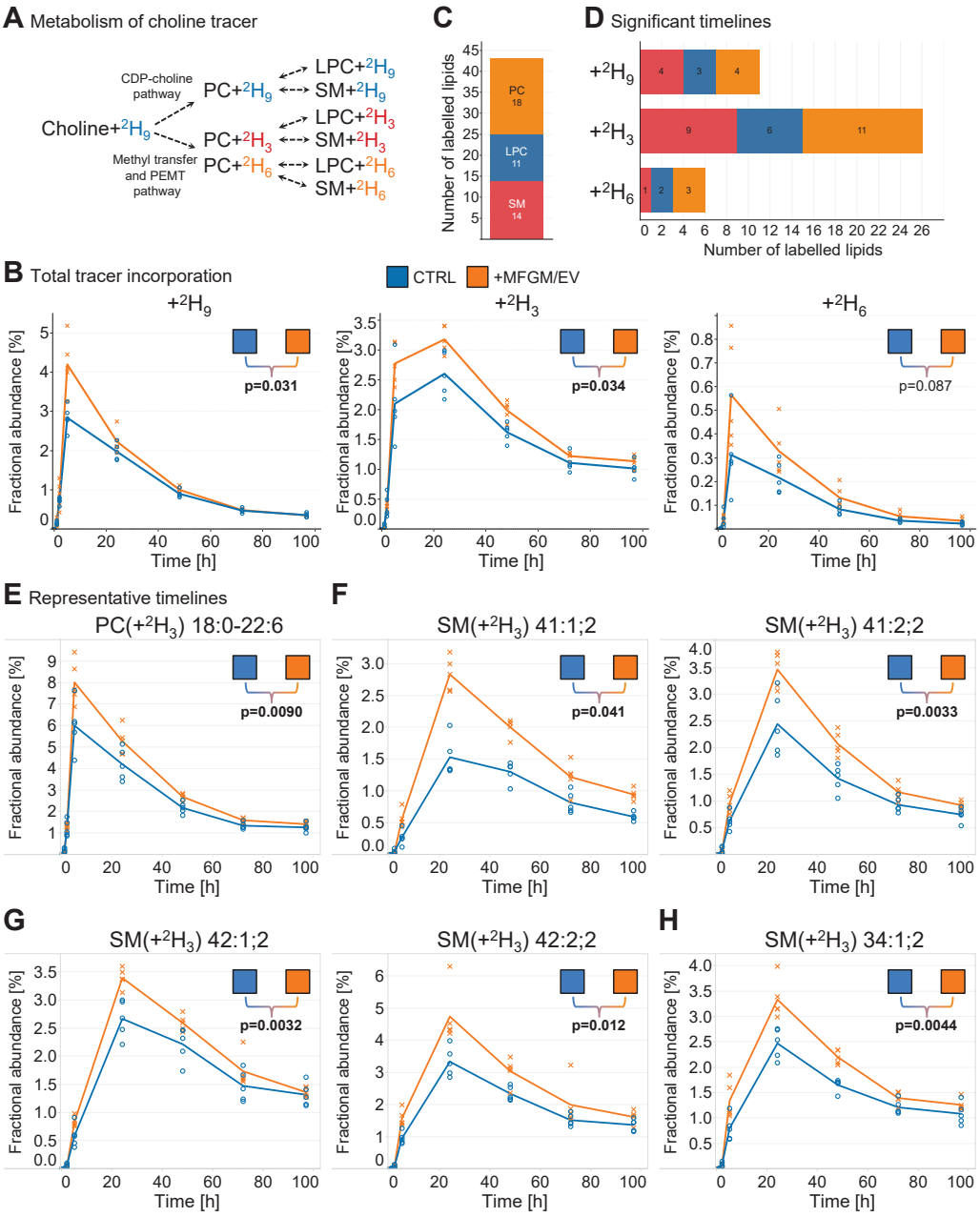


Figure 7. Intake of the MFGM/EV concentrate increases lipid metabolic activity. (**A**) Overview of the metabolic fates of the $^{2}H_{9}$-labeled choline tracer. (**B**) Fractional abundances of all choline-containing lipids in plasma. Lines represent means, and crosses and circles show individual measurements (n = 3–5 rats/timepoint/dietary group). (**C**) Total number of lipid molecular timelines with significant differences between the dietary groups. Statistical analysis was performed by repeated-measures ANOVA and post hoc t-tests. Timelines with ANOVA F-test and two or more t-test p-values < 0.05 are considered significant. (**D**) Number of lipid molecular timelines showing significant differences between the indicated dietary groups and per label (i.e., $^{2}H_{9}$, $^{2}H_{6}$, and $^{2}H_{3}$). (**E**–**H**) Representative timelines for indicated PC and SM molecules. Statistical analyses in (**B**,**E**–**H**) were performed by repeated-measures ANOVA F-test. Significant timelines are highlighted in bold ($p < 0.05$).

Altogether, these data demonstrate that intake of the MFGM/EV concentrate increases lipid metabolic activity at various levels. This includes increased flux through pathways involving PEMT that produce polyunsaturated phosphatidylcholine molecules (e.g., PC 18:0–22:6), which are used by hepatocytes to decorate the surface of secreted VLDL particles. Altogether, this suggests that the aged MFGM/EV-fed rats have metabolically healthier livers than the aged control rats, similar to what we previously observed in mice [20].

4. Conclusions

Age-related metabolic dysfunction increases the risk of cardiometabolic disorders, such as obesity, steatohepatitis, type 2 diabetes, and coronary artery disease, and represents an increasing health burden for elderly individuals and healthcare systems worldwide. Here, we investigated whether dietary supplementation with a milk sphingolipid-rich MFGM/EV concentrate can ameliorate age-related metabolic decline using aged sedentary male rats as a preclinical model (i.e., quiescent rats housed under regular conditions and without additional exercise regimes). Overall, our study unequivocally demonstrates that MFGM/EV supplementation leads to accretion of very-long-odd-chain ceramide and sphingomyelin molecules that are structurally identical to sphingolipid constituents in the concentrate [28]. Moreover, we showed that this lowers clinically relevant ceramide biomarkers, as well as more common lipid markers of cardiometabolic disease risk. Finally, our study reveals that the health-promoting effects are coupled to improved lipoprotein metabolism and liver function in the aged MFGM/EV-supplemented rats. Altogether, our study contributes to the emerging paradigm that the intake of MFGM/EV-containing food can lead to the build-up of bioactive very-long-chain-containing sphingolipids that have beneficial effects on whole-body metabolism in elderly individuals.

The molecular underpinnings of how milk sphingolipids impinge on metabolic health are multifaceted and poorly understood. Several intervention studies in humans, pigs, and rodents, and the present study, consistently show that intake of MFGM/EV-containing food results in accretion of very-long-odd-chain sphingolipids, as revealed by lipidomic analysis of steady-state lipid levels [20–23,50]. This, in turn, points to intestinal digestion and uptake as being a key determinant for the build-up of the unique milk sphingolipid signatures. While the digestion and uptake of long-chain (e.g., 16:0) sphingolipids have been studied [51–53], it is unclear how the digestive pathways handle the uptake and systemic incorporation of very-long-chain sphingolipids. A major pathway purportedly involves complete hydrolysis to the main breakdown products, a sphingoid base and a very-long-chain fatty acid, which are absorbed and can be reassembled by the endogenous metabolic machinery to produce identical sphingolipid molecules. Moreover, an alternative pathway that facilitates the direct uptake of intact very-long-chain sphingolipids and/or their partially hydrolyzed ceramide intermediates also seems to be at play. Although this remains to be tested in future studies, there are indications in the published literature that this process is taking place. For example, a study on mice with disrupted intestinal sphingomyelinase showed that a substantial proportion of a sphingomyelin-based radiotracer still accumulates in the circulation after 1 h [51]. In addition, a study with lymph-cannulated rats showed that ingestion of milk sphingomyelin leads to the build-up of ceramide intermediates with identical backbone structures in chylomicrons [54]. Intriguingly, this study also highlighted a preferential uptake of very-long-chain ceramides, including the 18:1;2/23:0 signature that is consistently found to be elevated in animals and humans fed MFGM/EV-containing food (Figure 3F) [20–23]. Altogether, this underscores the need for additional studies to dissect the molecular origins of the sphingolipid signatures that accumulate following MFGM/EV consumption.

Further work is also needed to deepen our understanding of the mechanisms that govern the health-promoting effects of milk sphingolipids [55]. One commonly suggested mechanism is that sphingolipids associate with cholesterol in the intestinal lumen and inhibit its uptake, which reduces the amount of cholesterol lipids and leads to a hypocholesterolemic effect. While our study on sedentary aged male rats supports the previous findings on the hypocholesterolemic effect of milk sphingolipids, it also sheds light on the mechanisms underlying their additional hypotriglyceridemic effect [14]. Specifically, our more detailed lipoprotein profiling data, together with the analysis of whole-body lipid metabolic flux, suggest that increased turnover of triglyceride-rich lipoprotein particles, accompanied by higher clearance as well as hepatic and intestinal production, is also at play. Specifically, our data show that aged rats have elevated CM levels during fasting and that this is lowered by MFGM/EV supplementation (Figure 6A). Furthermore, the supplementation also reduces the level of triglyceride-rich lipoprotein particles that reflect liver-derived VLDL and CM remnants. In agreement with this, the elevated lipid metabolic flux, specifically through the hepatic PEMT pathway that generates phosphorylcholine lipids for the production of VLDL particles [29], suggests a higher rate of VLDL production. How milk-derived very-long-chain sphingolipids modulate lipoprotein metabolism at the whole-body level to maintain a lower steady-state level of triglyceride-rich particles is currently unclear and warrants further mechanistic investigations. Furthermore, while this study was limited to aged male rats, potential gender-specific effects also deserve attention in future studies. Notably, however, our findings concur with results from a clinical intervention study in overweight postmenopausal women [14,15], raising the possibility that a conserved pathophysiological mechanism is at play across gender and species.

In summary, our study demonstrates that dietary supplementation with an MFGM/EV-rich concentrate is a viable strategy for modulating levels of bioactive very-long-chain ceramide biomarkers at the whole-body level. We further show that MFGM/EV supplementation can be used to ameliorate cardiometabolic risk markers in aged sedentary male rats with metabolic dysfunction. Furthermore, our findings define a framework for further exploring the molecular underpinnings of the health-promoting effects of milk sphingolipids rich in MFGM/EV-based food, as well as improving the therapeutic utility of nutraceuticals to treat cardiometabolic disorders in elderly individuals.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu17152529/s1. Figure S1: Frequencies of significantly different lipids in plasma; Figure S2: The MFGM/EV ingredient promotes accretion of very-long-odd-chain sphingolipids at the whole-body level; Figure S3: Plasma levels of ceramides; Figure S4: Lipid-lowering effects of the MFGM/EV ingredient; Figure S5: The MFGM/EV ingredient increases lipid metabolic activity; Table S1: Overview of lipid class abbreviations used for lipid molecules; Table S2: Nutritional details related to the supplements; Table S3: Multiple reaction monitoring parameters used for peptide detection; Table S4: Lipidomics data for plasma; Table S5: Lipidomics data for duodenum, liver, lungs, and kidneys; Table S6: Lipidomic flux data for plasma.

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Institutional Review Board Statement: This in vivo study was conducted in accordance with European standards and the Danish law on Animal Experiments and approved by the Danish Animal Experiment Inspectorate (protocol 2020-15-0201-00728, date of approval: 21 December 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data can be found at the GNPS/massIVE repository with accession numbers MSV000097591 (plasma), MSV000097593 (liver), MSV000097595 (duodenum), MSV000097597 (kidneys), and MSV000097596 (lungs). Any additional information is available upon request.

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Conflicts of Interest: A.B. and M.S.O. were full-time employees of Arla Food Ingredients during the time period of this study. Other authors declare that they have no conflicts of interest.

Abbreviations

The following abbreviations were used in this manuscript:

ANOVA	Analysis of variance
Cer	Ceramide; other lipid shorthand notations are specified in Table S1
CM	Chylomicron
CVD	Cardiovascular disease
EV	Extracellular vesicle
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
MFGM	Milk fat globule membrane
MS	Mass spectrometry
OCTT	Oral choline tracer test
VLDL	Very-low-density lipoprotein

References

1. GBD 2021 Demographics Collaborators. Global age-sex-specific mortality, life expectancy, and population estimates in 204 countries and territories and 811 subnational locations, 1950–2021, and the impact of the COVID-19 pandemic: A comprehensive demographic analysis for the Global Burden of Disease Study 2021. *Lancet* **2024**, *403*, 1989–2056. [CrossRef]
2. Tchkonia, T.; Morbeck, D.E.; Von Zglinicki, T.; Van Deursen, J.; Lustgarten, J.; Scrable, H.; Khosla, S.; Jensen, M.D.; Kirkland, J.L. Fat tissue, aging, and cellular senescence. *Aging Cell* **2010**, *9*, 667–684. [CrossRef]
3. He, Q.J.; Li, Y.F.; Zhao, L.T.; Lin, C.T.; Yu, C.Y.; Wang, D. Recent advances in age-related metabolic dysfunction-associated steatotic liver disease. *World J. Gastroenterol.* **2024**, *30*, 652–662. [CrossRef]
4. GBD 2021 Forecasting Collaborators. Burden of disease scenarios for 204 countries and territories, 2022-2050: A forecasting analysis for the Global Burden of Disease Study 2021. *Lancet* **2024**, *403*, 2204–2256. [CrossRef]
5. Holst, J.J. GLP-1 physiology in obesity and development of incretin-based drugs for chronic weight management. *Nat. Metab.* **2024**, *6*, 1866–1885. [CrossRef]
6. Berisha, H.; Hattab, R.; Comi, L.; Giglione, C.; Migliaccio, S.; Magni, P. Nutrition and Lifestyle Interventions in Managing Dyslipidemia and Cardiometabolic Risk. *Nutrients* **2025**, *17*, 776. [CrossRef]

7. Raza, G.S.; Herzig, K.H.; Leppaluoto, J. Invited review: Milk fat globule membrane-A possible panacea for neurodevelopment, infections, cardiometabolic diseases, and frailty. *J. Dairy Sci.* **2021**, *104*, 7345–7363. [CrossRef]
8. Pan, J.; Chen, M.; Li, N.; Han, R.; Yang, Y.; Zheng, N.; Zhao, S.; Zhang, Y. Bioactive Functions of Lipids in the Milk Fat Globule Membrane: A Comprehensive Review. *Foods* **2023**, *12*, 3755. [CrossRef]
9. Torres-Gonzalez, M.; Rice Bradley, B.H. Whole-Milk Dairy Foods: Biological Mechanisms Underlying Beneficial Effects on Risk Markers for Cardiometabolic Health. *Adv. Nutr.* **2023**, *14*, 1523–1537. [CrossRef]
10. Hannun, Y.A.; Obeid, L.M. Sphingolipids and their metabolism in physiology and disease. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 175–191. [CrossRef]
11. Kuo, A.; Hla, T. Regulation of cellular and systemic sphingolipid homeostasis. *Nat. Rev. Mol. Cell Biol.* **2024**, *25*, 802–821. [CrossRef]
12. Pokala, A.; Kraft, J.; Taormina, V.M.; Michalski, M.C.; Vors, C.; Torres-Gonzalez, M.; Bruno, R.S. Whole milk dairy foods and cardiometabolic health: Dairy fat and beyond. *Nutr. Res.* **2024**, *126*, 99–122. [CrossRef]
13. Calzada, C.; Vors, C.; Penhoat, A.; Cheillan, D.; Michalski, M.C. Role of circulating sphingolipids in lipid metabolism: Why dietary lipids matter. *Front. Nutr.* **2022**, *9*, 1108098. [CrossRef]
14. Vors, C.; Joumard-Cubizolles, L.; Lecomte, M.; Combe, E.; Ouchchane, L.; Drai, J.; Raynal, K.; Joffre, F.; Meiller, L.; Le Barz, M.; et al. Milk polar lipids reduce lipid cardiovascular risk factors in overweight postmenopausal women: Towards a gut sphingomyelin-cholesterol interplay. *Gut* **2020**, *69*, 487–501. [CrossRef]
15. Le Barz, M.; Vors, C.; Combe, E.; Joumard-Cubizolles, L.; Lecomte, M.; Joffre, F.; Trauchessec, M.; Pesenti, S.; Loizon, E.; Breyton, A.E.; et al. Milk polar lipids favorably alter circulating and intestinal ceramide and sphingomyelin species in postmenopausal women. *JCI Insight* **2021**, *6*, e146161. [CrossRef]
16. Tarasov, K.; Ekroos, K.; Suoniemi, M.; Kauhanen, D.; Sylvanne, T.; Hurme, R.; Gouni-Berthold, I.; Berthold, H.K.; Kleber, M.E.; Laaksonen, R.; et al. Molecular lipids identify cardiovascular risk and are efficiently lowered by simvastatin and PCSK9 deficiency. *J. Clin. Endocrinol. Metab.* **2014**, *99*, E45–E52. [CrossRef]
17. Laaksonen, R.; Ekroos, K.; Sysi-Aho, M.; Hilvo, M.; Vihervaara, T.; Kauhanen, D.; Suoniemi, M.; Hurme, R.; Marz, W.; Scharnagl, H.; et al. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. *Eur. Heart J.* **2016**, *37*, 1967–1976. [CrossRef]
18. Taskinen, M.R.; Boren, J. New insights into the pathophysiology of dyslipidemia in type 2 diabetes. *Atherosclerosis* **2015**, *239*, 483–495. [CrossRef]
19. Taskinen, M.R.; Matikainen, N.; Bjornson, E.; Soderlund, S.; Inkeri, J.; Hakkarainen, A.; Parviainen, H.; Sihlbom, C.; Thorsell, A.; Andersson, L.; et al. Contribution of intestinal triglyceride-rich lipoproteins to residual atherosclerotic cardiovascular disease risk in individuals with type 2 diabetes on statin therapy. *Diabetologia* **2023**, *66*, 2307–2319. [CrossRef]
20. Sprenger, R.R.; Bilgin, M.; Ostenfeld, M.S.; Bjornshave, A.; Rasmussen, J.T.; Ejsing, C.S. Dietary intake of a MFGM/EV-rich concentrate promotes accretion of very long odd-chain sphingolipids and increases lipid metabolic turnover at the whole-body level. *Food Res. Int.* **2024**, *190*, 114601. [CrossRef]
21. Davies, R.; van Diepen, J.A.; Brink, L.R.; Bijlsma, S.; Neufeld, K.M.; Cryan, J.F.; O'Mahony, S.M.; Bobeldijk, I.; Gross, G. Lipidome Analysis in Brain and Peripheral Plasma Following Milk Fat Globule Membrane Supplementation in Rodents. *Mol. Nutr. Food Res.* **2022**, *66*, e2200177. [CrossRef]
22. Grip, T.; Dyrlund, T.S.; Ahonen, L.; Domellof, M.; Hernell, O.; Hyotylainen, T.; Knip, M.; Lonnerdal, B.; Oresic, M.; Timby, N. Serum, plasma and erythrocyte membrane lipidomes in infants fed formula supplemented with bovine milk fat globule membranes. *Pediatr. Res.* **2018**, *84*, 726–732. [CrossRef]
23. Calzada, C.; Cheillan, D.; Ritsch, N.; Vors, C.; Durand, A.; Pesenti, S.; Pettazzoni, M.; Meugnier, E.; Michalski, M.C.; Penhoat, A. Intestinal absorption of sphingosine: New insights on generated ceramide species using stable isotope tracing in vitro. *J. Lipid Res.* **2024**, *65*, 100557. [CrossRef]
24. Blans, K.; Hansen, M.S.; Sorensen, L.V.; Hvam, M.L.; Howard, K.A.; Moller, A.; Wiking, L.; Larsen, L.B.; Rasmussen, J.T. Pellet-free isolation of human and bovine milk extracellular vesicles by size-exclusion chromatography. *J. Extracell. Vesicles* **2017**, *6*, 1294340. [CrossRef]
25. McManaman, J.L. Formation of milk lipids: A molecular perspective. *Clin. Lipidol.* **2009**, *4*, 391–401. [CrossRef]
26. Jensen, R.G. The composition of bovine milk lipids: January 1995 to December 2000. *J. Dairy. Sci.* **2002**, *85*, 295–350. [CrossRef]
27. Liu, Z.; Rochfort, S.; Cocks, B. Milk lipidomics: What we know and what we don't. *Prog. Lipid Res.* **2018**, *71*, 70–85. [CrossRef]
28. Sprenger, R.R.; Ostenfeld, M.S.; Bjornshave, A.; Rasmussen, J.T.; Ejsing, C.S. Lipidomic Characterization of Whey Concentrates Rich in Milk Fat Globule Membranes and Extracellular Vesicles. *Biomolecules* **2023**, *14*, 55. [CrossRef]
29. Noga, A.A.; Zhao, Y.; Vance, D.E. An unexpected requirement for phosphatidylethanolamine N-methyltransferase in the secretion of very low density lipoproteins. *J. Biol. Chem.* **2002**, *277*, 42358–42365. [CrossRef]
30. Phillips, M.C. Is ABCA1 a lipid transfer protein? *J. Lipid Res.* **2018**, *59*, 749–763. [CrossRef]

31. Sprenger, R.R.; Hermansson, M.; Neess, D.; Becciolini, L.S.; Sorensen, S.B.; Fagerberg, R.; Ecker, J.; Liebisch, G.; Jensen, O.N.; Vance, D.E.; et al. Lipid molecular timeline profiling reveals diurnal crosstalk between the liver and circulation. *Cell Rep.* **2021**, *34*, 108710. [CrossRef]
32. Sprenger, R.R.; Kiilerich, K.F.; Palner, M.; Ostenfeld, M.S.; Bjornshave, A.; Knudsen, G.M.; Ejsing, C.S. Aging predominates over an MFGM/EV-rich supplement in modulating the brain lipidome and cognitive decline of aged rats. *Food Biosci.* **2025**, *68*, 106689. [CrossRef]
33. Almeida, R.; Pauling, J.K.; Sokol, E.; Hannibal-Bach, H.K.; Ejsing, C.S. Comprehensive lipidome analysis by shotgun lipidomics on a hybrid quadrupole-orbitrap-linear ion trap mass spectrometer. *J. Am. Soc. Mass. Spectrom.* **2015**, *26*, 133–148. [CrossRef]
34. Gallego, S.F.; Hojlund, K.; Ejsing, C.S. Easy, Fast, and Reproducible Quantification of Cholesterol and Other Lipids in Human Plasma by Combined High Resolution MSX and FTMS Analysis. *J. Am. Soc. Mass. Spectrom.* **2018**, *29*, 34–41. [CrossRef]
35. Usui, S.; Hara, Y.; Hosaki, S.; Okazaki, M. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J. Lipid Res.* **2002**, *43*, 805–814. [CrossRef]
36. Okazaki, M.; Yamashita, S. Recent Advances in Analytical Methods on Lipoprotein Subclasses: Calculation of Particle Numbers from Lipid Levels by Gel Permeation HPLC Using "Spherical Particle Model". *J. Oleo Sci.* **2016**, *65*, 265–282. [CrossRef] [PubMed]
37. Husen, P.; Tarasov, K.; Katafiasz, M.; Sokol, E.; Vogt, J.; Baumgart, J.; Nitsch, R.; Ekroos, K.; Ejsing, C.S. Analysis of lipid experiments (ALEX): A software framework for analysis of high-resolution shotgun lipidomics data. *PLoS ONE* **2013**, *8*, e79736. [CrossRef] [PubMed]
38. Pauling, J.K.; Hermansson, M.; Hartler, J.; Christiansen, K.; Gallego, S.F.; Peng, B.; Ahrends, R.; Ejsing, C.S. Proposal for a common nomenclature for fragment ions in mass spectra of lipids. *PLoS ONE* **2017**, *12*, e0188394. [CrossRef]
39. Ellis, S.R.; Paine, M.R.L.; Eijkel, G.B.; Pauling, J.K.; Husen, P.; Jervelund, M.W.; Hermansson, M.; Ejsing, C.S.; Heeren, R.M.A. Automated, parallel mass spectrometry imaging and structural identification of lipids. *Nat. Methods* **2018**, *15*, 515–518. [CrossRef] [PubMed]
40. Blanchard, V.; Garcon, D.; Jaunet, C.; Chemello, K.; Billon-Crossouard, S.; Aguesse, A.; Garfa, A.; Famchon, G.; Torres, A.; Le May, C.; et al. A high-throughput mass spectrometry-based assay for large-scale profiling of circulating human apolipoproteins. *J. Lipid Res.* **2020**, *61*, 1128–1139. [CrossRef]
41. Rodrigues Oliveira, A.; Chevalier, C.; Wargny, M.; Pakulska, V.; Caradeuc, C.; Cloteau, C.; Letertre, M.P.M.; Giraud, N.; Bertho, G.; Bigot-Corbel, E.; et al. Methylglyoxal-Induced Glycation of Plasma Albumin: From Biomarker Discovery to Clinical Use for Prediction of New-Onset Diabetes in Individuals with Prediabetes. *Clin. Chem.* **2025**, *71*, 688–699. [CrossRef]
42. Hilvo, M.; Vasile, V.C.; Donato, L.J.; Hurme, R.; Laaksonen, R. Ceramides and Ceramide Scores: Clinical Applications for Cardiometabolic Risk Stratification. *Front. Endocrinol.* **2020**, *11*, 570628. [CrossRef] [PubMed]
43. Feraco, A.; Gorini, S.; Camajani, E.; Filardi, T.; Karav, S.; Cava, E.; Strollo, R.; Padua, E.; Caprio, M.; Armani, A.; et al. Gender differences in dietary patterns and physical activity: An insight with principal component analysis (PCA). *J. Transl. Med.* **2024**, *22*, 1112. [CrossRef]
44. Mensenkamp, A.R.; Jong, M.C.; van Goor, H.; van Luyn, M.J.; Bloks, V.; Havinga, R.; Voshol, P.J.; Hofker, M.H.; van Dijk, K.W.; Havekes, L.M.; et al. Apolipoprotein E participates in the regulation of very low density lipoprotein-triglyceride secretion by the liver. *J. Biol. Chem.* **1999**, *274*, 35711–35718. [CrossRef]
45. Hsu, C.C.; Kanter, J.E.; Kothari, V.; Bornfeldt, K.E. Quartet of APOCs and the Different Roles They Play in Diabetes. *Arterioscler. Thromb. Vasc. Biol.* **2023**, *43*, 1124–1133. [CrossRef]
46. Sarkar, S.; Morris, J.; You, Y.; Sexmith, H.; Street, S.E.; Thibert, S.M.; Attah, I.K.; Hutchinson Bunch, C.M.; Novikova, I.V.; Evans, J.E.; et al. APOA2 increases cholesterol efflux capacity to plasma HDL by displacing the *C*-terminus of resident APOA1. *J. Lipid Res.* **2024**, *65*, 100686. [CrossRef] [PubMed]
47. Greeve, J.; Altkemper, I.; Dieterich, J.H.; Greten, H.; Windler, E. Apolipoprotein B mRNA editing in 12 different mammalian species: Hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. *J. Lipid Res.* **1993**, *34*, 1367–1383. [CrossRef] [PubMed]
48. Zhao, Y.; Su, B.; Jacobs, R.L.; Kennedy, B.; Francis, G.A.; Waddington, E.; Brosnan, J.T.; Vance, J.E.; Vance, D.E. Lack of phosphatidylethanolamine N-methyltransferase alters plasma VLDL phospholipids and attenuates atherosclerosis in mice. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 1349–1355. [CrossRef]
49. Pynn, C.J.; Henderson, N.G.; Clark, H.; Koster, G.; Bernhard, W.; Postle, A.D. Specificity and rate of human and mouse liver and plasma phosphatidylcholine synthesis analyzed in vivo. *J. Lipid Res.* **2011**, *52*, 399–407. [CrossRef]
50. Henriksen, N.L.; Asmussen, K.S.; Pan, X.; Jiang, P.P.; Mori, Y.; Christiansen, L.I.; Sprenger, R.R.; Ejsing, C.S.; Pankratova, S.; Thymann, T. Brain lipidomics and neurodevelopmental outcomes in intrauterine growth restricted piglets fed dairy or vegetable fat diets. *Sci. Rep.* **2022**, *12*, 3303. [CrossRef] [PubMed]
51. Zhang, Y.; Cheng, Y.; Hansen, G.H.; Niels-Christiansen, L.L.; Koentgen, F.; Ohlsson, L.; Nilsson, A.; Duan, R.D. Crucial role of alkaline sphingomyelinase in sphingomyelin digestion: A study on enzyme knockout mice. *J. Lipid Res.* **2011**, *52*, 771–781. [CrossRef] [PubMed]

52. Kono, M.; Dreier, J.L.; Ellis, J.M.; Allende, M.L.; Kalkofen, D.N.; Sanders, K.M.; Bielawski, J.; Bielawska, A.; Hannun, Y.A.; Proia, R.L. Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. *J. Biol. Chem.* **2006**, *281*, 7324–7331. [CrossRef] [PubMed]
53. Kobayashi, T.; Suzuki, K. The glycosylceramidase in the murine intestine. Purification and substrate specificity. *J. Biol. Chem.* **1981**, *256*, 7768–7773. [CrossRef] [PubMed]
54. Morifuji, M.; Higashi, S.; Oba, C.; Ichikawa, S.; Kawahata, K.; Yamaji, T.; Itoh, H.; Manabe, Y.; Sugawara, T. Milk Phospholipids Enhance Lymphatic Absorption of Dietary Sphingomyelin in Lymph-Cannulated Rats. *Lipids* **2015**, *50*, 987–996. [CrossRef]
55. Norris, G.H.; Milard, M.; Michalski, M.C.; Blesso, C.N. Protective properties of milk sphingomyelin against dysfunctional lipid metabolism, gut dysbiosis, and inflammation. *J. Nutr. Biochem.* **2019**, *73*, 108224. [CrossRef]

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Lipid-Modulating Effects of *Sargassum fulvellum* Fermented by *Lactococcus lactis* KCCM12759P and *Leuconostoc mesenteroides* KCCM12756P in Ovariectomized Mice

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Abstract

Background/Objectives: Estrogen deficiency contributes to dyslipidemia and visceral adiposity, increasing cardiovascular risk in postmenopausal women. *Sargassum fulvellum* (Sf), a brown seaweed rich in bioactive compounds, possesses lipid-regulating properties that may be enhanced by lactic acid bacteria fermentation. This study aimed to evaluate the effects of fermented *S. fulvellum* (SfLlLm), prepared using *Lactococcus lactis* and *Leuconostoc mesenteroides*, on lipid metabolism and adipose tissue remodeling in an ovariectomized (OVX) mouse model of estrogen deficiency. **Methods**: Female C57BL/6 mice underwent ovariectomy and were fed an AIN-76A diet supplemented with either unfermented Sf or SfLlLm for eight weeks. Sham-operated and 17β-estradiol-treated OVX groups served as controls. Serum lipid levels—total cholesterol, triglycerides, LDL-C, and HDL-C—were assessed, and histological analysis of visceral adipose tissue was conducted to evaluate adipocyte morphology. **Results**: OVX-induced estrogen deficiency led to increased total cholesterol, triglycerides, and LDL-C, along with hypertrophic changes in visceral adipocytes. Supplementation with fermented *Sargassum fulvellum* (SfLlLm) markedly improved these parameters, reducing total cholesterol by 6.7%, triglycerides by 9.3%, and LDL-C by 52.9%, while increasing HDL-C by 17.5% compared to the OVX controls. SfLlLm also normalized visceral adipocyte size and distribution. These effects were comparable to or exceeded those of 17β-estradiol treatment. **Conclusions**: Fermented SfLlLm ameliorated dyslipidemia and visceral adiposity under estrogen-deficient conditions. These findings support its potential as a functional dietary intervention for managing postmenopausal lipid disorders and associated metabolic complications.

Keywords: *Sargassum*; fermentation; estrogen; dyslipidemias; vitamin K2

1. Introduction

Menopause is accompanied by a decline in circulating estrogen levels, which contributes to a range of metabolic disturbances, including dyslipidemia, hepatic steatosis, and visceral adiposity. These changes increase the risk of developing cardiovascular diseases, non-alcoholic fatty liver disease, and obesity-related complications [1,2]. The ovariectomized (OVX) mouse model, which mimics the hormonal milieu of postmenopausal women, is widely employed in preclinical research to evaluate the efficacy

of dietary and pharmacological interventions targeting estrogen deficiency-associated metabolic dysfunction [3].

Marine algae have recently garnered substantial interest as sustainable and potent sources of functional food ingredients [4–6]. As photosynthetic organisms, seaweeds possess an impressive repertoire of bioactive compounds, including polysaccharides (e.g., fucoidan, laminarin), phlorotannins, carotenoids (e.g., fucoxanthin), soluble fibers, and essential micronutrients such as vitamins A, C, E, and K [4,5,7]. These components contribute to diverse biological activities, including antioxidant, anti-inflammatory, lipid-lowering, anti-obesity, and hepatoprotective effects. Consequently, seaweeds are being increasingly explored for their applications in the food, nutraceutical, and pharmaceutical industries. Additionally, the marine ecosystem harbors a vast diversity of underutilized algae species with unique metabolic profiles and therapeutic potential [4,5,7]. Among these, *Sargassum fulvellum*, an edible brown macroalga traditionally consumed in East Asia, is recognized for its health-promoting properties [5,8]. Rich in phylloquinone (vitamin K1), polyphenols, and other antioxidative constituents, *S. fulvellum* reportedly exerts antioxidant, anti-inflammatory, and osteoprotective effects [5,8–10]. However, its functional potential in metabolic health remains underexplored due to the low bioavailability of its active compounds and the limited understanding of their mechanisms of action.

While *Sargassum fulvellum* has been primarily studied for its benefits in bone health—attributed to its anti-inflammatory and antioxidant actions—its relevance to metabolic regulation, particularly lipid metabolism under estrogen-deficient conditions, remains largely unexplored. Menopause-associated dyslipidemia and adipose tissue dysfunction share underlying pathophysiological mechanisms with osteoporosis, including chronic low-grade inflammation, oxidative stress, and impaired mitochondrial function. These overlapping pathways suggest that interventions promoting bone health may exert systemic metabolic benefits as well.

Among the promising candidates for such intervention are menaquinones (vitamin K2 homologs), which are produced during lactic acid bacterial fermentation. These compounds have been shown to support bone mineralization and to play emerging roles in lipid metabolism and vascular homeostasis by suppressing inflammatory cytokines, enhancing mitochondrial efficiency, and modulating the expression of genes involved in lipid transport and oxidation [11,12].

Fermentation has emerged as a promising strategy for enhancing the nutritional and functional value of plant-based ingredients. Lactic acid bacteria (LAB), particularly *Lactococcus lactis* (*L. lactis*) and *Leuconostoc mesenteroides* (*L. mesenteroides*), have demonstrated the ability to not only improve the digestibility and palatability of plant matrices but also biosynthesize menaquinones (vitamin K2), a class of fat-soluble vitamins known to support bone, liver, and lipid metabolism [11,13,14]. Notably, vitamin K2 (menaquinone-4, -7, and -9) has been shown to modulate mitochondrial activity and inflammatory signaling, suggesting its potential ability to ameliorate estrogen deficiency-induced metabolic disturbances [12,15].

Building on this mechanistic rationale, previous work has shown that *S. fulvellum* fermented with *L. lactis* KCCM12759P and *L. mesenteroides* KCCM12756P significantly enhances the production of long-chain menaquinones and increases antioxidant and anti-inflammatory capacity [10]. Given the overlapping mechanisms among inflammation, mitochondrial dysfunction, and lipid dysregulation observed in menopause, the fermented form (SfLlLm) may hold promise as a multifunctional dietary intervention. While prior research has highlighted its bone-protective properties in estrogen-deficient animal models [10], its potential role in regulating lipid metabolism, preventing hepatic lipid accumulation, and remodeling adipose tissue remains to be elucidated [16,17]. In addition to

metabolic dysfunction, estrogen deficiency has been associated with adverse psychosocial outcomes and reduced quality of life. Polycystic ovary syndrome (PCOS), a condition characterized by hormonal and metabolic dysregulation, shares features similar to those of postmenopausal states. A study by Panico et al. [18] emphasized the physical and psychological burden of such conditions, highlighting the need for functional interventions that address both metabolic health and overall well-being.

Therefore, the aim of this study was to investigate the effects of *S. fulvellum* and its LAB-fermented derivative (SfLlLm) on lipid profiles, hepatic steatosis, and visceral adipose tissue remodeling in ovariectomized (OVX) mice as a model of estrogen-deficient metabolic dysfunction. This study also contributes to the broader understanding of fermented marine bioresources as candidate ingredients for the dietary management of postmenopausal health.

2. Materials and Methods

2.1. Preparation of fermented S. fulvellum

Dried *S. fulvellum* was obtained from a domestic commercial supplier (Parajeju, Jeju, Republic of Korea; http://www.parajeju.com) and ground finely into powder. As illustrated in Figure 1a, fermentation was performed using *L. lactis* KCCM12759P and *L. mesenteroides* KCCM12756P, following the protocol described in a previous study [10]. Each strain was inoculated at 1% (*v/v*) into a sterile suspension of *S. fulvellum* (5% *w/v* in distilled water), which had been autoclaved to ensure sterility. The mixture was incubated at 30 °C for 24 h under static conditions. After fermentation, the culture was centrifuged to separate solids and liquids, which were then recombined and freeze-dried to produce the fermented product (SfLlLm). For the non-fermented control (Sf), dried *S. fulvellum* powder was directly incorporated into the experimental diet without undergoing fermentation.

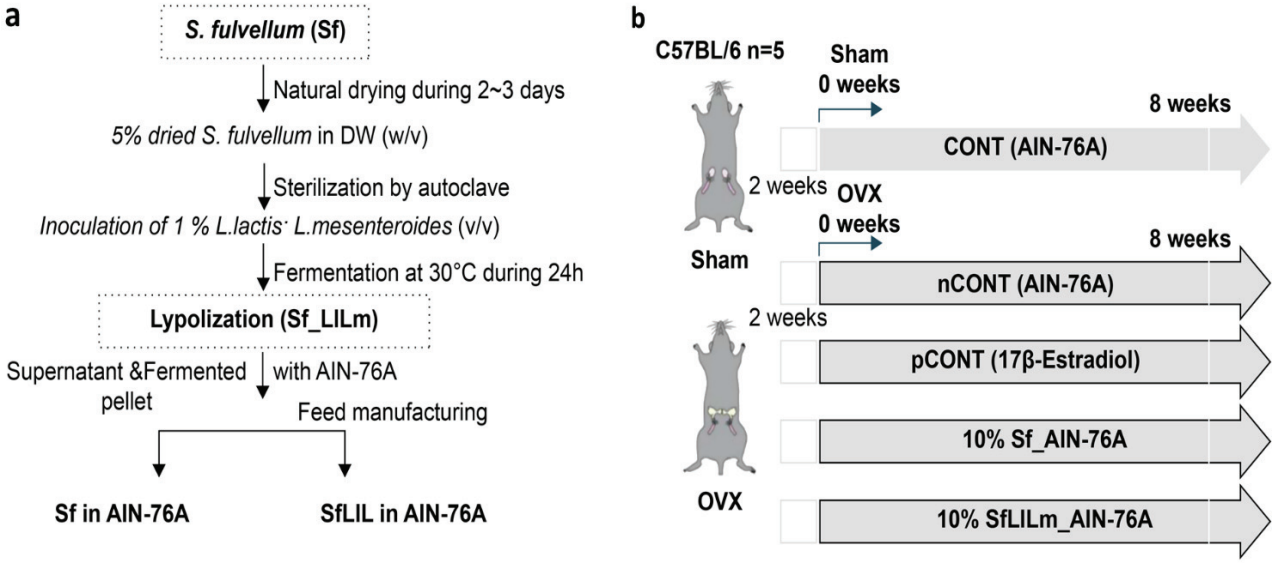


Figure 1. Sample preparation and experimental timeline for this in vivo study using *Sargassum fulvellum* (Sf). (**a**) Flowchart illustrating the feed preparation process using Sf and its fermented form (Sf_LlLm); (**b**) Schematic overview of the animal experiment involving sham-operated (CONT) and ovariectomized (OVX) C57BL/6 mice. Mice were assigned to the following groups (n = 5 per group): CONT, sham-operated mice fed a standard AIN-76A diet; nCONT, OVX mice fed the AIN-76A diet; pCONT, OVX mice fed AIN-76A supplemented with 17β-estradiol; Sf, OVX mice fed AIN-76A containing 10% Sf; and SfLlLm, OVX mice fed AIN-76A containing 10% SfLlLm. All treatments were administered for 8 weeks following a 2-week recovery period after sham or OVX surgery.

2.2. Ethics Statement

All animal procedures, including the OVX-induced osteoporosis mouse model and subsequent experimental protocols, were reviewed and approved by the Institutional Animal Care and Use Committee of Andong National University (Approval No. 2024-4-1111-06-01; Approval Date: 11 November 2024).

2.3. Experimental Animals and Design

As shown in Figure 1b, female C57BL/6 mice (8 weeks old) were purchased from Orient Bio Inc. (Seongnam, Republic of Korea) and acclimated for 1 week under standard laboratory conditions (22 ± 2 °C, 12 h light/dark cycle, 55 ± 10% relative humidity). Anesthesia was induced using tribromoethanol, after which the mice were subjected either to sham surgery (without ovariectomy) or to bilateral OVX to establish a model of estrogen deficiency-induced menopause. Precisely 2 weeks post-surgery, the mice were randomly assigned to five experimental groups (n = 5 per group): CONT (sham-operated, AIN-76A diet); nCONT (OVX, AIN-76A); pCONT (OVX, AIN-76A + 17β-estradiol at 0.01%); Sf (OVX, AIN-76A + 10% *S. fulvellum*); and SfLlLm (OVX, AIN-76A + 10% fermented *S. fulvellum*). Throughout the 8-week dietary intervention, body weight was recorded weekly to monitor metabolic changes. At the end of the study, the mice were fasted overnight and then euthanized under anesthesia. All surgical procedures were performed under anesthesia using tribromoethanol to minimize pain during the ovariectomy. Postoperative analgesia was not provided, as minimal discomfort was expected under tribromoethanol anesthesia, and no signs of pain or distress were observed. However, we acknowledge that analgesia is recommended and will apply this in future studies. However, no specific post-operative analgesics or pain monitoring protocols were described. No adverse events were observed, and all animals completed the study. Humane endpoints were not established, and no signs of distress or morbidity were reported during the 8-week dietary intervention. Blood and tissue samples were collected for biochemical and histological analyses. No inclusion or exclusion criteria were explicitly established a priori, and no animals or data points were excluded from the analysis in any experimental group. All animals assigned to the five groups (n = 5 per group) completed the study and were included in the final data analysis. Animals were randomly assigned to experimental groups following a 2-week recovery from sham or ovariectomy surgery. However, the method of random sequence generation was not specified. No specific strategies were described to control for potential confounding variables such as cage location, handling order, or measurement sequence. Randomization was performed using a simple random number generator in Microsoft Excel to assign animals to each treatment group. However, blinding was not implemented during sample collection or analysis owing to personnel limitations, which may introduce detection bias. This placement ensures it is reported transparently alongside other design-related details (e.g., randomization, group allocation, ethical approval). Outcome assessments, including biochemical analyses and histological evaluations, were not performed under blinded conditions. All analyses were conducted by researchers aware of the group allocations.

2.4. Tissue and Serum Collection

Liver, epididymal fat, and retroperitoneal fat pads were carefully excised, rinsed with sterile saline (0.75% NaCl; Sigma-Aldrich, St. Louis, MO, USA), blotted dry using filter paper (No. 2, Advantec, Tokyo, Japan), and weighed using an analytical balance (GR-202, A&D Company, Tokyo, Japan). Blood was collected via cardiac puncture under anesthesia, and serum was separated by centrifugation at 3000× g for 10 min at 4 °C using a refrigerated centrifuge (Gyrozen 1236R, Daejeon, Republic of Korea). The serum was

aliquoted into microtubes (Eppendorf, Hamburg, Germany) and stored at −80 °C until further analysis.

2.5. Serum Biochemical Analysis

Serum concentrations of triglycerides (TG; Triglyceride Quantification Kit, BioVision, Seoul, Republic of Korea), total cholesterol (TC; Total Cholesterol Colorimetric Assay Kit, Abcam, Cambridge, UK), high-density lipoprotein cholesterol (HDL-C; Abcam, Cambridge, UK), low-density lipoprotein cholesterol (LDL-C; Abcam, Cambridge, UK), aspartate aminotransferase (AST; Abcam, Cambridge, UK), and alanine aminotransferase (ALT; Abcam, Cambridge, UK) were determined using commercial enzymatic colorimetric assay kits, according to the manufacturers' instructions. Absorbance was measured using a microplate reader (SpectraMax iD3, Molecular Devices, San Jose, CA, USA).

2.6. Histological Analysis

Liver and epididymal adipose tissues were fixed in 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The fixed tissues were then dehydrated through a graded ethanol series (70%, 80%, 90%, 95%, and 100%; 10 min each; Sigma-Aldrich), cleared in xylene (Sigma-Aldrich), and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 5 μm using a rotary microtome (RM2235, Leica Biosystems, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (H&E; Merck, Darmstadt, Germany) according to standard histological protocols. Histological features, including hepatic lipid accumulation and adipocyte morphology, were examined using a light microscope (BX51, Olympus, Tokyo, Japan). Representative images were captured at 200× magnification using a digital camera (DP74, Olympus, Tokyo, Japan). The primary outcome was not explicitly defined, and no formal sample size calculation was conducted. A sample size of $n = 5$ per group was used based on precedents from similar preclinical studies.

2.7. Statistical Analyses

All data are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS Statistics for Windows, version 27.0 (IBM Corp., Armonk, NY, USA; https://www.ibm.com/products/spss-statistics, accessed on 30 June 2024). Group differences were assessed using one-way analysis of variance, followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant. The sample size ($n = 5$ per group) was based on similar exploratory studies using OVX mice. Although no formal a priori power calculation was performed, post hoc analysis indicated sufficient power (>80%) for key metabolic outcomes. We acknowledge the small sample size as a limitation and recommend larger, adequately powered studies in the future. No formal study protocol was pre-registered.

3. Results and Discussion

3.1. Effects of Fermented S. fulvellum on Body and Organ Weights in OVX Mice

Representative dorsal images of mice from each group after 8 weeks of dietary intervention are shown in Figure 2a. Mice in the OVX control group (nCONT) exhibited markedly increased body size and visible abdominal expansion compared to the sham-operated group (CONT), reflecting the typical weight gain and fat accumulation associated with estrogen deficiency. In contrast, the Sf and SfLlLm groups displayed visibly smaller abdominal girth and overall body size relative to nCONT, suggesting an amelioration of OVX-induced metabolic alterations. Notably, the SfLlLm group exhibited body contours

closely resembling those of the pCONT group (OVX + 17β-estradiol), indicating potential protective effects of the fermented product against adiposity and weight gain in OVX mice.

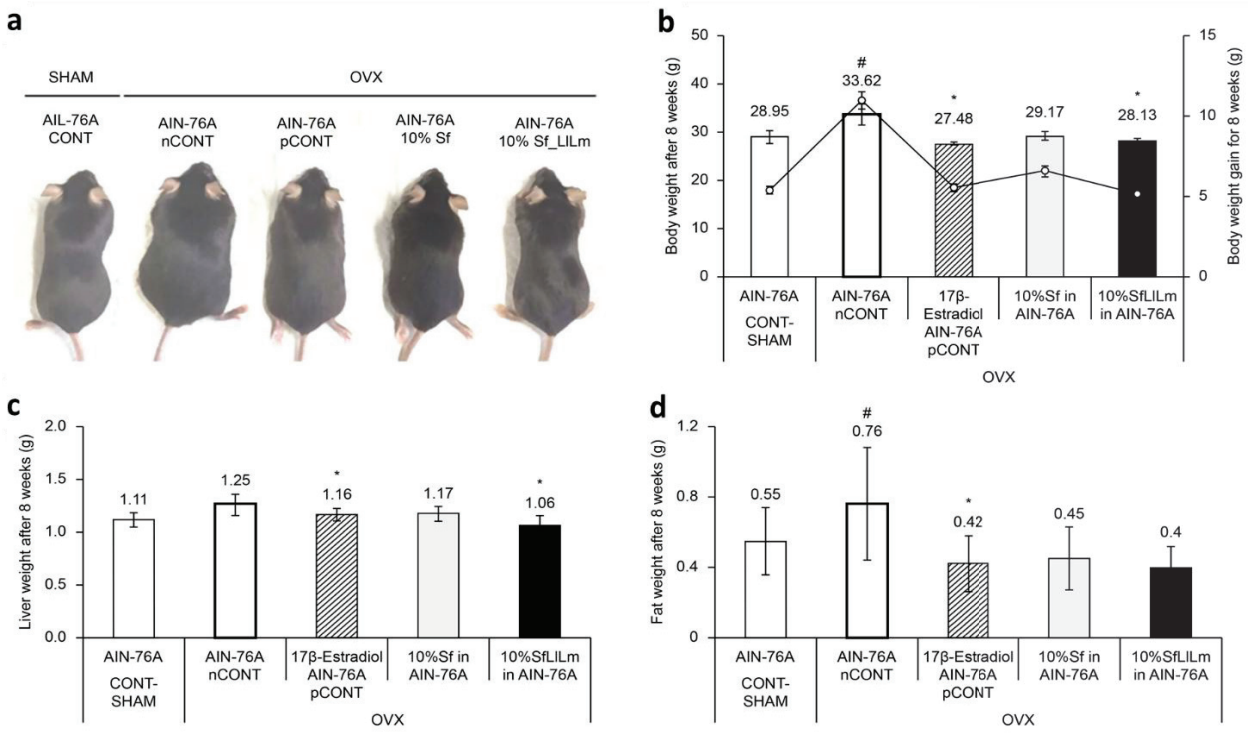


Figure 2. Effects of *Sargassum fulvellum* (Sf) and its fermented form (SfLlLm) on body, liver, and fat weights in ovariectomized (OVX) mice. (**a**) Representative dorsal images of mice from each group after 8 weeks of dietary intervention. (**b**) Final body weight (bar graph) and weight gain (line graph) in mice. (**c**) Relative liver weight normalized to body weight. (**d**) Relative fat weight, including epididymal and retroperitoneal fat, normalized to body weight. Data are presented as mean ± standard error of the mean ($n = 5$). # $p < 0.05$ vs. CONT (sham-operated control); * $p < 0.05$ vs. nCONT (OVX control), indicating significant effects of 17β-estradiol, Sf, or SfLlLm treatment.

These visual findings were consistent with the quantitative measurements. As shown in Figure 2b, administration of 10% Sf or SfLlLm for 8 weeks significantly suppressed body weight gain compared to the nCONT. Additionally, both Sf and SfLlLm led to notable reductions in liver weight and retroperitoneal fat pad weight (Figure 2c,d). Among the dietary groups, SfLlLm showed the most substantial improvements in these metabolic indicators. Although the SfLlLm group exhibited slightly greater reductions in cumulative body weight, liver weight, and fat mass than pCONT, these findings should be interpreted cautiously given the exploratory nature of the study and limited sample size. These results align with previous reports demonstrating the efficacy of dietary seaweed in mitigating obesity-related outcomes in estrogen-deficient animal models [17,19].

The enhanced metabolic efficacy of SfLlLm compared to unfermented *S. fulvellum* may be attributed to the formation of fermentation-enriched bioactives, including long-chain menaquinones (e.g., MK-4, MK-7, MK-9), short-chain fatty acids (SCFAs), and microbial exopolysaccharides produced by *L. lactis* and *L. mesenteroides* [10,20–22]. These metabolites are reported to influence antioxidant activity, lipid metabolism, mitochondrial function, and systemic inflammation, which are central to adiposity and metabolic regulation [23]. However, while these hypotheses are supported by prior research, molecular validation (e.g., gene or protein expression analyses) was not conducted in the present study. Therefore, the involvement of these compounds in mediating the observed outcomes remains speculative and warrants further investigation.

While hormone replacement therapy (HRT) restores systemic estrogen levels to ameliorate menopausal symptoms, the metabolic benefits of SfLlLm appear to be exerted through non-hormonal pathways involving fermentation-derived bioactives. This distinction may offer potential advantages in safety and tolerability, particularly for populations for whom HRT is contraindicated. Nonetheless, the long-term efficacy and safety of SfLlLm require validation in future preclinical and clinical studies.

Given the growing demand for safe, food-based interventions to address menopausal metabolic dysfunction, fermented *S. fulvellum* represents a promising candidate as a functional dietary supplement to manage weight gain and hepatic steatosis in postmenopausal populations.

3.2. Hepatoprotective and Hypolipidemic Effects of Fermented S. fulvellum

As shown in Figure 3a, ALT levels were significantly elevated in the OVX control group (nCONT) compared to the sham-operated group (CONT), indicating hepatic dysfunction associated with estrogen deficiency. However, administration of either Sf or SfLlLm for 8 weeks markedly reduced ALT levels. Among the treatment groups, SfLlLm exhibited the most pronounced ALT-lowering effect, comparable to that of the 17β-estradiol-treated group (pCONT). Similarly, Figure 3b shows that serum AST levels were also significantly increased in the nCONT group relative to CONT. Both Sf and SfLlLm effectively reduced AST levels, with SfLlLm again demonstrating greater efficacy than the unfermented form. These biochemical improvements were further corroborated by histological analysis. As presented in Figure 3c, liver sections stained with H&E revealed prominent lipid droplet accumulation in the livers of the nCONT mice, indicative of hepatic steatosis. In contrast, mice treated with Sf or SfLlLm exhibited markedly fewer lipid vacuoles, suggesting reduced hepatic fat deposition. Notably, the SfLlLm group displayed a histological profile most similar to that of pCONT, indicating a strong protective effect against OVX-induced fatty liver. Collectively, these findings demonstrate that fermented *S. fulvellum* not only improves serum lipid profiles but also alleviates hepatic lipid accumulation and injury in estrogen-deficient mice. In particular, the normalization of ALT and AST levels suggests hepatoprotective effects, which may be partially mediated by fermentation-enriched bioactive compounds such as vitamin K2.

Vitamin K2 reportedly attenuates hepatic inflammation and fibrosis by modulating oxidative stress, mitochondrial function, and expression of proinflammatory cytokines [11]. Given that lactic acid fermentation of *S. fulvellum* significantly increases MK-4, MK-7, and MK-9 levels [10], these menaquinones may play a key role in restoring liver enzyme balance and mitigating liver damage. Additionally, SCFAs and microbial exopolysaccharides produced during fermentation are also known to improve hepatic lipid metabolism and reduce the steatotic burden [21,22]. Importantly, the hepatoprotective and lipid-lowering effects of SfLlLm were comparable to—or in some cases exceeded—those of 17β-estradiol. This suggests that fermented *S. fulvellum* may exert its metabolic benefits through estrogen-independent mechanisms while offering a favorable safety profile. These findings are particularly relevant, considering the well-documented risks associated with long-term HRT, such as stroke and thromboembolism [24,25]. Therefore, SfLlLm may serve as a promising food-derived alternative for managing dyslipidemia, hepatic steatosis, and liver enzyme imbalance in postmenopausal women.

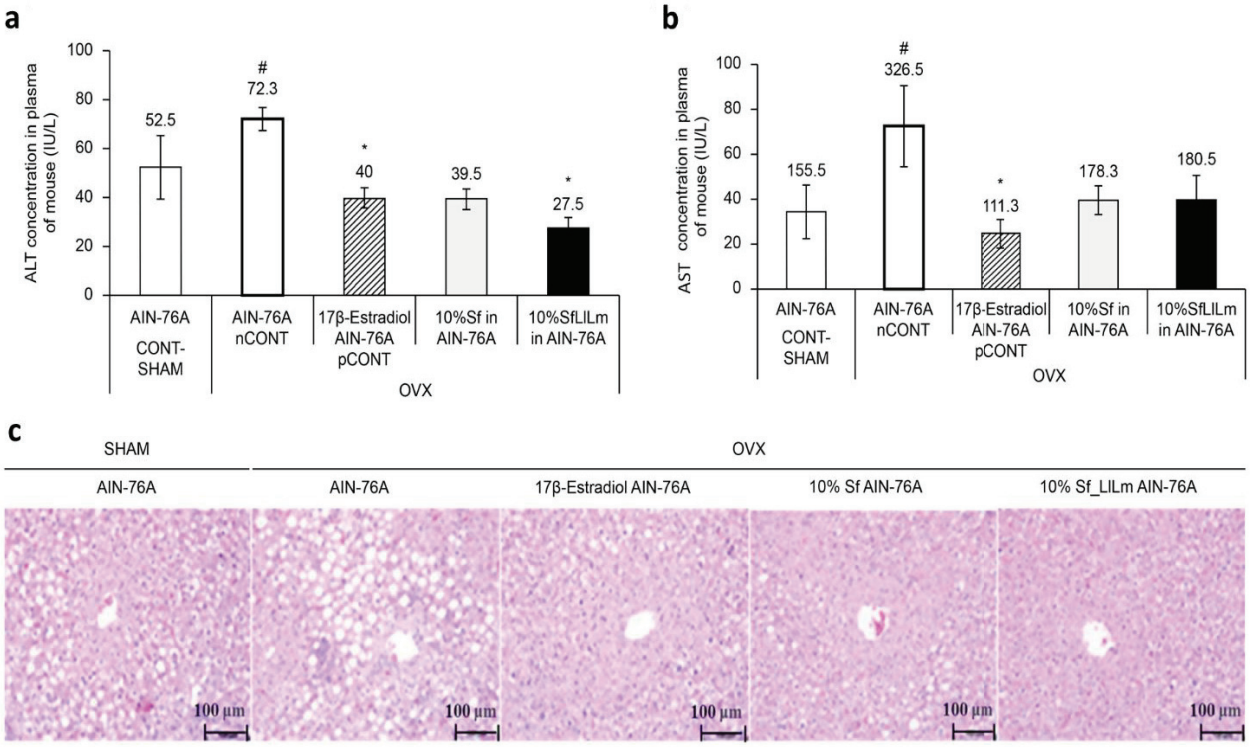


Figure 3. Effects of *Sargassum fulvellum* (Sf) and its fermented form (SfLlLm) on hepatic lipid accumulation and serum biochemical parameters in ovariectomized (OVX) mice. (**a**) Serum alanine aminotransferase (ALT) levels. (**b**) Serum aspartate aminotransferase (AST) levels. (**c**) Representative histological images of liver tissues stained with hematoxylin and eosin (H&E), showing lipid accumulation in each group (scale bar = 100 μm). Data are expressed as mean ± standard error of the mean (n = 5). # $p < 0.05$ vs. CONT (sham-operated control); * $p < 0.05$ vs. nCONT (OVX control), indicating significant effects of 17β-estradiol, Sf, or SfLlLm treatment.

3.3. Effects of Fermented S. fulvellum on Serum Lipid and Adipose Tissue

As shown in Figure 4a,b, serum TC and TG levels were significantly elevated in the OVX control group (nCONT) compared to the sham-operated group (CONT), reflecting dyslipidemia induced by estrogen deficiency. Dietary supplementation for 8 weeks with either Sf or SfLlLm markedly reduced TC and TG levels. Among the treatments, SfLlLm exhibited lipid-lowering effects, comparable to those of 17β-estradiol (pCONT). Regarding lipoprotein profiles (Figure 4c,d), LDL-C levels were moderately elevated in the nCONT group, indicating impaired lipid transport and increased cardiovascular risk. All treatment groups—Sf, SfLlLm, and 17β-estradiol—significantly reduced LDL-C levels, with SfLlLm showing the most notable reduction. HDL-C levels were also significantly increased following all treatments. Interestingly, unfermented Sf led to the highest HDL-C elevation, suggesting that native bioactive compounds within *S. fulvellum* may promote reverse cholesterol transport. Histological analysis of epididymal adipose tissue (Figure 4e) revealed that the nCONT group displayed enlarged, irregularly distributed adipocytes typical of visceral fat hypertrophy. In contrast, both Sf and SfLlLm treatments led to smaller, more uniform adipocytes. The SfLlLm group, in particular, exhibited an adipocyte morphology closely resembling that of the pCONT group, indicating a reversal of OVX-induced adipose remodeling. These data collectively suggest that SfLlLm exerts beneficial effects on systemic lipid homeostasis and adipose tissue architecture under estrogen-deficient conditions. The simultaneous reduction of LDL-C and normalization of adipocyte morphology, alongside increased HDL-C levels, underscore the dual regulatory potential of SfLlLm on lipid transport and storage.

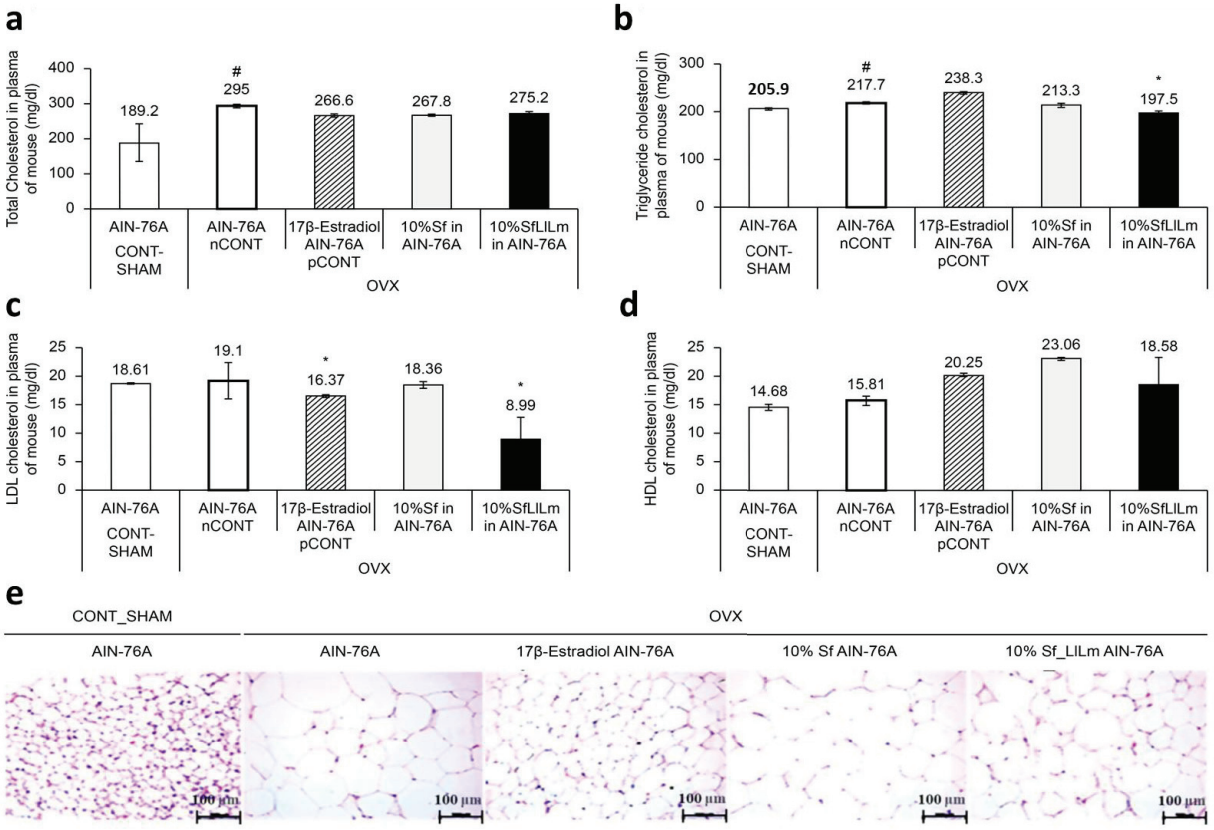


Figure 4. Effects of *Sargassum fulvellum* (Sf) and its fermented form (SfLlLm) on serum lipid levels and adipocyte morphology in ovariectomized (OVX) mice. (**a**) Serum total cholesterol (TC) levels. (**b**) Serum triglyceride (TG) levels. (**c**) Serum low-density lipoprotein cholesterol (LDL-C) levels. (**d**) Serum high-density lipoprotein cholesterol (HDL-C) levels. (**e**) Representative histological images of epididymal adipose tissue stained with hematoxylin and eosin (H&E), showing adipocyte size and distribution (scale bar = 100 μm). Data are presented as mean ± standard error of the mean (n = 5). # $p < 0.05$ vs. CONT (sham-operated control); * $p < 0.05$ vs. nCONT (OVX control), indicating significant effects of 17β-estradiol, Sf, or SfLlLm treatment.

The enhanced metabolic efficacy of SfLlLm appears to be associated with fermentation-derived metabolites, including vitamin K2 homologs (menaquinones MK-4, MK-7, MK-9), short-chain fatty acids (SCFAs), and microbial exopolysaccharides. These compounds are known to modulate adipogenesis, promote lipid oxidation, and attenuate inflammation in adipose tissue [21,22]. In particular, menaquinones have demonstrated potential for reducing adipocyte hypertrophy, improving insulin sensitivity, and suppressing proinflammatory cytokine expression [10], which aligns with the histological and biochemical improvements observed in this study. While unfermented *S. fulvellum* also improved the lipid profiles, its effects were relatively modest. This may be due to its native content of marine bioactives such as fucoidans, polyphenols, and sterols, which are associated with cholesterol metabolism and vascular health.

Our findings support the hypothesis that fermentation enhances the lipid-modulating efficacy of *S. fulvellum* under estrogen-deficient conditions, likely by generating additional or synergistic bioactive compounds. This aligns with prior reports on the metabolic benefits of vitamin K2, SCFAs, and fermented seaweed components. Nevertheless, several limitations should be acknowledged. First, the small sample size (n = 5 per group) may limit the statistical power and generalizability. Second, the absence of blinding during data collection and histological evaluation could introduce observer bias. Third, although the OVX mouse is a well-established model of postmenopausal metabolic dysfunction,

it does not fully recapitulate the complexity of human physiology. Finally, mechanistic pathways were not directly evaluated in this study, and future investigations should include molecular analyses to elucidate the underlying mechanisms. Additionally, the outcome assessments were not blinded, which may introduce potential observer bias. Although objective assay kits and standardized histological protocols were used, the lack of blinding remains a methodological limitation.

Despite these limitations, SfLlLm effectively mitigated dyslipidemia and adipose tissue hypertrophy induced by estrogen deficiency in OVX mice. Its superior efficacy over unfermented *S. fulvellum* suggests that fermentation significantly enhances the functional potential of this marine resource. Through its modulation of lipid profiles and normalization of adipocyte morphology, SfLlLm is a promising candidate for dietary intervention in postmenopausal women and others at risk of metabolic syndrome. While the OVX model provides translational relevance, caution is warranted in extrapolating the findings to humans due to species-specific differences in metabolism. In addition, while this study demonstrated the beneficial effects of SfLlLm on lipid metabolism and adipose tissue remodeling, the absence of molecular analyses limits mechanistic interpretation. Moreover, our study did not include mechanistic assays to identify the molecular targets or signaling pathways involved in the observed effects. Future studies should incorporate gene and protein expression analyses, such as those related to PPARγ (peroxisome proliferator-activated receptor gamma), adipogenic regulators, and lipid metabolism enzymes, to elucidate the underlying mechanisms of action. Future investigations should incorporate gene or protein expression analyses related to lipid metabolism, inflammation, and estrogen signaling to substantiate the proposed bioactive effects. Further clinical studies are needed to validate the efficacy and safety of fermented *S. fulvellum* as a nutraceutical for improving cardiovascular and metabolic health in postmenopausal populations.

4. Conclusions

This study demonstrates that fermented *S. fulvellum* (SfLlLm) significantly improves serum lipid profiles and mitigates adipose tissue hypertrophy in an estrogen-deficient OVX mouse model. Compared to its unfermented counterpart, SfLlLm exhibited superior efficacy in reducing triglycerides and LDL-C levels. Histological analysis further revealed that SfLlLm supplementation restored adipocyte size and distribution to levels comparable to those observed in the 17β-estradiol-treated group. These metabolic improvements are likely attributable to fermentation-derived bioactive compounds such as menaquinones, SCFAs, and microbial exopolysaccharides. Collectively, these findings support the potential application of fermented *S. fulvellum* as a functional dietary intervention for mitigating postmenopausal metabolic dysfunction. However, given the preclinical nature of the study, further molecular and clinical investigations are warranted to validate these effects in humans.

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References

1. Abildgaard, J.; Ploug, T.; Al-Saoudi, E.; Wagner, T.; Thomsen, C.; Ewertsen, C.; Bzorek, M.; Pedersen, B.K.; Pedersen, A.T.; Lindegaard, B. Changes in abdominal subcutaneous adipose tissue phenotype following menopause is associated with increased visceral fat mass. *Sci. Rep.* **2021**, *11*, 14750. [CrossRef]
2. Fasero, M.; Coronado, P.J. Cardiovascular disease risk in women with menopause. *J. Clin. Med.* **2025**, *14*, 3663. [CrossRef]
3. Medina-Contreras, J.M.L.; Villalobos-Molina, R.; Zarain-Herzberg, A.; Balderas-Villalobos, J. Ovariectomized rodents as a menopausal metabolic syndrome model: A minireview. *Mol. Cell. Biochem.* **2020**, *475*, 261–276. [CrossRef]
4. Holdt, S.L.; Kraan, S. Bioactive compounds in seaweed: Functional food applications and legislation. *J. Appl. Phycol.* **2011**, *23*, 543–597. [CrossRef]
5. Liu, J.; Luthuli, S.; Wu, Q.; Wu, M.; Choi, J.I.; Tong, H. Pharmaceutical and nutraceutical potential applications of *Sargassum fulvellum*. *BioMed Res. Int.* **2020**, *2020*, 2417410. [CrossRef]
6. Kim, S.-H.; Lee, Y.-M.; Moon, S.-W.; Ahn, J.; Oh, S.; Lee, Y.-W.; Jeon, Y.-J. How do brown seaweeds work on biomarkers of dyslipidemia? A meta-analysis. *Mar. Drugs* **2022**, *21*, 220. [CrossRef]
7. Peng, J.; Yuan, J.P.; Wu, C.F.; Wang, J.H. Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: Metabolism and bioactivities relevant to human health. *Mar. Drugs* **2011**, *9*, 1806–1828. [CrossRef]
8. Han, P.; Cho, S.-Y.; Kang, M.-C.; Lee, H.-G.; Lim, Y.; Ko, S.-C.; Kang, S.-M.; Kim, A.-D.; Kim, D.; Jeon, Y.-J. In vitro and in vivo anti-inflammatory activities of sulfated polysaccharides isolated from Celluclast-extract of an edible brown seaweed, *Sargassum fulvellum*. *Antioxid* **2019**, *8*, 493. [CrossRef]
9. Kim, K.N.; Heo, S.J.; Kang, S.M.; Ahn, G.; Jeon, Y.J. Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated Bcl-xL pathway. *Toxicol. Vitr.* **2010**, *24*, 1648–1654. [CrossRef]
10. Sim, Y.; Jo, H.S.; Kim, C.G.; Cho, Y.E.; Yang, J.; Hong, S.M. Anti-inflammatory and osteogenic effects of vitamin K from *Sargassum fulvellum* fermented by *Lactococcus lactis* KCCM12759P and *Leuconostoc mesenteroides* KCCM12756P. *Fermentation* **2024**, *10*, 569. [CrossRef]
11. Morishita, T.; Tamura, N.; Makino, T.; Kudo, S. Production of menaquinones by lactic acid bacteria. *J. Dairy Sci.* **1999**, *82*, 1897–1903. [CrossRef]
12. Shea, M.K.; Booth, S.L. Update on the role of vitamin K in skeletal health. *Nutr. Rev.* **2008**, *66*, 549–557. [CrossRef]
13. Liu, Y.; van Bennekom, E.O.; Zhang, Y.; Abee, T.; Smid, E.J. Long-chain vitamin K2 production in *Lactococcus lactis* is influenced by temperature, carbon source, aeration and mode of energy metabolism. *Microb. Cell Factories* **2019**, *18*, 129. [CrossRef] [PubMed]
14. Fu, X.; Harshman, S.G.; Shen, X.; Haytowitz, D.B.; Karl, J.P.; Wolfe, B.E.; Booth, S.L. Multiple vitamin K forms exist in dairy foods. *Curr. Dev. Nutr.* **2017**, *1*, e000638. [CrossRef]
15. Harshman, S.G.; Shea, M.K. The role of vitamin K in chronic aging diseases: Inflammation, cardiovascular disease, and osteoarthritis. *Curr. Nutr. Rep.* **2016**, *5*, 90–98. [CrossRef] [PubMed]
16. Mansour, S.W.; Selim, S.A.; Salama, S.A.; Hussein, S.; Abozaid, E.R. Anti-apoptotic effect of menaquinone-7 protects the brain of ovariectomized rats. *Beni Suef Univ. J. Basic Appl. Sci.* **2023**, *12*, 22. [CrossRef]
17. Yamaguchi, M.; Taguchi, H.; Gao, Y.H.; Igarashi, A.; Tsukamoto, Y. Effect of vitamin K2 (menaquinone-7) in fermented soybean (natto) on bone loss in ovariectomized rats. *J. Bone Miner. Metab.* **1999**, *17*, 23–29. [CrossRef] [PubMed]
18. Jang, H.R.; Park, H.-J.; Kang, D.; Chung, H.; Nam, M.H.; Lee, Y.; Park, J.-H.; Lee, H.-Y. A protective mechanism of probiotic Lactobacillus against hepatic steatosis via reducing host intestinal fatty acid absorption. *Exp. Mol. Med.* **2019**, *51*, 1–14. [CrossRef]
19. Panico, A.; Messina, G.; Lupoli, G.A.; Lupoli, R.; Cacciapuoti, M.; Moscatelli, F.; Esposito, T.; Villano, I.; Valenzano, A.; Monda, V.; et al. Quality of life in overweight (obese) and normal-weight women with polycystic ovary syndrome. *Patient Prefer. Adherence* **2017**, *11*, 423–429. [CrossRef]

20. LeBlanc, J.G.; Milani, C.; de Giori, G.S.; Sesma, F.; van Sinderen, D.; Ventura, M. Bacteria as vitamin suppliers to their host: A gut microbiota perspective. *Curr. Opin. Biotechnol.* **2013**, *24*, 160–168. [CrossRef]
21. Markowiak-Kopeć, P.; Śliżewska, K. The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients* **2020**, *12*, 1107. [CrossRef]
22. Nagpal, R.; Wang, S.; Ahmadi, S.; Hayes, J.; Gagliano, J.; Subashchandrabose, S.; Kitzman, D.W.; Becton, T.; Read, R.; Yadav, H. Human-origin probiotic cocktail increases short-chain fatty acid production via modulation of mice and human gut microbiome. *Sci. Rep.* **2018**, *8*, 12649. [CrossRef] [PubMed]
23. Kim, J.Y.; Jang, S.; Song, H.J.; Lee, S.; Cheon, S.; Seo, E.J.; Choi, Y.H.; Kim, S.H. *Sargassum horneri* extract fermented by *Lactiplantibacillus pentosus* SH803 mediates adipocyte metabolism in 3T3-L1 preadipocytes by regulating oxidative damage and inflammation. *Sci. Rep.* **2024**, *14*, 15064. [CrossRef] [PubMed]
24. Yao, C.-C.; Tong, Y.-X.; Jiang, H.; Yang, D.-R.; Zhang, X.-J.; Zhang, P.; Su, L.; Zhao, Y.-Y.; Chen, Z.-W. Native polypeptide vglycin prevents nonalcoholic fatty liver disease in mice by activating the AMPK pathway. *J. Funct. Foods* **2020**, *73*, 104110. [CrossRef]
25. Shang, L.-Y.; Zhang, S.; Zhang, M.; Sun, X.-D.; Wang, Q.; Liu, Y.-J.; Zhao, Y.-N.; Zhao, M.; Wang, P.-J.; Gao, X.-L. Natto alleviates hyperlipidemia in high-fat diet-fed mice by modulating the composition and metabolic function of gut microbiota. *J. Funct. Foods* **2024**, *112*, 105968. [CrossRef]

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Glycemic Control and Quality of Life Among People with Type 1 Diabetes: Relationships with Insulin Therapy and Carbohydrate Counting

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Abstract

Background/Objectives: Diabetes mellitus has physical, psychological and behavioral implications related to glycemic control and quality of life. Our aims were to evaluate glycemic control and disease-related quality of life among adults with type 1 diabetes mellitus and to study their relationships with sociodemographic, clinical and anthropometric characteristics. **Methods**: Eighty-five participants (49.4% females, mean age 36 years, mean BMI 25.3 kg/m^2) attending endocrinology and/or nutrition consultations at ULS São João (Porto, Portugal) were assessed regarding quality of life (Diabetes Health Profile) and social desirability (Marlowe–Crowne's scale). Anthropometric (weight and height), biochemical (A1c) and metabolic control data (time at glycemic target) were collected, as well as information on type and duration of insulin therapy, carbohydrate counting, use of mobile applications for carbohydrate counting and perceived health status. **Results**: Older patients had higher BMI (r = 0.227, p = 0.037) and worse quality of life in the 'barriers to activity' domain (r = 0.290, p = 0.007). In the multivariate analysis, sociodemographic, clinical and quality of life characteristics significantly explained A1c (p = 0.046, η^2_p = 0.271) and perceived health status (p = 0.012, η^2_p = 0.313). Higher eating self-efficacy significantly explained better perceived health status (p = 0.006, η^2_p = 0.105), but no variable independently had a significant effect on glycemic control. **Conclusions**: Glycemic control results from the interaction between various clinical features that must be considered in personalized approaches. Eating self-efficacy significantly explained perceived health status, and may represent a potential target for intervention among people with type 1 diabetes mellitus.

Keywords: glycemic control; quality of life; glycosylated hemoglobin; Diabetes Health Profile; eating self-efficacy

1. Introduction

Diabetes mellitus (DM) is a complex, chronic condition in which glucose is not used effectively as a source of energy, resulting in hyperglycemia [1]. It can be categorized as type 1 diabetes mellitus (DM1), type 2 diabetes mellitus (DM2), gestational diabetes mellitus, and other specific types arising from various causes. DM1 (including latent autoimmune

diabetes of the adult, LADA) results from the autoimmune destruction of pancreatic beta cells, which generally leads to absolute insulin deficiency and, therefore, the need to obtain it exogenously. It is thought that DM1 represents around 10% of all DM cases and, although there is currently no effective measure to prevent it, it has been hypothesized that, in addition to other factors unrelated to diet, excess weight may represent a risk factor [2,3].

Poor glycemic control in DM is associated with the onset of acute complications, such as diabetic ketoacidosis and severe hypoglycemia. The aim of treatment and management of DM is to prevent the onset of these complications and to reduce the risk of medium-to-long-term complications. Glycemic control can be assessed by measuring glycosylated hemoglobin (A1c), by capillary glucose monitoring or through continuous monitoring systems using the 'time in range' metric or through average glucose. A1C values correspond to the proportion of glycosylated (i.e., 'coated' with sugar) hemoglobin, and measures the average blood sugar levels over the previous 2 to 3 months. It is a valuable tool for diagnosing and monitoring diabetes and prediabetes, as it indicates the overall blood glucose control. The A1c value is strongly related to the risk of complications from DM [4,5] and current recommendations target a maximum A1c of 7% [6].

Individuals with DM1 require the administration of exogenous insulin. Within intensive insulin therapy, the use of continuous subcutaneous insulin infusion (CSII) seems to be more effective in reducing A1c levels [7–9], with patients who had worse control on a regimen involving the multiple administration of insulin analogs benefiting more from the transition to CSII [7–10]. In addition, the use of CSII is associated with a lower risk of hypoglycemia [7–11] and less glycemic variability [7,8].

In patients with DM1, carbohydrate counting (CHC) is a safe dietary strategy to achieve adequate glycemic control [12], and is associated with improvements in control compared to dietary education strategies alone [13–15]. As far as diet therapy is concerned, applications that enable CHC are intended to help patients make a better estimate of the carbohydrate content and, consequently, to administer the correct amount of insulin. The use of applications that enable CHC and the calculation of the insulin bolus to be administered seem to show promising results [15].

DM has various physical, psychological and behavioral implications for quality of life [16]. Good management of this disease requires the active participation of the patient, and it is becoming increasingly important to assess the impact of the disease on the patient's behavioral profile and quality of life, which seems to be negatively affected by diabetes. Within the insulin therapy available for the treatment of DM, the use of CSII compared to the multiple administration of insulin analogs seems to be associated with better glycemic control [7,17] and a better quality of life [11,18]. The quality of life of those who use the CHC method seems to be better compared to those who do not.

Eating self-efficacy refers to beliefs in the ability to implement the actions necessary to achieve a certain result regarding food and eating, as well as the feeling of control over one's dietary behaviors and environment [19,20]. This dimension is particularly important because patients with diabetes mellitus are expected to take on a series of behaviors and activities aimed at self-care and good management of this chronic disease on a daily basis. Lower levels of eating self-efficacy are associated with greater resistance to treatment or the adoption of measures [21], which could have implications for glycemic control.

Social desirability corresponds to the tendency of individuals to try to convey a culturally accepted image, in accordance with social norms [22], so this is a potential source of bias when self-report instruments are used.

Despite glycemic control being a fundamental feature of diabetes treatment, the literature is scarce regarding its relationship with eating behavior, namely eating self-efficacy, which may play a major role in determining the ability to comply with dietetic recom-

mendations. On the other hand, a multidimensional perspective of health care provision implies the concomitant consideration of objective measures (such as glycemic control) and subjective measures (namely quality of life) as clinical outcomes. Moreover, the relationships between such features may vary between patients with different sociodemographic and clinical characteristics (namely regarding the use of CHC, CSII and CHC apps), and the knowledge of these differences is of high relevance for the implementation of strategies aimed at improving the efficacy of dietary therapy and glycemic control.

The objectives of this study were (1) to assess glycemic control and quality of life, defined in terms of disease-related psychosocial and behavioral dysfunction (psychological strain, barriers to activity and disinhibition in eating) of adults with DM1; (2) to compare sociodemographic characteristics (sex, age, education), clinical characteristics, glycemic control, quality of life and eating self-efficacy according to the use of CHC, CSII and CHC apps; (3) to study the relationships between sociodemographic and clinical characteristics, glycemic control, quality of life and eating self-efficacy; and (4) to study the effect of sociodemographic, clinical characteristics, quality of life and eating self-efficacy on glycemic control and perceived health status. Based on the current body of knowledge, we expect that younger and more educated participants present better glycemic control and perceived health status. We also hypothesize that these two outcomes are associated with higher eating self-efficacy and better disease-related psychosocial profile.

2. Methods

This study used a convenience sample of diabetic patients attending the Nutrition and/or Endocrinology consultation at the Local Health Unit of São João (Porto, Portugal). All the study procedures were approved by the Ethics Committee of the University Hospital Centre of São João/Faculty of Medicine of the University of Porto (Reference 85/2023) and it was carried out under the parameters of the Declaration of Helsinki.

2.1. Sample and Procedures

Recruitment took place between August 2023 and January 2024. Inclusion criteria were (i) age between 18 and 65; and (ii) diagnosis of DM1 at least one year previously. Exclusion criteria were the presence of a pathology or clinical condition that implied dietary therapy other than that recommended for the treatment of diabetes and/or that affected the ability to make a free and informed decision or to participate. After providing written informed consent, participants answered a questionnaire collecting sociodemographic (sex, age and education) and clinical data (current glycosylated hemoglobin, time in range, co-morbidities, duration of illness, type of insulin therapy, use of CSII, CHC and mobile applications for CHC), which was verified and complemented with the information available in each patient's electronic clinical record. Finally, participants were assessed regarding their health-related quality of life, perceived health status, eating self-efficacy and social desirability.

2.2. Instruments and Measures

Height was measured using a SECA® 213 portable stadiometer (SECA, Hamburg, Germany) (precision of 0.1 cm). Weight was measured using the InBody body composition analyser model 230 (Seoul, Republic of Korea) (precision of 100 g).

The Portuguese 18-item version of the Diabetes Health Profile [23] was used to assess the impact of living with DM on the emotional and social state. It is an 18-question instrument that aims to outline the psychosocial profile of patients who use insulin, across different dimensions: (1) psychological distress, which comprises 6 items referring to problems such as depressive symptoms, more arguments and hassles at home or feelings of

hopelessness and irritability due to diabetes; (2) barriers to activity, which comprises 7 items focusing on issues such as food controlling life or avoiding going out when sugar is low; and (3) disinhibited eating, which comprises 5 items and refers to issues such as finding it easy to stop eating after having started, or wishing there were not so many good things to eat. The items are answered in a Likert-type scale with 4 response options, and the answers in each dimension are summed and transformed into a score between 0 and 100, where 0 indicates no dysfunction and 100 the worst perception of quality of life. The Portuguese version of this instrument presented adequate psychometric properties, namely construct validity, content validity, and reliability (Cronbach's alpha values for the psychological distress, barriers to activity and disinhibited eating dimensions of, respectively, 0.834, 0.645 and 0.732.

As for the assessment of perceived health status, a visual analog scale from 0 to 100 was used, in which the higher the value, the better the subjective state of health: 0 corresponds to the 'worst state of health' and 100 to the 'best state of health' [24]. Participants were asked to indicate the number that best described their state of health at the time.

Eating self-efficacy was assessed using the General Eating Self-Efficacy Scale [25], which comprises 5 items with 5 response options ranging from 'I don't agree' to 'I agree very much'. Each item is graded from 0 to 4 points and the total score (0 to 20 points) is obtained by the sum of the scores of individual items. Higher scores correspond to higher eating self-efficacy. This scale has shown good internal consistency, with Cronbach's alpha values above 0.85, as well as convergent and discriminant validities, both among higher education students [25] and in a clinical sample [26].

The Portuguese composite version of Marlowe–Crowne's Social Desirability Scale [27] comprises 12 dichotomous response items ('Yes'/'No'), to each of which 1 point is awarded to the response that corresponds to the attitude that reveals social desirability. The total score (0 to 12 points) results from the sum of the scores on the items, and higher scores correspond to greater social desirability. This scale has shown adequate psychometric properties, including internal consistency (Kuder–Richardson index of 0.60), temporal stability (three months test–retest: $r = 0.76$, $p < 0.01$), discriminant and divergent validity [27].

2.3. Statistical Analysis

The statistical analysis was carried out using IBM SPSS version 29.0 for Windows. Descriptive statistics consisted of absolute (n) and relative (%) frequencies, means and standard deviations (SDs).

The normality of quantitative variables was assessed using asymmetry and kurtosis. When the variables had a non-normal distribution, a 2-parameter logarithmic transformation was applied along with a linear transformation so that the median was not altered. The transformed variables were used in the inferential analysis, but descriptive statistics are presented for the non-transformed variables.

All inferential statistics were carried out with 95% confidence. Pearson's correlation coefficient (r) was used to measure the degree of association between pairs of variables, and Student's t-tests and one-way ANOVA were used to compare means of independent samples (post hoc tests were performed with Sidak's correction). A MANCOVA model was carried out to study the effect of various characteristics on A1c and perceived health status.

3. Results

Sample characterization and comparison between sexes:

The final sample consisted of 85 participants, 49.4% of whom were women ($n = 42$). Table 1 shows the characterization of the sample and comparison between sexes. Partic-

ipants had a mean age of 37 years (SD = 13), mean BMI of 25.8 kg/m^2 (SD = 3.9), mean education of 14 years (SD = 4) and mean duration of illness of 20.3 years (SD = 12.7).

Table 1. Sample characterization and comparison between sexes.

	Total Sample (*n* = 85)	**Women (*n* = 42)**	**Men (*n* = 43)**	***p***
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	
Age (years)	37 (13)	35 (12)	38 (13)	0.356
Education (years)	14 (4)	14 (3)	14 (4)	0.914
BMI (kg/m^2)	25.8 (3.9)	25.8 (2.7)	24.8 (3.7)	0.169
A1C (%)	7.7 (1.2)	7.8 (1.1)	7.6 (1.2)	0.510
Time in range (%) [*n* = 64]	57.0 (20.1)	54.9 (20.1)	59.1 (20.2)	0.402
Diabetes duration (years)	20.3 (12.7)	21.5 (12.1)	19.1 (13.3)	0.385
CSII duration (years) [*n* = 34]	6.6 (4.0)	7.2 (4.6)	5.8 (2.9)	0.300
CHC duration (years) [*n* = 69]	7.7 (5.6)	8.3 (5.3)	7.0 (6.0)	0.334
App duration (years) [*n* = 33]	1.7 (1.4)	1.2 (1.1)	2.0 (1.5)	0.119
Health status	71.6 (17.6)	74.4 (3.8)	75.3 (3.4)	0.254
Barriers to activity	32.0 (17.0)	32.7 (17.6)	31.5 (16.6)	0.747
Disinhibited eating	29.7 (16.8)	32.5 (16.5)	27.0 (16.9)	0.128
Psychological distress	18.7 (15.5)	17.3 (3.9)	15.6 (3.7)	0.039
Eating self-efficacy	12.9 (3.6)	12.7 (3.5)	13.0 (3.7)	0.692
Social desirability	8.2 (2.3)	8.3 (2.3)	8.1 (2.3)	0.598

A1C: Glycosylated hemoglobin. BMI: Body mass index. CHC: Carbohydrate counting. CSII: Continuous subcutaneous insulin infusion. Health status, barriers to activity, disinhibited eating and psychological distress: score between 0 and 100. Eating self-efficacy: score between 0 and 20. Social desirability: score between 0 and 12.

With regard to analytical values and disease-related data, the average A1C value was 7.7% (SD = 1.2) and the average time in range was 57% (SD = 20). Forty percent of the participants used CSII, with a mean duration of use of 6.6 years (SD = 4.0), 81% performed CHC, with a mean duration of CHC of 7.6 years (SD = 5.6) and 39% used applications, with a mean duration of use of 1.7 years (SD = 1.4).

The average score on the eating self-efficacy scale was 12.9 (SD = 3.6). With regard to the score on the DHP, the quality of life dimension was highest for barriers to activity (mean = 32.0; SD = 17.0), followed by disinhibited eating (mean = 29.7; SD = 16.8) and psychological distress (mean = 18.7; SD = 15.5). The average perceived health status was 72 (SD = 18), and the mean score on the social desirability scale was 8.2 (SD = 2.3).

Regarding the comparison between sexes, significant differences were found only for the 'Psychological distress' dimension of the DHP, with women scoring higher than men (mean = 17.3 vs. 15.6; $p = 0.039$).

Use of CSII, CHC and Apps

Table 2 compares the groups defined by the use of CSII, CHC and apps for CHC in terms of age and education, BMI, clinical parameters, the dimensions of the DHP, eating self-efficacy and social desirability. There were no significant differences between groups regarding health status perception, glycemic control (A1c or time in range), DM duration or eating self-efficacy.

Participants not on CSII and who did not perform CHC were on average 13 to 14 years older than those on CSII with ($p = 0.039$) or without apps ($p = 0.016$) and those using

apps but not on CSII ($p = 0.015$). Despite the overall significant difference between groups in terms of education ($p = 0.017$), the post hoc comparisons did not identify significant differences between pairs of groups. The tendency was for participants that were not on CSII and did not perform CHC, or who used apps to perform it, to have lower education than the remaining.

Table 2. Comparison of sociodemographic, clinical, glycemic control, anthropometric, quality of life and eating self-efficacy according to the use of CSII, CHC and apps.

	No CSII, No CHC (*n* = 16)	**No CSII, CHC, No Apps (*n* = 16)**	**No CSII, CHC with Apps (*n* = 19)**	**CSII, CHC, No Apps (*n* = 20)**	**CSII, CHC with Apps (*n* = 14)**	***p***
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Age (years)	47 (14) ^{a}	33 (11) ^{b}	37 (11) ^{ab}	34 (11) ^{b}	34 (13) ^{b}	0.007
Education (years)	12 (5) ^{a}	14 (4) ^{a}	12 (3) ^{a}	15 (3) ^{a}	15 (3) ^{a}	0.017
BMI (kg/m^2)	27.2 (3.5) ^{a}	25.0 (1.9) ^{ab}	24.5 (4.2) ^{ab}	26.2 (2.9) ^{ab}	23.3 (1.9) ^{b}	0.007
Health status	74.1 (3.5) ^{a}	75.7 (4.3) ^{a}	74.7 (2.7) ^{a}	75.0 (4.4) ^{a}	74.7 (3.3) ^{a}	0.842
A1C (%)	8.1 (1.4) ^{a}	7.3 (0.8) ^{a}	7.9 (1.3) ^{a}	7.9 (1.3) ^{a}	7.3 (0.6) ^{a}	0.123
Time in range (%) (*n* = 64)	45.8 (22.2) ^{a}	58.5 (15.2) ^{a}	54.4 (23.1) ^{a}	57.8 (22.5) ^{a}	64.8 (14.3) ^{a}	0.328
DM duration (years)	25.8 (16.9) ^{a}	14.3 (8.4) ^{a}	17.8 (11.9) ^{a}	22.6 (13.0) ^{a}	20.7 (9.4) ^{a}	0.089
Disinhibited eating	29.2 (22.0) ^{a}	26.3 (15.1) ^{a}	35.1 (16.0) ^{a}	32.7 (15.7) ^{a}	22.9 (13.0) ^{a}	0.234
Psychological distress	16.5 (3.7) ^{a}	15.9 (4.2) ^{a}	16.5 (3.9) ^{a}	16.2 (4.4) ^{a}	17.3 (3.4) ^{a}	0.903
Barriers to activity	40.5 (17.2) ^{a}	33.3 (15.2) ^{ab}	31.6 (16.1) ^{ab}	32.9 (15.7) ^{ab}	20.4 (17.8) ^{b}	0.028
Eating self-efficacy	12.6 (4.0) ^{a}	13.9 (3.2) ^{a}	12.2 (3.4) ^{a}	12.2 (3.5) ^{a}	14.1 (3.6) ^{a}	0.358
Social desirability	8.3 (1.8) ^{ab}	8.8 (2.0) ^{ab}	9.1 (2.6) ^{a}	7.8 (2.4) ^{ab}	6.8 (2.0) ^{b}	0.038

The presence of the same letter in superscript indicates the absence of significant differences in the post hoc tests. A1C: Glycosylated hemoglobin. BMI: Body mass index. CHC: Carbohydrate counting. CSII: Continuous subcutaneous insulin infusion. Health status, barriers to activity, disinhibited eating and psychological distress: score between 0 and 100. Eating self-efficacy: score between 0 and 20. Social desirability: score between 0 and 12.

With regard to BMI, participants who were not on CSII and did not perform CHC had on average a BMI 3.9 kg/m^2 higher than those on CSII and performing CHC with apps ($p = 0.008$).

Concerning the dimensions assessed by the DHP, there were no differences between the groups for disinhibited eating or psychological distress. Patients who were not on CSII and did not perform CHC presented greater dysfunction in terms of barriers to activity than the group with CSII and CHC with apps (mean score = 40.5 vs. 20.4; $p = 0.012$).

Participants without CSII and who performed CHC with apps had the highest mean social desirability score, their mean score being significantly higher than those with CSII and performing CHC with apps (mean difference of 2.3).

Relationships of age and education with anthropometric, clinical and behavioral characteristics:

Table 3 presents the associations between clinical, anthropometric, psychological and behavioral characteristics and age and education. Older individuals had worse perceived health (r = −0.220; $p = 0.043$), higher BMI (r = 0.227; $p = 0.037$), longer duration of illness (r = 0.668; $p < 0.001$), longer duration of CSII use (r = 0.459; $p = 0.006$) and more barriers to activity (r = 0.290; $p = 0.007$). On the other hand, people with a higher level of education had lower A1C values (r = -0.329; $p = 0.002$), fewer barriers to activity (r = −0.224; $p = 0.040$) and lower social desirability (r = −0.310; $p = 0.004$).

Effects of sociodemographic and clinical characteristics, glycemic control, quality of life and eating self-efficacy on glycemic control and health status:

Table 4 shows the effects of sex, age, level of education, BMI, duration of illness, eating self-efficacy, barriers to activity, disinhibited eating, psychological distress and social desirability on the A1C value and perceived health status. Both A1C value and health

status were significantly explained by all the variables included in the model, with the effect size being greater for health status (η^2_p = 0.313, p = 0.012 vs. η^2_p = 0.271, p = 0.046 for A1c).

Table 3. Relationship between sociodemographic (age and education) and clinical characteristics, glycemic control, quality of life, eating self-efficacy and social desirability.

	Age r (*p*)	**Education r (*p*)**
BMI (kg/m^2)	0.227 (0.037)	−0.083 (0.105)
A1C (%)	0.034 (0.755)	−0.329 (0.002)
Time in range (%) [*n* = 64]	0.081 (0.526)	0.224 (0.075)
Health status	−0.220 (0.043)	−0.014 (0.900)
DM duration (years)	0.668 (<0.001)	−0.030 (0.785)
Pump duration (years) [*n* = 34]	0.459 (0.006)	0.027 (0.879)
CHC duration (years) [*n* = 69]	0.013 (0.917)	0.147 (0.227)
App duration (years) [*n* = 33]	−0.112 (0.535)	0.321 (0.069)
Disinhibited eating	−0.084 (0.446)	−0.136 (0.214)
Psychological distress	−0.034 (0.756)	−0.122 (0.267)
Barriers to activity	0.290 (0.007)	−0.224 (0.040)
Eating self-efficacy	−0.152 (0.165)	0.199 (0.068)
Social desirability	0.085 (0.437)	−0.310 (0.004)

A1C: Glycosylated hemoglobin. BMI: Body mass index. CHC: Carbohydrate counting. Health status, barriers to activity, disinhibited eating and psychological distress: score between 0 and 100. Eating self-efficacy: score between 0 and 20. Social desirability: score between 0 and 12.

Table 4. Multivariate effects of sociodemographic and clinical characteristics on A1C and perceived health status (*n* = 85).

	A1C (%)			**Perceived Health Status**		
	β	***p***	**η^2_p**	**β**	***p***	**η^2_p**
Corrected model		0.046	0.271		0.012	0.313
Female sex	0.008	0.977	0.000	−0.641	0.435	0.009
Age (years)	−0.023	0.109	0.036	−0.020	0.643	0.003
Education (years)	−0.063	0.109	0.036	−0.131	0.276	0.017
Group		0.455	0.050		0.837	0.020
CSII, CHC with apps	(Ref.)			(Ref.)		
CSII, CHC without apps	0.308	0.478		0.941	0.482	
No CSII, CHC with apps	0.201	0.650		−0.371	0.786	
No CSII, CHC without apps	−0.309	0.500		0.101	0.943	
No CSII, no CHC	0.373	0.441		−0.323	0.829	
BMI (kg/m^2)	−0.004	0.925	0.000	−0.041	0.758	0.001
DM duration (years)	0.018	0.198	0.024	−0.039	0.352	0.012
Barriers to activity	0.017	0.072	0.045	−0.005	0.861	0.000
Disinhibited eating	−0.001	0.895	0.005	−0.000	0.998	0.000
Psychological distress	0.022	0.571	0.005	−0.214	0.083	0.042
Eating self-efficacy	−0.025	0.547	0.005	0.358	0.006	0.105
Social desirability	0.065	0.312	0.015	0.220	0.267	0.018

A1C: Glycosylated hemoglobin. BMI: Body mass index. CHC: Carbohydrate counting. CSII: Continuous subcutaneous insulin infusion. Health status, barriers to activity, disinhibited eating and psychological distress: score between 0 and 100. Eating self-efficacy: score between 0 and 20. Social desirability: score between 0 and 12.

Only eating self-efficacy significantly explained perceived health status (p = 0.006, η^2_p = 0.105), with higher levels of eating self-efficacy being related to a better perception of health status. With regard to A1C, none of the variables independently significantly explained the value.

4. Discussion

In this study, we aimed to assess glycemic control, relating it to different dimensions of quality of life and anthropometric and sociodemographic parameters, in order to understand how the quality of life of individuals can be affected by the presence of DM1 and which factors contribute most to glycemic control.

We hypothesized that better glycemic control and perceived health status were related to lower age and higher education. Education level appears to be an important predictor of glycemic control in patients with DM [28,29]. Thus, participants with lower levels of education tend to have worse glycemic control, while higher levels of education appear to be associated with greater skills and knowledge about the disease and its consequences, which may lead patients to make the necessary lifestyle changes, namely improving eating habits, practicing physical activity, adhering to therapy and regularly monitoring blood glucose levels. However, in the bivariate analysis, age only presented a relationship with perceived health status, and education with glycemic control.

Following previous findings [29,30], it was found that older patients had a higher BMI and more barriers to activity. One possible explanation for these findings is that, with advancing age and living with DM (a chronic disease that requires a high level of self-care from the patient) for several years, there may be lower adherence, namely to an adequate CHC or correct administration of insulin, which may result in poorer glycemic control. On the other hand, the fact that the multivariate analysis adjusted the results for possible confounders, namely age, may have influenced these associations.

We did not find any association between longer disease duration and higher A1C values, contrary to what some studies have indicated, where individuals with longer disease duration had higher A1C values compared to those with a more recent diagnosis [28]. Although, in some studies, younger individuals with shorter disease duration, lower BMI and higher education levels had better A1C values [28], in other studies, older age did not appear to be necessarily associated with higher A1C values among people with type 1 diabetes [31].

Eating self-efficacy can be defined as the belief that the individual will be able to perform a certain task required to produce the desired result and the feeling of control over behaviors and the environment [19,32]. No relationship was observed between eating self-efficacy and glycemic control, although some studies suggest that greater eating self-efficacy is associated with better glycemic control [29]. In our study, eating self-efficacy significantly explained perceived health status in the multivariate analysis. The feeling or belief that an individual is not capable of adopting measures to achieve a certain glycemic goal can lead them to have a more negative perception of their health status compared to what their health status actually is in practice.

Patients not on CSII and who did not perform CHC presented greater dysfunction in terms of barriers to activity than the group with CSII and CHC using apps. However, these groups also presented significant age differences, which may explain the lack of significant effect of barriers to activity when age is considered simultaneously. Despite the interest in performing multivariate analysis, namely to clarify possible causal relationships, we highlight that the bivariate relationships may also be useful for guiding future research. Considering the scarce research on how eating self-efficacy and disease-related psychosocial profile may interact in terms of glycemic control and perceived health status in people with type 1 diabetes with different clinical features, future research should try to address the study of such relationships, namely with larger samples, which will allow more accurate comparisons.

This study has some limitations that should be considered when interpreting the results, as well as for future studies, such as the fact that the medication used by the

patients was not collected, which may have obscured some altered analytical values. The fact that a convenience sample was used should be seen as a limitation, but this aspect is partially offset by the fact that the sample was collected in a public hospital, where people with diverse sociodemographic characteristics are treated and where access is provided to a wider group of the population [33]. The lack of time in range data for all patients prevented a more complete analysis with regard to glycemic control and its relationships with other variables. The main strengths of our work are the sample size, an identical proportion of men and women in our sample, as well as a combined assessment of several clinical, sociodemographic, behavioral and quality of life features.

5. Conclusions

This work contributes to a broader understanding of the factors that may impact the quality of life of people with DM1, as well as to understanding the determinants of glycemic control in this population. Our results indicate that glycemic control, as defined by A1C values, is the result of the interaction between various clinical features that patients with DM1 should consider, aiming for optimal metabolic control and prevention or delay of the onset of complications. Furthermore, eating self-efficacy was the only variable that significantly explained perceived health status, which may represent a focus for intervention among people with DM1.

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References

1. Sacks, D.B.; Arnold, M.; Bakris, G.L.; Bruns, D.E.; Horvath, A.R.; Lernmark, Å.; Metzger, B.E.; Nathan, D.M.; Kirkman, M.S. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. *Diabetes Care* **2023**, *46*, e151–e199. [CrossRef] [PubMed]
2. American Diabetes Association Professional Practice Committee. Diagnosis and Classification of Diabetes: Standards of Care in Diabetes—2024. *Diabetes Care* **2024**, *47* (Suppl. S1), S20–S42. [CrossRef] [PubMed]
3. Dahlquist, G. Can we slow the rising incidence of childhood-onset autoimmune diabetes? The overload hypothesis. *Diabetologia* **2006**, *49*, 20–24. [CrossRef] [PubMed]
4. Mohr, D.C.; Zhang, L.; Prentice, J.C.; E Nelson, R.; Li, D.; Pleasants, E.; Conlin, P.R. Association of hemoglobin A1c time in range with risk for diabetes complications. *BMJ Open Diabetes Res. Care* **2022**, *10*, e002738. [CrossRef]
5. Little, R.R.; Rohlfing, C.L.; Sacks, D.B. Status of hemoglobin A1c measurement and goals for improvement: From chaos to order for improving diabetes care. *Clin. Chem.* **2011**, *57*, 205–214. [CrossRef]
6. Glycemic Goals and Hypoglycemia: Standards of Care in Diabetes-2024. *Diabetes Care* **2024**, *47* (Suppl. S1), S111–S125. [CrossRef]

7. Benkhadra, K.; Alahdab, F.; Tamhane, S.U.; McCoy, R.G.; Prokop, L.J.; Murad, M.H. Continuous subcutaneous insulin infusion versus multiple daily injections in individuals with type 1 diabetes: A systematic review and meta-analysis. *Endocrine* **2017**, *55*, 77–84. [CrossRef]
8. Pickup, J.; Mattock, M.; Kerry, S. Glycaemic control with continuous subcutaneous insulin infusion compared with intensive insulin injections in patients with type 1 diabetes: Meta-analysis of randomised controlled trials. *BMJ* **2002**, *324*, 705. [CrossRef]
9. Fatourechi, M.M.; Kudva, Y.C.; Murad, M.H.; Elamin, M.B.; Tabini, C.C.; Montori, V.M. Clinical review: Hypoglycemia with intensive insulin therapy: A systematic review and meta-analyses of randomized trials of continuous subcutaneous insulin infusion versus multiple daily injections. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 729–740. [CrossRef]
10. Roze, S.; Smith-Palmer, J.; Valentine, W.; de Portu, S.; Nørgaard, K.; Pickup, J.C. Cost-effectiveness of continuous subcutaneous insulin infusion versus multiple daily injections of insulin in Type 1 diabetes: A systematic review. *Diabet. Med.* **2015**, *32*, 1415–1424. [CrossRef]
11. Cummins, E.; Royle, P.; Snaith, A.; Greene, A.; Robertson, L.; McIntyre, L.; Waugh, N. Clinical effectiveness and cost-effectiveness of continuous subcutaneous insulin infusion for diabetes: Systematic review and economic evaluation. *Health Technol. Assess.* **2010**, *14*, iii–iv, xi–xvi. 1–181. [CrossRef] [PubMed]
12. Witkow, S.; Liberty, I.F.; Goloub, I.; Kaminsky, M.; Otto, O.; Rabia, Y.; Boehm, I.H.; Golan, R. Simplifying carb counting: A randomized controlled study—Feasibility and efficacy of an individualized, simple, patient-centered carb counting tool. *Endocrinol. Diabetes Metab.* **2023**, *6*, e411. [CrossRef] [PubMed]
13. Bell, K.J.; Barclay, A.W.; Petocz, P.; Colagiuri, S.; Brand-Miller, J.C. Efficacy of carbohydrate counting in type 1 diabetes: A systematic review and meta-analysis. *Lancet Diabetes Endocrinol.* **2014**, *2*, 133–140. [CrossRef] [PubMed]
14. Centenaro, A.; do Nascimento, C.; Beretta, M.V.; da Costa Rodrigues, T. Carbohydrate counting as a strategy to optimize glycemic control in type 1 diabetes mellitus. *Arch. Endocrinol. Metab.* **2023**, *67*, 385–394. [CrossRef]
15. AlBabtain, S.A.; AlAfif, N.O.; AlDisi, D.; AlZahrani, S.H. Manual and application-based carbohydrate counting and glycemic control in type 1 diabetes subjects: A narrative review. *Healthcare* **2023**, *11*, 934. [CrossRef]
16. Porojan, M.; Poantă, L.; Dumitraşcu, D.L. Assessing health related quality of life in diabetic patients. *Rom. J. Intern. Med.* **2012**, *50*, 27–31.
17. Pickup, J.C.; Kidd, J.; Burmiston, S.; Yemane, N. Determinants of glycaemic control in type 1 diabetes during intensified therapy with multiple daily insulin injections or continuous subcutaneous insulin infusion: Importance of blood glucose variability. *Diabetes Metab. Res. Rev.* **2006**, *22*, 232–237. [CrossRef]
18. Pouwer, F.; Hermanns, N. Insulin therapy and quality of life. A review. *Diabetes Metab. Res. Rev.* **2009**, *25* (Suppl. S1), S4–S10.
19. Glasofer, D.R.; Haaga, D.A.; Hannallah, L.; Field, S.E.; Kozlosky, M.; Reynolds, J.; Yanovski, J.A.; Tanofsky-Kraff, M. Self-efficacy beliefs and eating behavior in adolescent girls at-risk for excess weight gain and binge eating disorder. *Int. J. Eat. Disord.* **2013**, *46*, 663–668. [CrossRef]
20. Senécal, C.; Nouwen, A.; White, D. Motivation and dietary self-care in adults with diabetes: Are self-efficacy and autonomous self-regulation complementary or competing constructs? *Health Psychol.* **2000**, *19*, 452–457. [CrossRef]
21. Mishali, M.; Omer, H.; Heymann, A.D. The importance of measuring self-efficacy in patients with diabetes. *Fam. Pr. Pract.* **2011**, *28*, 82–87. [CrossRef] [PubMed]
22. Leite, W.; Beretvas, S. Validation of Scores on the Marlowe-Crowne Social Desirability Scale and the Balanced Inventory of Desirable Responding. *Educ. Psychol. Meas.* **2005**, *65*, 140–154. [CrossRef]
23. Cruz, R.S.; Leitão, C.E.; Lopes Ferreira, P. Determinantes do estado de saúde dos diabéticos. *Rev. Port. Endocrinol. Diabetes E Metab.* **2016**, *11*, 188–196. [CrossRef]
24. Shmueli, A. Subjective health status and health values in the general population. *Med. Decis. Mak.* **1999**, *19*, 122–127. [CrossRef]
25. Poínhos, R.; Canelas, H.; Oliveira, B.M.P.M.; Correia, F. Desenvolvimento e validação de uma escala de auto-eficácia alimentar. *Aliment. Humana* **2013**, *9*, 65–72.
26. Poínhos, R.; Caetano, E.; Guimarães, J.M.; Pinhão, S.; Oliveira, B.M.P.M.; Correia, F. Psychometric properties of the General Eating Self-Efficacy Scale among females treating overweight. *Acta Port. Nutr.* **2022**, *29*, 60–63.
27. Pechorro, P.; Vieira, R.; Poiares, C.; Maroco, J. Contributos para a validação duma versão curta da Escala de Desejabilidade Social de Marlowe-Crowne com adolescentes portugueses. *Arq. Med.* **2012**, *26*, 103–108.
28. Cooper, J.G.; Bakke, Å.; Dalen, I.; Carlsen, S.; Skeie, S.; Løvaas, K.F.; Sandberg, S.; Thue, G. Factors associated with glycaemic control in adults with Type 1 diabetes: A registry-based analysis including 7601 individuals from 34 centres in Norway. *Diabet. Med.* **2020**, *37*, 828–837. [CrossRef]
29. Cheng, L.J.; Wang, W.; Lim, S.T.; Wu, V.X. Factors associated with glycaemic control in patients with diabetes mellitus: A systematic literature review. *J. Clin. Nurs.* **2019**, *28*, 1433–1450. [CrossRef]
30. Aghili, R.; Polonsky, W.H.; Valojerdi, A.E.; Malek, M.; Keshtkar, A.A.; Esteghamati, A.; Heyman, M.; Khamseh, M.E. Type 2 Diabetes: Model of Factors Associated with Glycemic Control. *Can. J. Diabetes* **2016**, *40*, 424–430. [CrossRef]

31. Miller, K.M.; Beck, R.W.; Foster, N.C.; Maahs, D.M. HbA1c Levels in Type 1 Diabetes from Early Childhood to Older Adults: A Deeper Dive into the Influence of Technology and Socioeconomic Status on HbA1c in the T1D Exchange Clinic Registry Findings. *Diabetes Technol. Ther.* **2020**, *22*, 645–650. [CrossRef] [PubMed]
32. Bandura, A. Human agency in social cognitive theory. *Am. Psychol.* **1989**, *44*, 1175–1184. [CrossRef] [PubMed]
33. Kruse, F.M.; Stadhouders, N.W.; Adang, E.M.; Groenewoud, S.; Jeurissen, P.P.T. Do private hospitals outperform public hospitals regarding efficiency, accessibility, and quality of care in the European Union? A literature review. *Int. J. Health Plan. Manag.* **2018**, *33*, e434–e453. [CrossRef] [PubMed]

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Article

UK Patient Access to Low-Protein Prescription Foods in Phenylketonuria (PKU): An Uneasy Path

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Abstract

Background: Special low-protein foods are essential in the dietary treatment of phenylketonuria (PKU). In the UK, these are available on prescription through the General Practitioners (GPs) and distributed via nutritional home delivery companies or pharmacies. Methods: A 58-item online non-validated semi-structured questionnaire was emailed to British Inherited Metabolic Disease Group (BIMDG) dietitians and dietetic support workers (DSW)/administrators working in PKU to ascertain the main system issues and errors with the supply of low-protein prescription foods (LPPF). Results: 73% (n = 53/73) of dietitians and 72% (n = 18/25) of DSW/administrators responded. A total of 80 questionnaires (representing 44 paediatric and 36 adult PKU centres) were completed. A total of 50% (n = 40/80) of respondents reported patient/caregiver problems accessing LPPF at least weekly. The most common problems were unavailable products (82%), missing LPPF in deliveries (79%), and delayed deliveries (66%). For 64% of respondents, >25% of their patients had recurring problems accessing LPPF, and 69% of respondents spent ≥1 h/week and 11%

>5 h/week correcting LPPF patient supply issues. The most common foods patients experienced supply issues with were bread (96%), pasta/rice (41%) and milk replacements (35%). This was associated with GP prescription errors (65%), LPPF prescriptions sent to incorrect dispensers/suppliers (60%), and manufacturer supply issues (54%). Problems with patients/caregivers included not ordering LPPF in a timely way (81%), not responding to messages from home delivery companies (73%) and poor understanding of the ordering process (70%). The majority (93%) of respondents reported that prescription issues impacted their patients' blood Phe control. Suggestions for improving access to LPPF included centralisation of the system to one supplier (76%) and apps for ordering LPPF (69%). Conclusions: The supply of LPPF for PKU in the UK is problematic; it may adversely affect the ability of patients to adhere to dietary management, and a review investigating patient access to LPPF is urgently required.

Keywords: phenylketonuria; PKU; low-protein foods; dietitian

1. Introduction

Phenylketonuria (PKU) is a rare inherited disorder of metabolism caused by a deficiency or absence of the hepatic enzyme phenylalanine (Phe) hydroxylase, resulting in the partial or complete inability to catalyse the hydroxylation of the essential amino acid Phe to tyrosine [1]. This leads to accumulation of Phe in the blood and brain, tyrosine deficiency, and neurological impairment. Whilst there are some pharmacological treatments such as pegvaliase (a subcutaneously administered Phe ammonium lyase enzyme), and sapropterin (an orally administered tetrahydrobiopterin dihydrochloride that acts as a chaperone), their use is limited to subgroups of patients. Pegvaliase is aimed at patients aged ≥16 years with a blood Phe ≥600 µmol/L, and sapropterin will only benefit approximately 30–40% of patients with mild or moderate PKU [2–4]. Even with sapropterin, patients will rarely attain an unrestricted diet [5]. Therefore, the main treatment is a lifelong Phe-restricted diet supplemented with Phe-free amino acids or low Phe casein glycomacropeptide, usually with added vitamins and minerals [6]. Foods high in protein such as meat, fish, eggs, cheese, nuts, seeds, regular bread, cereals, pasta and flours must be avoided in patients with classical PKU.

An important part of a Phe-restricted diet is the provision of naturally low-protein foods (protein < 0.5 g/100 g or Phe < 25 mg/100 g), mostly vegetables, fruit, fats and sugars. These are accompanied by specially modified low-protein foods, e.g., low-protein bread, pasta, flours, milk replacements, cereals, pizza bases, burger and sausage mixes, biscuits, crackers, and cakes. These foods help to avoid catabolism (and subsequent elevation of blood Phe) through the provision of energy, they aid satiety, and they improve the variety of foods available in an otherwise monotonous and limited diet [7]. There are approximately 150 individual low-protein prescription foods (LPPF) available in the UK [8]. In Italy, Germany and the United Kingdom (UK), the percentage of mean daily energy intake from LPPF is reported as 47, 39 and 33% respectively [9–11]. The range and amount consumed varies depending on individual preferences, product availability, and PKU severity, with patients with milder phenotypes being less reliant on LPPF due to a higher natural protein tolerance.

In the UK, LPPF are classified as Foods for Special Medical Purpose (FSMP) and are approved by the government Advisory Committee on Borderline Substances (ACBS) based on clinical need, their efficacy, and price [12]. They are not available in retail shops or pharmacies and are usually obtained via National Health Service (NHS) prescriptions

through the General Practitioner (GP). In England, there are 2359 patients with PKU (923 paediatric and 1436 adult patients) who need access to LPPFs [12]. Prescriptions for LPPF are issued monthly and dispensed by specialist home delivery companies or local pharmacists. In Scotland, Wales and Northern Ireland (NI), all prescriptions for all patient age groups are issued free of charge through the NHS and Health and Social Care (NI). In England, prescription products are without cost to children under the age of 16 years or those in fulltime education aged ≤18 years, but most adults (aged ≥19 y to 60 y) are expected to pay a prescription fee for each LPPF item requested unless they are exempt or they have purchased a prescription prepayment certificate at a subsidised rate [13]. In order to control expenditure, guidance on the maximum number of units (1 unit = 1 pack/box as sold) of any particular product to prescribe each month are available through the National Society for PKU (NSPKU) for different age groups [14]. See Figure 1 for flowchart of LPPF ordering and acquisition process.

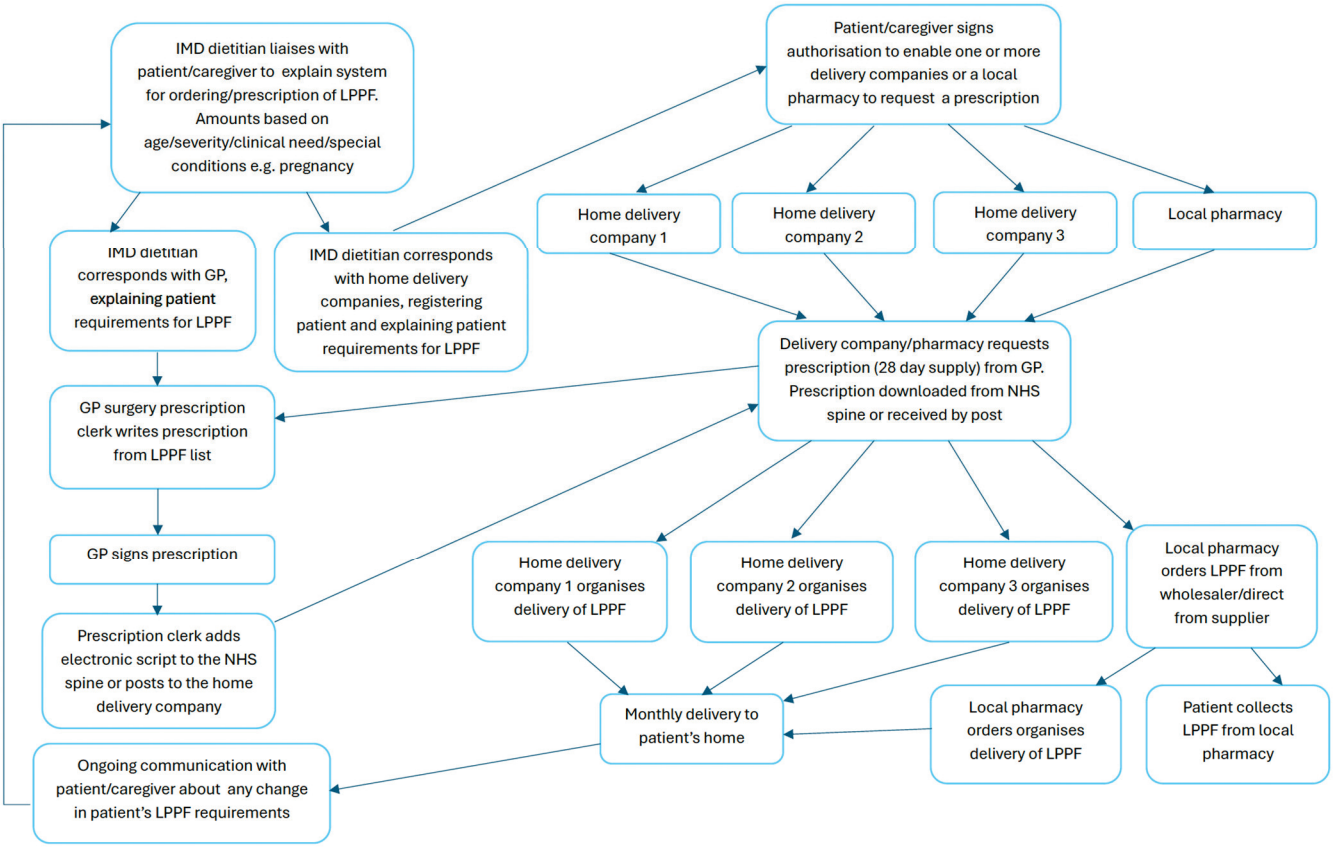


Figure 1. Low-protein prescription food ordering and acquisition process. IMD—Inherited Metabolic Disease; LPPF—Low-protein prescription food; GP—General Practitioner; NHS—National Health Service.

In PKU, evidence from studies in Scotland and England [7,12] suggest that the majority of LPPF prescribed by the NHS are staple foods such as bread, pasta, flour mixes and cereals, with sweet foods such as cakes, biscuits and desserts used in smaller amounts. Numerous studies have acknowledged significant issues with the current system for distribution of LPPF in the UK [7,12,15]. These include but are not limited to difficulty in patients obtaining GP prescriptions, incorrect prescriptions, incorrect product quantity received, delayed delivery of products, out-of-stock products, GPs declining requests or restricting prescription amounts, out-of-date, damaged or poor-quality products. Of the 252 patients with PKU or their caregivers completing an online questionnaire, 59% reported problems with accessing basic LPPF, such as bread and pasta, and over a third had recurring

problems [15]. Patients/caregivers reported stress or anxiety associated with the process of obtaining LPPF [7,15], and there was an impression that PKU was not taken seriously by GP practices (clerks, managers, pharmacists and GPs), prescribers and suppliers. In addition, over 50% of patients/caregivers felt that this affected their health or the health of their child [15].

It is well-established that LPPF are more expensive than their equivalent regular or 'free from' products [12], leading to a GP's reluctance to prescribe them. In addition, prescription charges in England may discourage patient use of LPPF, potentially causing termination of dietary treatment [12]. In children who receive free LPPF, the amounts prescribed per patient are less than 50% of the maximum amounts recommended by the NSPKU [7,12], and the cost to the NHS (<GBP 1000/patient/year) [12] is well below previously estimated costs (GBP 2000–GBP 2500/patient/year) [15]. The underutilisation of LPPF may well be associated with the difficulties inherent in the prescribing and dispensing system.

Whilst issues with the current system for prescribing and dispensing LPPF have been reported from the patient perspective [7,12,15], any challenges from a dietetic perspective have not been previously described. Problems with deliveries of LPPF are generally referred to the patient's dietitian and/or dietetic support workers (DSW). This survey aimed to investigate the extent of any problems experienced by UK dietitians, DSWs and dietetic administrators in organising LPPF for their patients and correcting errors in their prescription, dispensing and delivery.

2. Materials and Methods

2.1. Study Group

In February/March 2024, an online questionnaire via Microsoft Forms (Microsoft 365) was emailed to all British Inherited Metabolic Disease Group (BIMDG) dietitians (n = 100) and cascaded to DSWs and administrative staff, inviting individuals involved in managing LPPF ordering for PKU to respond.

2.2. Questionnaire

The non-validated questionnaire (see Supplementary Materials S1) was designed and piloted with a focus group of 4 dietitians acting in an advisory capacity for the NSPKU and 20 BIMDG dietitians and was further modified before distribution. The questionnaire comprised nine demographic multiple choice/short-answer questions identifying workplace, UK location, role (clinical dietitian, DSW, administrator), days worked per week, specialising in adult or paediatric care (or both), dietetic prescribers, and any patient prescription charges. The questionnaire was divided into paediatric and adult sections and respondents were directed to each section based on which patient group they work with. Each section comprised 27 multiple choice questions and 21 extended response questions inviting the respondent to comment on their previous answers. A summary of the questionnaire content is given in Table 1.

2.3. Data Analysis and Statistics

Responses were analysed, and the quantitative data were presented as a percentage of responses and analysed statistically using nonparametric Fisher's exact test (statistical significance $p < 0.05$, 95% confidence) to compare differences between paediatric and adult dietetic responses. Qualitative data from open comments was grouped into themes and tabulated. Statistical analysis was carried out using GraphPad Prism version 10.2.2 for Windows, GraphPad Software, Boston, MA, USA.

Table 1. Topics addressed by the questionnaire regarding the supply of LPPF for patients with PKU.

Questionnaire Topics:
• Number of patients with PKU in respondent's dietetic care;
• Who prescribes and supplies LPPF for their patients;
• Frequency and nature of communication from patients regarding issues with accessing LPPF;
• Proportion of patients experiencing access issues to LPPF;
• Dietetic time spent in organising and correcting issues with LPPF supply;
• Type of LPPFs most difficult to access and why;
• Description of issues with LPPF manufacturers, GP prescriptions, pharmacies and home delivery companies;
• Frequency of communication with home delivery companies regarding patient supply of LPPFs;
• Frequency of GP LPPF refusal or any restrictions on supply;
• Issues with the NHS spine (the digital platform that connects healthcare information technology systems to enable secure sharing of prescriptions with pharmacy suppliers);
• Frequency of the need to issue emergency supplies of LPPF from the specialist PKU hospitals;
• Most common problems associated with patients/caregiver behaviour that affect the supply of LPPF;
• Impact of LPPF issues on patient blood Phe control;
• Suggestions for improving the current system for supply of LPPF.

LPPF—low-protein prescription food.

2.4. Ethical Approval

Ethical approval was not required as there was no patient involvement, no change in patient treatment, and no collection of respondent personal or identifiable data. Ethical review and approval were not required according to the NHS Health Research Authority and Medical Research Council "Is my study research" online decision tool [16] (Supplementary Materials S2).

3. Results

3.1. Study Group

In the UK, 72 inherited metabolic disease (IMD) dietitians and 25 DSWs/administrators were identified by the BIMDG as working with patients with PKU. Participants responding to this survey consisted of 53/72 (74%) clinical dietitians and 18/25 (72%) DSWs/administrators from 24 hospitals across the UK. All areas of the UK were represented. See Supplementary Materials (Table S1) for details of respondents. Some respondents cared for both adult and paediatric patients with PKU (n = 3 dietitians and n = 6 DSWs), so they completed both the paediatric and the adult questionnaire. A total of 80 questionnaires were completed (44 paediatric and 36 adult). All hospitals had a minimum of 10 patients with PKU, and 82% (n = 58) cared for >50 patients with PKU. Over half (52%, 37/71) of the respondents worked full-time (n = 28 dietitians and n = 9 DSWs).

3.2. Prescribing LPPF

Although six dietitians (11%) were dietetic prescribers (i.e., NHS approved supplementary prescribers), all respondents said that their patients' LPPFs were prescribed by the patient's GP (except patients in the area of Rotherham, Yorkshire where an independent pharmacist issued prescriptions). Sixty-three percent (45/71) of respondents said that all of their patients were exempt from prescription charges. This included all NI, Scottish and Welsh centres (n = 14) and English paediatric centres (n = 31). However, patients aged > 16 y in Scotland, Wales and NI paid a prescription charge if their LPPF was dispensed and supplied via an English home delivery/dispensing company.

For patients in England, dietitians were concerned about the transition from paediatric to adult care as prescription charges were considered a deterrent to lifetime dietary treatment.

3.3. How LPPF Are Dispensed

The majority of respondents (84%, 67/80) reported that LPPF were supplied to patients via a combination of home delivery companies and their local pharmacy. This was determined by the patients or their caregivers.

3.4. Dietetic Time Managing LPPF Supply

Fifty percent (40/80) of respondents received communication from patients/caregivers at least weekly about problems accessing LPPF, and at least 20% (n = 8) said they were contacted daily or several times a week.

Twenty-nine percent (23/80) of respondents spent 3 or more hours/week organising supplies of LPPF for their patients (Table 2). Paediatric dietitians/DSWs spent more time than adult dietitians/DSWs co-ordinating LPPF supplies although this was not statistically significant (Table 2). Similarly, 24% (19/80) spent 3 or more hours/week correcting problems with LPPF for their patients with more time expended on this task by paediatric than adult dietitians/DSWs (Table 2). This could cost the NHS up to GBP 6500 annually per respondent in lost dietetic resources.

Table 2. Dietetic time per week spent on organising LPPF and correcting LPPF problems n (%).

Hours/Week	Organising LPPF *n*(%)		*p* Value *	Correcting LPPF Problems *n* (%)		*p* Value *
	Paediatric	Adult		Paediatric	Adult	
<1 h	13 (30)	13 (36)		12 (27)	19 (53)	
1–2 h	15 (34)	16 (44)	0.08	21 (48)	9 (25)	0.09
3–5 h	12 (27)	2 (6)		7 (16)	4 (11)	
>5 h	4 (9)	5 (14)		4 (9)	4 (11)	

LPPF—Low-protein prescription food * Fisher's exact test (paediatric vs. adult).

3.5. Main Issues with LPPF Supply

The most common issues for which paediatric and adult patients sought help from their dietitian/DSWs were unavailable LPPF (81%, n = 65/80), missing delivery items (79%, 63/80) and delayed LPPF deliveries (commonly without communication) (66%, 53/80). Paediatric patients were more likely than adult patients to have missing, damaged or inedible (e.g., mouldy bread or broken crackers), or out-of-date LPPF, whilst adults were more likely than children to be without LPPF supplies, experience out-of-stock items, and require information about the process for re-ordering LPPF. However, these differences were not statistically significant.

Thirty-one percent (25/80) of respondents reported that up to 25% of their patients had recurring LPPF supply problems and 33% (26/80) said up to 50% of patients had repeated problems. There was no significant difference between paediatric and adult patients. Respondents reported that the most common items in which patients experience supply issues were staple foods, including low-protein bread (96%, 77/80), pizza bases (34%, 27/80), pasta and rice (41%, 33/80), milk replacements (35%, 28/80) and flour or bread mix (33%, 26/80) (Figure 2). This was similar for paediatric and adult patients, where no differences were statistically significant.

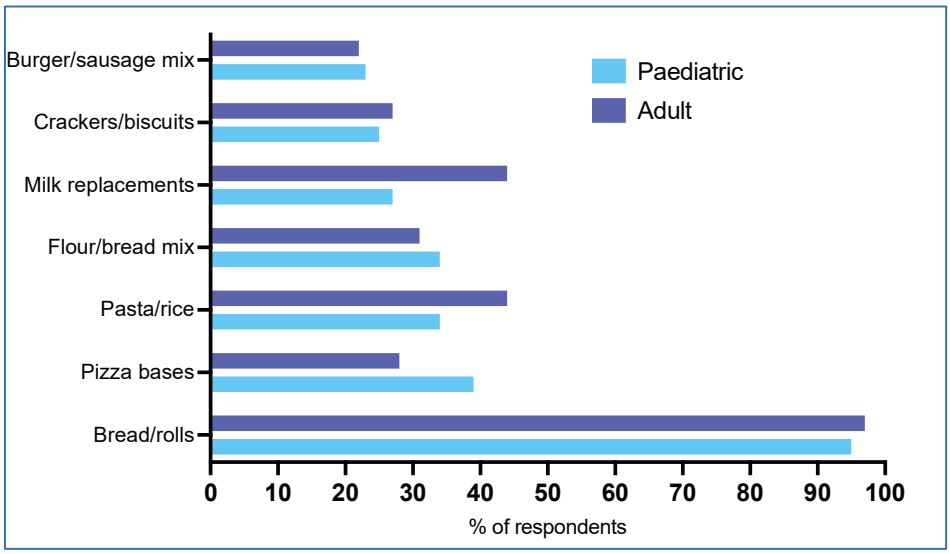


Figure 2. The most common LPPF supply issues patients experience.

The top five most common reasons reported for LPPF supply issues were: GPs prescribing incorrect items (65%, 52/80), prescriptions received by the wrong dispenser (home delivery company or pharmacy) (60%, 48/80), pharmacists unable to locate a LPPF on their online database that list all the ACBS LPPF (58%, 46/80), manufacturer supply issues (54%, 43/80), or the GP's were unable to locate LPPFs on their online drug database (53%, 42/80). There was no significant difference between paediatric and adult patients in terms of the reasons for supply issues.

As a consequence of supply problems, 38% (30/80) of respondents provided patients with some additional "bridging stock" of LPPF from their hospital's own supplies at least monthly and 23% (7/30) at least weekly. This was significantly more problematic for paediatric patients. Fifty-two percent (23/44) issued LPPF to children monthly compared with 19% (7/36) to adults with PKU ($p = 0.003$; Fisher's exact test).

3.6. Frequency of Issues with LPPF

Sixty percent (48/80) of respondents were informed about manufacturer delays in the supply of LPPF at least monthly; of these, 13% (6/48) were informed of problems at least weekly (Table 3). There were no differences between paediatric and adult respondents.

GP prescription issues (see Section 3.7 for main issues) occurred at least weekly for 38% (30/80) of respondents, and this was more common in adult patients (47%, 17/36) compared with paediatric patients (30%, 13/44) (Table 3). Nearly half of the respondents (48%, 38/80) had to resolve issues with GP refusal to send prescriptions directly to delivery companies at least monthly, with one quarter (26%, 10/38) occurring at least weekly. This was similar for paediatric and adult patients.

Respondents experienced issues with the pharmacy dispensing of LPPF at least monthly (98%, 78/80) and at least weekly for 25% (20/80) of the respondents (Table 3). There was no significant difference between paediatric and adult patients.

Eighty-one percent (65/80) of respondents reported delivery company issues with dispensing of LPPF at least monthly, and 28% (22/80) at least weekly (Table 3). This was similar for paediatric and adult patients, with no statistically significant differences for any of the contact issues.

Table 3. Frequency of patient dietetic contact regarding issues with LPPF supply *n*(%).

Contact Issue	At Least Weekly	Every 2 Weeks	Once a Month	Rarely	Unaware It Is an Issue
Manufacturer delays	6 (8)	11 (14)	31 (39)	31 (39)	1 (1)
GP prescription issues	30 (38)	25 (31)	21 (26)	4 (5)	0 (0)
GP prescription refusal	10 (13)	15 (19)	13(16)	24(43)	8 (10)
Pharmacy issues	20 (25)	25 (31)	33 (41)	2 (3)	0 (0)
Delivery company issues	22 (28)	24 (30)	19 (24)	14 (18)	0 (0)

3.7. Main Issues with GP Practice

The top five issues with GP prescription of LPPFs reported to dietetics were prescription delays (79%, 63/80), prescriptions being sent to the incorrect supplier (65%, 52/80), prescription items missing (50%, 40/80), the GP practice unable to locate a specific LPPF on their computer drug database (41%, 33/80), and the number of LPPF items was less than requested (40%, 32/80) (Figure 3). Issues for paediatric and adult patients were generally similar, although adult patients were twice as likely as paediatric patients to experience GP refusal of some LPPF items (50%, 18/36 vs. 25%, 11/44; $p = 0.03$ Fisher's exact test) (Figure 3).

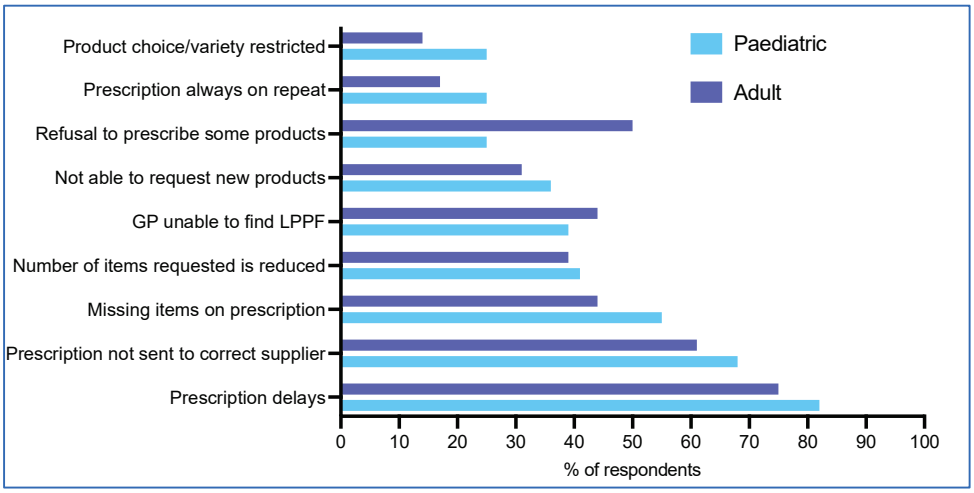


Figure 3. Main problems reported by caregivers/patients with GPs when obtaining LPPF.

Nearly 50% (38/80) of respondents said GP practices put some restrictions on the prescription of LPPFs (50%, 18/36 adults, 45%, 20/44 paediatric patients). In addition, half of the respondents (50%, 40/80) reported that GP prescriptions were commonly electronically downloaded by the wrong supplier (home delivery company or pharmacy) using the NHS digital spine platform at least monthly and 25% (10/40) at least weekly.

3.8. Main Issues with Pharmacies

The top five problems reported when pharmacies dispensed LPPFs were a lack of manufacturer supply (69%, 55/80), inability to source some of the LPPFs (50%, 40/80), lack of pharmacist knowledge about LPPF (45% (36/80), missing delivery items (44%, 35/80), and issues accessing the GP prescription (43%, 34/80) (Figure 4). One concern that was more prevalent for adults compared with paediatric patients, was lack of manufacturer supply (83%, 30/36 vs. 57%, 25/44; $p = 0.01$ Fisher's exact test) (Figure 4). Conversely, poor communication with caregiver/patient when products were unavailable was more problematic for paediatric than adult patients (39%, 17/44 vs. 17%, 6/36; $p = 0.05$) (Figure 4).

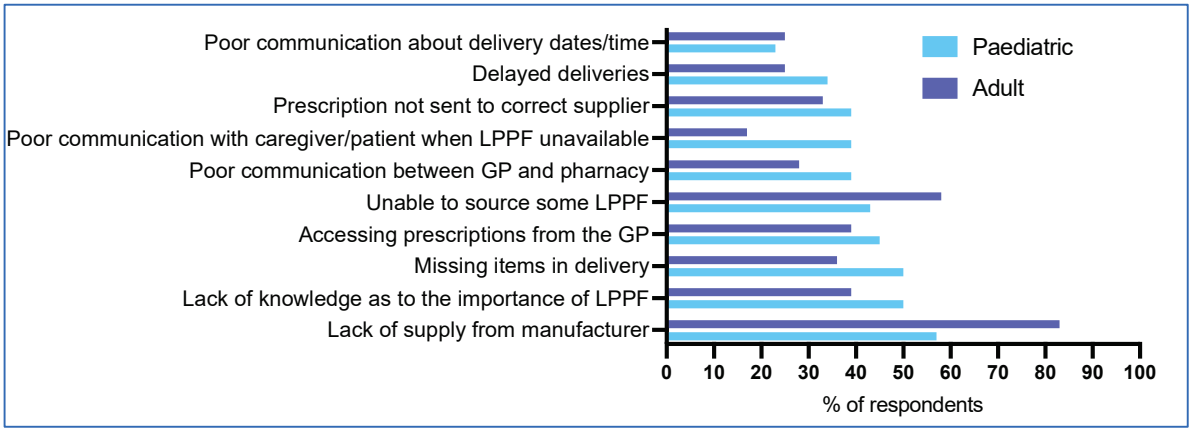


Figure 4. Main problems reported by caregivers/patients with pharmacies when obtaining LPPF.

3.9. Main Issues with Home Delivery Companies

All respondents (100%, $n = 80$) reported that most patients used at least one or more home delivery companies for deliveries of LPPF.

Twenty-nine percent (23/80) of respondents said that home delivery companies contacted them at least weekly for help in obtaining prescriptions from GPs and 79% (63/80) at least monthly.

The top five problems reported with home delivery companies for patients accessing LPPF were home delivery company difficulty obtaining the prescription from the GP (65%, 52/80), missing items in the delivery (60%, 48/80), poor communication between the GP and the home delivery company (59%, 47/80), inadequate communication with the patient or caregiver about delivery dates/times (44%, 35/80), and delayed deliveries (43%, 34/80) (Figure 5). Issues were similar for paediatric and adult patients, although adults were more likely than children to experience problems with home delivery companies accessing the prescription from the GP (78%, 28/36 vs. 55%, 24/44; $p = 0.04$), and locating the prescription on the NHS electronic spine (22%, 8/36 vs. 5%, 2/44; $p = 0.04$ Fisher's exact test) (Figure 5).

3.10. Patient/Caregiver Issues

The most common patient and caregiver issues influencing the supply of LPPF were late ordering of LPPF (81%, 65/80), not responding to messages about the delivery (73%, 58/80), patient and caregiver confusion about the ordering process (70%, 56/80), and 28% (10/36) of respondents said that adults with PKU could not afford to pay for LPPF prescriptions. Respondents reported a trend for adults with PKU to have more problems with understanding the ordering process than caregivers of children with PKU (81%, 29/36 vs. 61%, 27/44; $p = 0.08$ Fisher's exact test).

Thirty-nine percent (31/80) of respondents reported that prescription issues with LPPF frequently impacted metabolic control. There was no significant difference between adult and paediatric respondents. Figure 6 summarises the interdependent issues in the acquisition of LPPF for PKU that can adversely affect metabolic control.

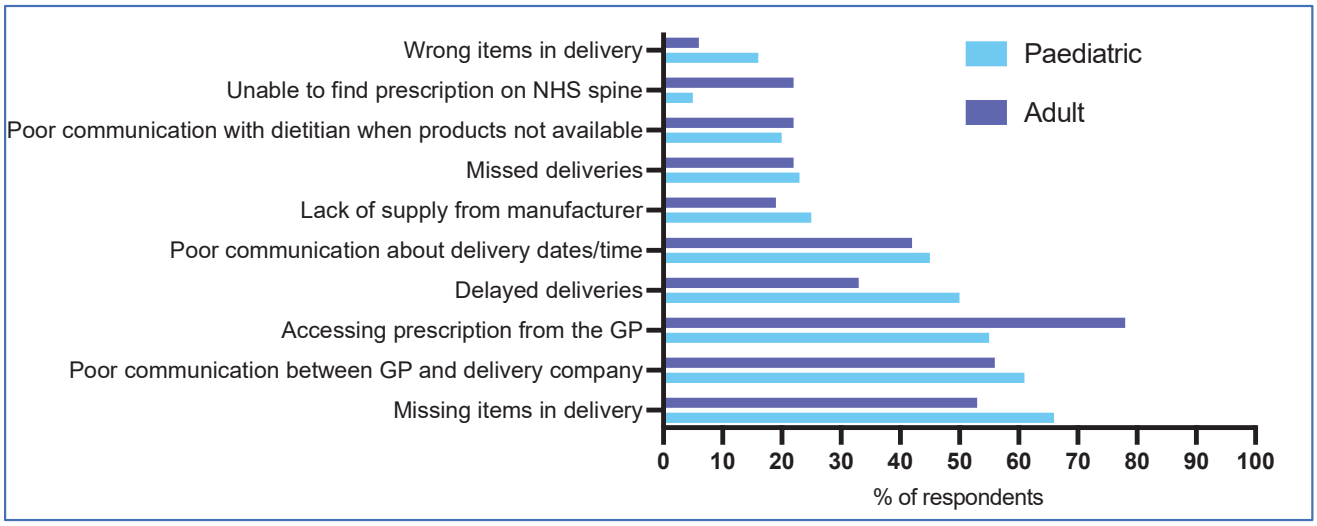


Figure 5. Main problems reported by caregivers/patients with home delivery companies when obtaining LPPF.

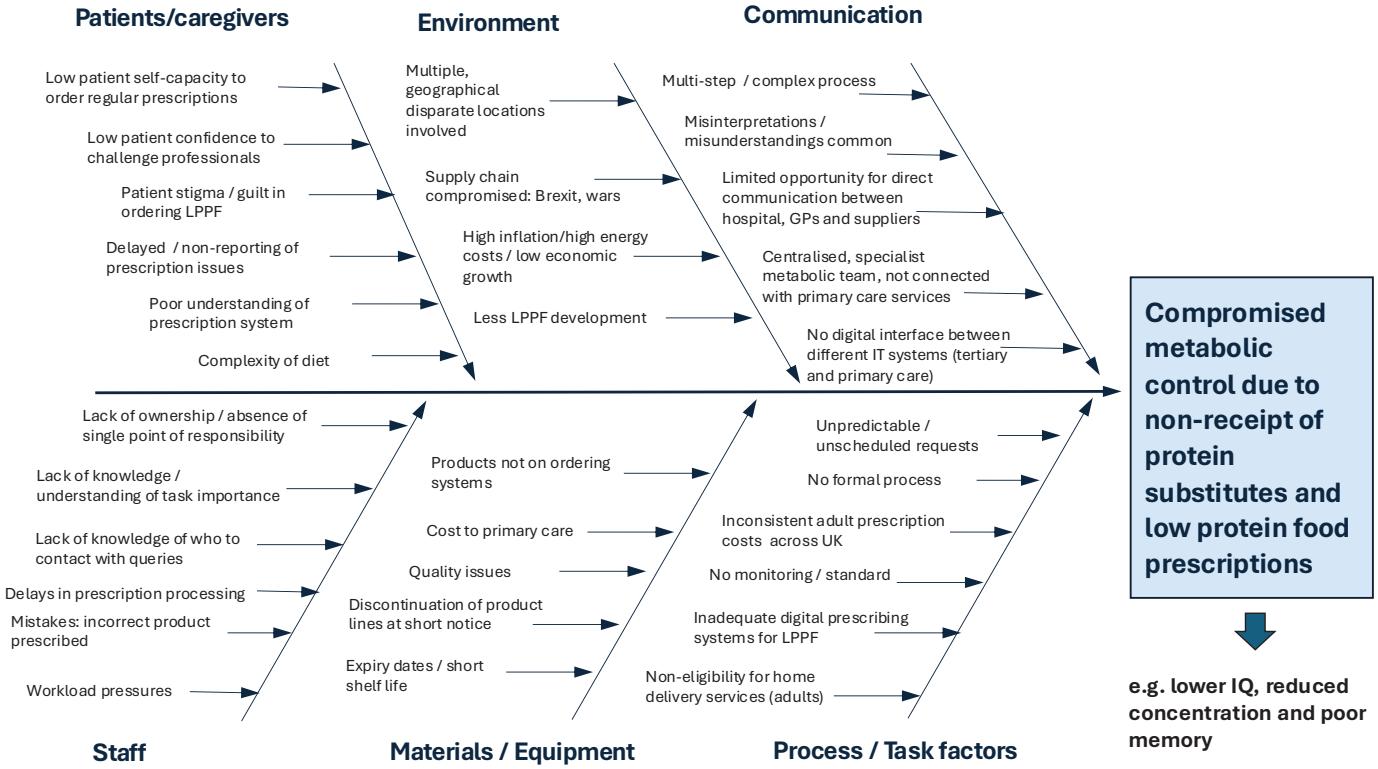


Figure 6. Interdependent issues associated with accessing LPPF for patients with PKU that may lead to ongoing supply issues and ultimately compromised metabolic control (LPPF—Low-protein prescription foods).

3.11. Summary of Issues with the Current System for Obtaining LPPF in the UK

Table 4 summarises the open comments from dietitians and DSWs divided into common themes with supporting quotes.

Table 4. Issues with the current system for obtaining low-protein prescription foods for PKU.

Issues	Supporting Respondent Quotes
Prescription charges • In England, prescription charges are exempt for children <16 y or in full-time education ≤18 y. Adult patients in England have to pay a prescription charge for each low-protein food item or obtain a subsidised pre-payment certificate; • In Scotland, Wales and NI, prescriptions are free for all patients; • Some delivery companies have dispensing pharmacies in England, so patients in Scotland, Wales and NI have to pay a prescription charge for deliveries; • NHS spine (electronic prescribing system) is not available in Scotland, Wales and NI so prescriptions are sent by post to home delivery companies.	*I worry for when patients become adults and how having to pay prescription charges may affect compliance to the very low-protein diet* [English response]. *Causes a lot of confusion for those turning 18* [English response]. *Prescriptions are free for all ages in Scotland. It does however cause issues with the home delivery companies who all have dispensing pharmacies based in England* [Scottish response]. *Adults in Wales are not eligible for some home delivery services, unless they pay a pre-payment prescription. As they get their prescriptions for free, most are reluctant to do this. LP foods predominantly supplied by local pharmacy* [Welsh response]. *In Northern Ireland, prescriptions do not go via 'the electronic spine' and need to be sent via post—there are often issues with prescriptions going to the wrong place/getting lost [Northern Ireland response].*
Dispensing of low-protein prescription foods • Different delivery companies deliver different LPPFs; • Use of more than one delivery service is confusing for GPs, delivery companies, pharmacies and patients; • Use of more than one delivery service increases the number of home deliveries each month, this is made worse by some companies splitting deliveries so they arrive at different times/days; • Some GPs operate a single nominated pharmacy policy so patients can only choose one delivery service/pharmacy.	*It is difficult for families to understand the prescribing process and know who/when to contact if there are problems. It varies from patient to patient depending on how organised they are, how good their pharmacy is and the range of products the child accepts.* *Having different suppliers of low-protein prescription foods is confusing for families, GPs and pharmacists. It can lead to prescription requests being sent to the wrong pharmacy or home delivery company and this ultimately results in a delay in the time it takes for patients to receive their low-protein foods. This can then have a knock-on effect on compliance as these patients cannot just go and buy these foods from the local supermarket.* *Adult patients need to be at home to accept deliveries.* *Most GPs seem to operate a single nominated pharmacy policy and this causes so many problems getting the prescriptions to the right company/pharmacy, so patients can no longer have three different home delivery companies and get non-PKU related items from the local pharmacy.*
Time dealing with low-protein prescription food issues • Dietitians suggest that problems are significantly under-reported; • The reason why a patient cannot obtain their products is not always clear, so it requires a lot of phone calls, waiting on hold and waiting for responses; • Most patients have issues at some point, and many have ongoing issues every month despite interventions; • Much time is spent trying to access prescriptions from the GP because the delivery companies may refuse to call GPs.	*It is so time-consuming sorting out problems with low-protein prescription deliveries.* *Sometimes the prescription issue is resolved, then after a few months, the issue arises again.* *I feel for the patients and the families as it is time-consuming for them and a big additional burden to try and resolve their low-protein food prescription issues.* *A lot of dietetic time is spent ringing and waiting on hold at GP surgeries and being the go-between for communication with GPs, delivery companies and patients.*

Table 4. *Cont.*

Issues	Supporting Respondent Quotes
Pharmacy issues • Out-of-stock foods; • Discontinued foods; • Missing items from delivery; • Delivery of short-shelf-life products; • Patients informed that the low-protein food is discontinued when the wholesaler is out-of-stock, or it has been ordered from the wrong wholesaler, or pharmacies have not tried an alternative wholesaler; • Pharmacies cannot locate low-protein foods in their prescription system.	*Often patients report: "pharmacy says that product is out-of-stock/product discontinued" when actually the pharmacy are trying to order from the wrong wholesaler or haven't tried an alternative wholesaler.* *A high street pharmacy will say an item is out-of-stock or no longer manufactured, when in fact it's that their suppliers don't stock it and they are unwilling to order it in through other means.* *Often, items are missing from their order but no information is given about why items are missing.*
Home delivery company issues • Lack of communication between the home delivery companies and GP regarding prescriptions; • Dietitians/dietetic support workers receive daily communication from home delivery companies asking them to obtain outstanding GP prescriptions on their behalf; • Delivery companies claim that GPs are more likely to listen to dietitians rather than themselves regarding prescriptions; • Patients are often unaware of when their delivery is due; • Deliveries are often delayed. • Damaged products are commonly received by patients/caregivers due to courier services; • Methods of ordering can be confusing for patients; • There is a lot of paperwork involved in registering patients with home delivery companies, and every time there is a change in prescription to add or remove a food item or change the quantity.	*Dietitians receive a lot of emails from delivery companies asking them to liaise with GPs to organise owed prescriptions.* *I routinely hear of requests not being delivered by a home delivery company because they are waiting for a prescription.* *Have daily communications from home delivery companies stating they have not got the prescription from the GP, so they are unable to deliver.* *Parents need to be organised about when they should send in their order forms and keep track of when they should be expecting deliveries.* *If patients are unaware when the delivery is due and are not home, there is a risk of failed deliveries or damaged food if left somewhere inappropriate.*
Electronic Prescribing System (EPS)/NHS Spine • Delivery companies and pharmacies may download the wrong prescription from the EPS (one intended for another company/pharmacy); • Many dietitians do not understand how the EPS works.	*Would hope that if delivery companies download the wrong prescription that they will put it straight back on the spine as they are aware it's not for them. This is not always the case with local pharmacies who may think it's for them or want to dispense it.*

Table 4. *Cont.*

Issues	Supporting Respondent Quotes
GP issues • Incorrect amount of food on prescriptions due to misconceived perceptions of what is appropriate; • Incorrect or missing foods on prescription; • GP refusal to prescribe some foods, e.g., "luxury" or "unhealthy" items such as low-protein cakes, biscuits or chocolate; • GP refusal to send prescriptions to third-party pharmacies (i.e., delivery companies), so instead paper prescriptions are given to patients to post to the delivery companies; • Some GPs operate a single nominated pharmacy policy so patients can only choose one delivery service/pharmacy; • Some GPs refuse to accept requests for repeat prescriptions from home delivery companies; • Some GPs cannot locate LPPFs on their computer system in order to prescribe them; • GP surgeries do not understand the complex ordering process.	*Our prescription letter has instructions for the GP on how to send the prescription to each of the home delivery companies, including via the post, spine and nominated pharmacy options. But still, they get this wrong.* *GPs sometimes refuse more 'luxury/convenience' foods.* *GPs are sometimes suspicious the whole family are using the products to save money.* *Items that should be on repeat prescription (and often are listed as such on the GP system) seem to 'drop off' and then have to be re-requested.* *GPs often struggle to find individual LPPFs on their system.* *It is difficult to explain to surgeries that patients can order any food from a long list of low-protein food items and that the prescription required will change each month.* *GPs do not understand the process and are confused as to where the prescription needs to be sent.* *Some GPs have a mistrust of home delivery companies and seem to believe that the home delivery companies are trying to rip them off.* *As PKU is a rare disorder, educating GP surgery staff is a relentless task due to the number of staff and staff turnover.* *Some GPs confuse LPPF with gluten-free foods which are available in the supermarkets.*
Contacting GP surgeries • The first contact is usually the receptionist, who does not have the authority to resolve issues; • The surgery pharmacist or prescription clerk is the preferred contact by most dietitians, but they are commonly unavailable (e.g., part-time); • If issues are long standing or not resolving, dietitians will request to speak to the GP or practice manager.	*GP reception staff often do not have the correct training and are not sure what we are asking about.* *Practice pharmacists often work part-time—so it can be a week until you can speak to them.* *It depends on the practice. The first contact point is always the receptionist. Can be difficult to get beyond this, but I try to get put through to the person involved in prescribing.* *Phone lines can be extremely busy, and not all practices have dedicated prescription lines.*
Manufacturer/wholesaler issues • Poor communication with patients and dietitians when foods are discontinued or out-of-stock; • Increasing numbers of LPPFs are being discontinued; • Some LPPF foods may be unavailable for periods of time.	*Dietitians are not always made aware that there are supply issues until we are chasing up why an order has not been received.* *A lot of popular items have been discontinued or the supplier has been having issues obtaining the products. These items are often staples (e.g., bread products).*

Table 4. *Cont.*

Issues	Supporting Respondent Quotes
* **Patient/caregiver issues** • Some patients do not understand the ordering process; • Patients may not order in a timely way; • The volume of LPPF required for 1 month is high, particularly for families with more than one child; • Adults may be less likely to discuss issues with delivery companies due to learning and cognitive difficulties; • The system is commonly overwhelming and difficult for patients; • Some patients run out of LPPFs.	*Families do not allow enough time when ordering low-protein foods—but they are expected to be very organised.* *For some of our vulnerable patients, we arrange a standing order of staple LPPF to ensure they always have a supply, however they may change their minds about food they enjoy* *Families with more than one child can find it hard to store a month's supply of LPPF.* *More problems tend to be in families with communication difficulties, e.g., due to language, level of education or in areas of lower socio-economic status.* *Many adult patients have learning difficulties and become frustrated with the system.* *The current system is clunky and inefficient and causes unacceptable stress and anxiety for patients. Many find it hard to engage with their GPs / pharmacies and they feel like they're being a nuisance having to go back and ask for something else. They often give up.* *Patients may come to clinic and report they haven't had any low-protein foods for the last 3 months.* *Accessing low-protein foods is a barrier to adults returning or staying on diet.* *Patients are frequently left without staple foods such as bread due to:* - *Missing a full delivery;* - *Missing products from their delivery;* - *Delayed deliveries;* - *Failure of GP to issue a prescription;* - *Damaged, mouldy or out-of-date food.*
Impact on blood Phe • Inadequate supplies of LPPF can lead to high blood Phe levels due to: - Eating the wrong foods (higher in protein, e.g., gluten-free foods) as they have nothing else to eat; - Insufficient energy intake and weight loss leading to catabolism; - Limited variety of foods, which impacts enjoyment of food and subsequently dietary adherence; - Adult demotivation to follow dietary treatment; - Increased stress and anxiety in adult patients.	*Patients running out of LPPFs can lead to high Phe levels as patients have no choice but to eat the wrong foods. It also leads to hungry patients and weight loss.* *If bread arrives mouldy or not at all, they may have to use supermarket gluten-free options, which are higher in protein.* *Adult patients can become demotivated by poor product access.* *Often impacts blood Phe levels as adult patients can go on/off diet depending on what stock they have available.* *Some patients make do and only tell you when blood Phe levels are high that they have not been getting basic low-protein foods.*

* Note: The term "GP" is used interchangeably to refer to staff at GP surgeries who may be dealing with patient prescriptions (may be prescription clerk, pharmacist, receptionist, practice manager, GP). The term "patient" is used interchangeably to refer to adult patients or caregivers of children with PKU.

3.12. Improvements Required

The most commonly reported recommendations by respondents for improving access to LPPF for patients with PKU included centralisation of the system to one supplier (76%, 61/80), and apps for ordering LPPF (69%, 55/80) (Figure 7). Paediatric respondents were more likely to suggest that improved GP surgery education may help (80%, 35/44 vs. 56%, 20/36 for adult dietitians/DSWs, $p = 0.03$ Fisher's exact test), and adult dietitians/DSWs were more likely to suggest direct dietetic prescription of LPPFs (67%, 24/36 vs. 48%, 21/44 paediatric dietitians/DSWs; $p = 0.11$) (Figure 7).

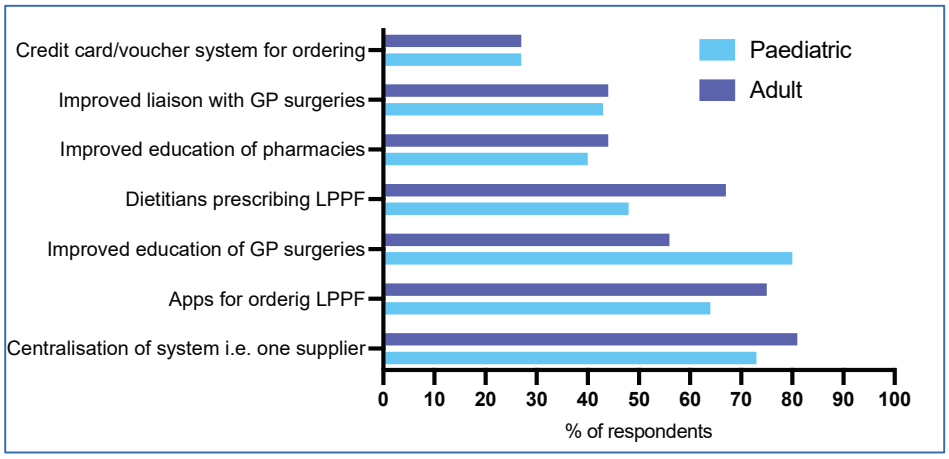


Figure 7. Suggestions for improving access to LPPF for patients with PKU in the future.

Based on open comments in the questionnaire, Table 5 shows a list of possible solutions or improvements to the current system.

Table 5. Possible solutions or improvements to the current system for accessing low-protein prescription foods for PKU.

Suggestions for Improvement
• A separate UK prescribing system is necessary for LPPFs as they are different to drugs/medication; • Centralisation of the system with one supplier/delivery company; • Giving patients a budget each month to spend on LPPFs; • Order LPPFs via a voucher or pre-loaded credit card; • An app for ordering—one list, one supplier; • Improve NHS App functionality for complex script handling; • In the UK, all patients (of all age groups) with PKU should have access to free NHS prescriptions; • Patients in Scotland, Wales and NI to have free access to specialist home delivery companies; • Consider dietetic prescribing of LPPFs.

4. Discussion

The results of this survey showed that organising and managing patient access to LPPF for PKU was time-intensive and arduous for dietitians/DSWs working in paediatric and adult services and challenging for patients. Fifty percent of dietitians/DSWs were notified at least weekly that some of their patients had problems accessing LPPFs. Common issues with LPPFs were unavailability, missing items in deliveries, delayed deliveries, and prescription refusal by GPs. A central NHS digital prescription service, not designed to manage access to LPPFs for rare IMD conditions, generated some of the supply difficulties, coupled with increasing issues in the availability of some LPPFs.

There was unequal access to 'free' prescriptions for patients across the UK. Although there are only 1436 adults with PKU in follow up in English hospitals [12], unlike patients in Scotland, Wales or NI, they had to pay for the cost of their prescriptions. The cost of an NHS prescription in 2024–2025 was GBP 9.90 per item. Alternatively, an adult could

purchase a certificate for prepayment for prescriptions (PPCs), in which, for a preset price of GBP 32.05 once every three months, or GBP 114.50 every twelve months, all prescriptions are covered, including LPPFs and protein substitutes [13]. This creates a substantial cost burden for patients with PKU and produces a barrier to treatment access. Inadequate access to LPPF may force patients to either abandon their treatment or go hungry. It has been previously suggested that prescription charges for working-age people may have a significant impact on adherence, self-management, quality of life and health outcomes [17].

Low-income or benefit-qualified patients may be eligible for help with medication costs. People entitled to free prescriptions in England include individuals aged 60 or over, aged under 16 years, aged 16 to 18 years and in full-time education, and pregnant or having had a baby in the previous 12 months. However, this can lead to confusion for patients, and some may be unaware of the need to pay for prescriptions. Some patients with PKU have mistakenly claimed free prescriptions (believing they were exempt) and have received financial penalty charges [18]. Furthermore, patients also need strong literacy and numeracy skills to be able to apply for some of the exemptions. They need to supply dated certificates to 'prove' exemption and reapply regularly for their exemption documents. It is possible that the cost to the NHS of providing exemptions to working-age people with PKU would be more than offset by reductions in healthcare resource use [17]. This would not only be through the cost savings associated with a reduction in administration and dietetic time but would enable patients to sustain satisfactory and stable metabolic control, potentially leading to fewer co-morbidities and lower demands on the health care system overall.

In addition, dietitians describe how some patients with PKU have difficulty accessing LPPFs on prescription, leaving them without adequate supplies. In this survey, respondents reported that some GPs did not prescribe sufficient quantities of LPPF or home delivery companies had issues accessing prescriptions for the LPPF from the GP practice, particularly in adulthood. Adult patients reluctantly tolerate these issues even though they may be left without LPPF. This leads to abandonment or relaxation of dietary treatment and the consumption of higher protein replacement foods purchased from supermarkets. Dietitians describe how some GP practices do not accept the seemingly high amounts of LPPFs that adult patients may need for a 28-day supply, leading to under prescription particularly of staple foods like bread. Patients/caregivers may also underestimate the amount of LPPF they need for a full four-week prescription cycle. Forecasting accurate amounts of necessary supplies is difficult, especially if there is more than one child in the family with PKU or LPPFs arrive damaged or mouldy and are inedible. Some patients/caregivers have difficulty understanding the ordering process and prescription timelines. Dietitians describe how some patients/caregivers are confused, frustrated and overwhelmed by the over complicated ordering systems leading to unnecessary stress and anxiety. Prescription problems commonly re-occur for the same patients and this can be demotivating for patients/caregivers, and may lead to food poverty and loss of metabolic control. Inadequate supply of LPPF may be a particular problem in maternal PKU where strict control of blood Phe is crucial for healthy foetal outcome.

A large amount of dietetic time was spent on unnecessary tracking of missing prescriptions for LPPF supplies. There were often delays in GPs issuing prescriptions for LPPFs. This was associated with prescriptions being issued to the wrong pharmacy/home delivery company, the wrong quantity of prescription items being given, some LPPF items being omitted, and mistakenly categorising LPPF as gluten-free and then refusing to issue a new prescription. Liaising with GP practices may be frustrating, with long telephone queues or untimely delays in responding to emails. Dietitians cannot access the GP systems to check if prescriptions have been completed, if all the items have been issued correctly or where

the prescription has been sent. In addition, home delivery companies frequently request dietitians to obtain prescriptions from the GP on their behalf due to frequent unsuccessful communications with a GP practice. It is recognized that GP practices are exceptionally busy and overextended, and safe administration of multiple item prescriptions for LPPFs is likely to be time-consuming. Many will have received little training on LPPFs, the amount that should be prescribed, and they may have difficulty identifying specific LPPF products on their computer systems.

Home delivery services for LPPFs have seen considerable expansion in recent years. Most respondents reported that their patients accessed their LPPFs by using one or more home delivery companies in combination with their local pharmacy. In theory, home delivery services should enable patients to receive their prescription items in a consistent, safe and timely fashion, but the NHS system for issuing prescriptions does not readily support the use of multiple home delivery companies to provide a smooth service to patients. In England, with the electronic prescription service (EPS), patients can nominate for their prescriptions to be sent electronically and directly from the GP surgery to one single pharmacy or dispenser of their choice. Nominations can be changed or removed, but only at the request of the patient. For any prescriptions without an EPS nomination, a token is issued containing a unique barcode which is uploaded to the NHS Spine. This can be downloaded, and the prescription details retrieved by any home delivery company or pharmacy.

In our survey, dietitians reported numerous problems with this service with prescriptions commonly being lost or downloaded by the incorrect delivery service, leading to frustration, stress and immeasurable loss of professional time tracking prescriptions. Better collaboration and understanding is necessary between the NHS digital and management teams, health professionals in primary and secondary care (including GP practice clerks, managers, pharmacists and GPs), and supply chain partners, including manufacturers, suppliers, distributors and logistics providers.

Patients can register to use the NHS App to request repeat prescriptions. Using this system, patients will know when their prescription is issued and can see all their prescription details in one secure place, giving them greater visibility and control of their healthcare. Each repeat prescription ordered electronically, saves GP practices three minutes of time [19]. However, this system does not work for non-repeat prescriptions. This is unfortunate, as the majority of LPPF prescriptions required by patients with PKU are non-repeat. With over 150 LPPFs on prescription, the amount and type of each LPPF ordered is likely to vary from month to month, meaning that non-repeat prescriptions must be ordered by directly contacting the GP surgery each month. In addition, patients with poor digital literacy, without appropriate technology or with learning disabilities may be disadvantaged by digital prescription ordering.

In the UK, each prescription is written by a registered prescriber, i.e., GP. Traditionally, the prescribing of human medicines has been perceived as a medical role, with only medical professionals and dentists having full prescribing rights. Two seminal reports challenged this view; the Cumberlege Report [20], which paved the way for limited prescribing by health visitors and district nurses, and the Crown Report [21], which recommended extending prescribing rights for the benefit of patients and to utilise the skills and extend the role of healthcare professionals. From February 2016, dietitians were able to become supplementary prescribers following the successful completion of a Health and Care Professions Council (HCPC)-approved prescribing programme [22]. However, only six dietitians in this survey were qualified prescribers. In addition, these dietitians did not have a financial budget to support the prescription of LPPF. Without the financial resources, it is unmanageable to further develop dietetic prescription services.

Some home delivery services did not meet the needs of patients with PKU. Commonly, delivery times were inflexible with a weekday service only, meaning that working adults or caregivers were not at home to accept deliveries. At times, supplies from the same company arrived in separate deliveries on different days, causing confusion for patients and caregivers. Adult patients living in Wales and Scotland (normally exempt from prescription charges) were expected to purchase a prescription certificate of pre-payment in order to access services from English home delivery services. Dietitians reported that most adult patients were reluctant to do this. They also report that patients were commonly unaware of expected delivery dates for their LPPF, and so contacted their specialty dietetic team for this information. Equally, there were issues with community pharmacists. Dietitians reported pharmacy delays in receiving LPPFs from multiple suppliers, dispensing of short shelf-life stock received from wholesalers, and patients/caregivers being informed that some LPPFs have been discontinued when there were stock issues only. Commonly, dietitians reported having to supply patients with 'bridging stock' or samples to ensure that they had sufficient LPPF to eat.

A frequent issue identified by dietitians was the interruptions to LPPF supply and manufacturing issues. Manufacturers of LPPF have significant challenges. They require alternative and suitable low-protein grains/starches requiring complex manufacturing processes. Since 2011, the UK NSPKU have identified that 43 LPPFs have been withdrawn by manufacturers and thereby from prescription [23]. Manufacturers may have fragile supply chain challenges, increasing energy costs, costs associated with inflation, delays in accessing key ingredients and raw materials, batch production failures, global supply chain challenges, and labour shortages. Goods imported may take longer to arrive in the country, and the introduction of full customs controls in the European Union has created additional administration. LPPF manufacturers supplying a range of products may have to use third-party suppliers who consider that it is not cost effective to produce goods in the small volumes required by patients with rare disorders such as PKU. Furthermore, outsourcing production means reliance on external partners to maintain the quality of LPPF.

For the future, dietitians considered that a single centralised, dedicated delivery company dispensing all LPPF would make the prescription service easier, although this would need service evaluation. In addition, the flexibility of patients and caregivers being permitted to directly request a set number of LPPF items each month, possibly through an app system monitored by their specialist dietetic team rather than the GP service, would improve the effectiveness and efficiency of LPPF access. A regulated age-related monthly financial allowance could be considered, giving patients the freedom to order the LPPF they need directly from the suppliers. Similar systems are implemented in countries such as Australia, Belgium, Denmark and Norway [24]. Although regular GP service education is important, staff turnover and the number of people involved make this unviable. As of November 2024, there are over 35,000 fully qualified GPs working in England alone, with less than 100 IMD dietitians to provide the education [25]. However, more educational resources, such as short videos, could be developed to assist patients with LPPF ordering.

Limitations

Respondents had varying levels of involvement in managing patient LPPF access issues, and this will have influenced their answers, particularly with respect to the amount of dietetic time spent in organising LPPFs and correcting problems, and the frequency of specific problems. Some clinical dietitians, particularly in adult services, reported that these issues were more likely to be dealt with by their DSW than by themselves. Some hospitals did not have DSWs, and not all dietitians and DSWs worked exclusively in PKU or IMD. The number of years of experience working in PKU, and therefore the degree of

exposure to the issues addressed by the questionnaire also varied. The average length of time to complete the questionnaire was 50 min (range: 6–198 min), which in turn may have led to respondent fatigue and accuracy of answers in later questions in the questionnaire. Some adults with PKU that may be less likely to report issues with LPPF supply compared with caregivers, masking the full extent of the problems observed.

5. Conclusions

Prescription of LPPF for patients with PKU in the UK is complex, inefficient, and involves multiple stakeholders, leading to suboptimal LPPF patient supplies. LPPF prescriptions are generally issued by GP practices who do not receive any training or guidance in this task. It is a cause of patient and caregiver anxiety, stress and a potential source of error leading to overall mistrust in the healthcare system. The current system requires a substantial amount of IMD dietetic resources to ensure that patients have adequate LPPF supplies. Easier and seamless channels are necessary to support communication between GPs, patients, caregivers and supporting health professionals. A review of patient access to LPPF is urgently required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu17030392/s1, S1. Patient access to low-protein prescription foods in PKU: a questionnaire for dietetics; S2. Result—NOT Research; Table S1. Demographics of respondents.

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References

1. van Spronsen, F.J.; Blau, N.; Harding, C.; Burlina, A.; Longo, N.; Bosch, A.M. Phenylketonuria. *Nat. Rev. Dis. Primers* **2021**, *7*, 36. [CrossRef] [PubMed]
2. Blau, N. Genetics of Phenylketonuria: Then and Now. *Hum. Mutat.* **2016**, *37*, 508–515. [CrossRef] [PubMed]
3. Longo, N.; Dimmock, D.; Levy, H.; Viau, K.; Bausell, H.; Bilder, D.A.; Burton, B.; Gross, C.; Northrup, H.; Rohr, F.; et al. Evidence- and consensus-based recommendations for the use of pegvaliase in adults with phenylketonuria. *Genet. Med.* **2019**, *21*, 1851–1867. [CrossRef] [PubMed]
4. Burton, B.K.; Longo, N.; Vockley, J.; Grange, D.K.; Harding, C.O.; Decker, C.; Li, M.; Lau, K.; Rosen, O.; Larimore, K.; et al. Pegvaliase for the treatment of phenylketonuria: Results of the phase 2 dose-finding studies with long-term follow-up. *Mol. Genet. Metab.* **2020**, *130*, 239–246. [CrossRef] [PubMed]
5. Longo, N.; Arnold, G.L.; Pridjian, G.; Enns, G.M.; Ficicioglu, C.; Parker, S.; Cohen-Pfeffer, J.L.; Phenylketonuria Demographics, O.; Safety, R. Long-term safety and efficacy of sapropterin: The PKUDOS registry experience. *Mol. Genet. Metab.* **2015**, *114*, 557–563. [CrossRef] [PubMed]
6. Talebi, S.; Eshraghi, P. Nutrition in phenylketonuria. *Clin. Nutr. ESPEN* **2024**, *64*, 307–313. [CrossRef] [PubMed]
7. Cochrane, B.; Schwahn, B.; Galloway, P.; Robinson, P.; Gerasimidis, K. A questionnaire survey on the usage of low protein staple foods by people with phenylketonuria in Scotland. *J. Hum. Nutr. Diet.* **2014**, *27*, 533–541. [CrossRef] [PubMed]
8. National Institute for Health and Care Excellence (NICE). British National Formulary (BNF). Available online: https://bnf.nice.org.uk/ (accessed on 19 December 2024).
9. Daly, A.; Evans, S.; Pinto, A.; Ashmore, C.; Rocha, J.C.; MacDonald, A. A 3 Year Longitudinal Prospective Review Examining the Dietary Profile and Contribution Made by Special Low Protein Foods to Energy and Macronutrient Intake in Children with Phenylketonuria. *Nutrients* **2020**, *12*, 3153. [CrossRef] [PubMed]
10. Moretti, F.; Pellegrini, N.; Salvatici, E.; Rovelli, V.; Banderali, G.; Radaelli, G.; Scazzina, F.; Giovannini, M.; Verduci, E. Dietary glycemic index, glycemic load and metabolic profile in children with phenylketonuria. *Nutr. Metab. Cardiovasc. Dis. NMCD* **2017**, *27*, 176–182. [CrossRef] [PubMed]
11. Thiele, A.G.; Rohde, C.; Mutze, U.; Arelin, M.; Ceglarek, U.; Thiery, J.; Baerwald, C.; Kiess, W.; Beblo, S. The challenge of long-term tetrahydrobiopterin (BH4) therapy in phenylketonuria: Effects on metabolic control, nutritional habits and nutrient supply. *Mol. Genet. Metab. Rep.* **2015**, *4*, 62–67. [CrossRef] [PubMed]
12. Wood, G.; Pinto, A.; Evans, S.; Daly, A.; Adams, S.; Costelloe, S.; Gribben, J.; Ellerton, C.; Emm, A.; Firman, S.; et al. Special Low Protein Foods Prescribed in England for PKU Patients: An Analysis of Prescribing Patterns and Cost. *Nutrients* **2021**, *13*, 3977. [CrossRef] [PubMed]
13. National Health Service (NHS) Business Services Authority (BSA). NHS Prescription Prepayment Certificate. Available online: https://www.nhsbsa.nhs.uk/help-nhs-prescription-costs/nhs-prescription-prepayment-certificate-ppc (accessed on 19 December 2024).
14. National Society for Phenylketonuria (NSPKU). Unit Guidelines for the Prescription of Low Protein Foods. Available online: https://nspku.org/wp-content/uploads/2020/09/Prescription-Guidelines-NT-Sept-2020.pdf (accessed on 19 December 2024).
15. Ford, S.; O'Driscoll, M.; MacDonald, A. Prescribing issues experienced by people living with phenylketonuria in the UK. *Mol. Genet. Metab. Rep.* **2019**, *21*, 100527. [CrossRef] [PubMed]
16. NHS Health Research Authority (HRA) and Medical Research Council (MRC). "Is My Study Research" Online Decision Tool. Available online: https://www.hra-decisiontools.org.uk/research/ (accessed on 8 January 2024).
17. York Health Economics Consortium. Economic Evaluation of the Benefits of Extending Free Prescriptions to People with Long-Term Conditions. Available online: https://www.prescriptionchargescoalition.org.uk/uploads/1/2/7/5/12754304/economic_evaluation_report.pdf (accessed on 19 December 2024).
18. UK Parliament Public Accounts Committee. Committee Take Evidence on Penalty Charge Notices in Healthcare. Available online: https://committees.parliament.uk/committee/127/public-accounts-committee/news/98292/penalty-charge-notices-in-healthcare-inquiry-launch-17-19/ (accessed on 19 December 2024).
19. National Heath Service (NHS) England. Digital Prescriptions Go Live in NHS App. Available online: https://www.england.nhs.uk/2024/01/digital-prescriptions-go-live-in-nhs-app (accessed on 19 December 2024).
20. Department of Health and Social Care. First Do No Harm. The Report of the Independent Medicines and Medical Devices Safety Review. Available online: https://www.immdsreview.org.uk/downloads/IMMDSReview_Web.pdf (accessed on 19 December 2024).
21. UK Parliament. Review of Prescribing, Supply and Administration of Medicines. Available online: https://www.publichealth.hscni.net/sites/default/files/directorates/files/Review%20of%20prescribing,%20supply%20and%20administration%20of%20medicines.pdf (accessed on 19 December 2024).

22. National Health Service (NHS) England. Supplementary Prescribing by Dietitians. Available online: https://www.england.nhs.uk/ahp/med-project/dietitians/ (accessed on 19 December 2024).
23. Ford, S.; (NSPKU). NSPKU UK Report 43 Low Protein Prescription Foods Have been Withdrawn by Manufacturers Since 2011. NSPKU, Ed. Personal Communication, 2024.
24. SBelanger-Quintana, A.; Dokoupil, K.; Gokmen-Ozel, H.; Lammardo, A.M.; MacDonald, A.; Motzfeldt, K.; Nowacka, M.; Robert, M.; van Rijn, M.; Ahring, K. Diet in phenylketonuria: A snapshot of special dietary costs and reimbursement systems in 10 international centers. *Mol. Genet. Metab.* **2012**, *105*, 390–394. [CrossRef] [PubMed]
25. British Medical Association (BMA). Pressures in General Practice Data Analysis. Available online: https://www.bma.org.uk/advice-and-support/nhs-delivery-and-workforce/pressures/pressures-in-general-practice-data-analysis (accessed on 19 December 2024).

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Review

Intermittent Fasting: A Metabolically Focused Therapeutic Strategy for Obesity

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Abstract

The global prevalence of obesity continues to rise and is a significant risk factor for the onset and progression of cardiovascular diseases. Despite the development of new pharmacological therapies, novel strategies are being explored to mitigate the impact of this disease. Intermittent fasting (IF) is a nutritional intervention that has gained popularity and shows potential as an innovative approach to weight management. This study aims to compile scientific evidence on various aspects of fasting, including its physiological effects, the molecular and thermogenic mechanisms involved, and recommendations regarding nutritional strategies during the refeeding period within the eating window. We conducted a narrative review, analyzing evidence available from PubMed/MEDLINE based on studies related to intermittent fasting, thermogenesis, and their associated outcomes. Our results demonstrate the existence of three commonly used IF protocols: alternate day fasting (ADF), periodic fasting (PF), and time-restricted eating (TRE). In addition to its effects on weight loss, IF has demonstrated notable benefits for cardiovascular health, oxidative stress, and metabolic function. Moreover, the interaction between the central nervous system and brown adipose tissue provides an alternative mechanism for the molecular regulation of thermogenesis. Nutritional patterns adopted during intermittent fasting play a crucial role in optimizing outcomes, with particular emphasis on the intake of proteins, fiber, bioactive compounds, and essential fatty acids during the feeding window. In summary, current evidence indicates that intermittent fasting provides a biologically robust framework for studying energy balance and holds promise for developing targeted nutritional interventions.

Keywords: intermittent fasting (IF); alternate day fasting (ADF); time-restricted eating (TRE); thermogenesis; brown adipose tissue (BAT); CNS-BAT communication; browning

1. Introduction

In recent decades, the global prevalence of obesity has substantially increased. Currently, an estimated 650 million adults and approximately 340 million children and adolescents aged 5–19 years are affected by obesity worldwide [1]. This condition arises from an imbalance between caloric intake and energy expenditure, leading to excessive accumulation of adipose tissue as a consequence of a sustained positive energy balance [2]. Obesity is associated with significant health risks, including type 2 diabetes mellitus (T2DM) [3], dyslipidemias [4], and various types of cancer [5], all of which are linked to insulin resistance and lipotoxicity [6]. In addition to genetic and environmental factors, various pathological cellular processes, such as chronic inflammation and mitochondrial dysfunction, contribute to the development and progression of the disease [7,8].

Although there have been remarkable advancements in pharmacological therapeutic interventions based on pharmacology [9], there has been increasing interest in improving dietary patterns through dietary interventions [10]. In this regard, intermittent fasting (IF) appears to be one of the most popular strategies for controlling caloric intake, showing benefits in weight management, cardiovascular health, oxidative stress, and general health and lifestyle outcomes [11]. IF is a dietary pattern that cycles between fasting periods and regular ad libitum eating on a scheduled basis, during which individuals severely restrict their caloric intake within a day or week (Table 1) [12,13]. It has been suggested that IF is less restrictive, simpler, and more flexible than traditional calorie restriction (CR) methods [14]. Studies in humans have reported several health benefits, including reductions in fat mass and body weight, improvements in glucose tolerance and insulin sensitivity, and lower risks of cardiovascular disease and cancer [12,14,15]. However, more evidence is needed to evaluate the risks, benefits, and adherence to IF, particularly in individuals with obesity [12,15–18]. Likewise, studies in animals have shown that IF can modify adipose tissue morphology and induce changes in tissue growth dynamics [19]. Notably, sexual dimorphisms in IF responses remain an important research gap, given the significant variations in inflammatory processes and diet-induced obesity between men and women [20,21].

IF induces long-term "metabolic adaptation", which can reduce the metabolic rate and may extend human lifespan [22]. In this context, during IF, a metabolic switch is activated, shifting energy utilization from glucose to fatty acids and ketone bodies, which represents an evolutionary mechanism that reprograms metabolism [23]. Therefore, IF may provide a targeted strategy for restoring metabolic flexibility, improving insulin sensitivity, and supporting healthy body composition [13]. In addition, the ability of IF to modulate glucoregulatory factors has been frequently assessed in clinical studies. In particular, subjects with T2DM have shown significant beneficial effects of IF in both diabetic animal models and T2DM individuals [24].

Therefore, this review aims to compile scientific information on the different IF schemes and their effects. In addition, the molecular mechanisms involved in IF, including those from the perspectives of neuroscience and thermogenesis, are discussed. Finally, some alternatives for refeeding during the eating window are reviewed, highlighting the use of diet-induced thermogenesis (DIT) as a novel nutritional strategy.

Table 1. Approaches and characteristics of intermittent fasting.

Features	Alternate-Day Fasting (ADF)	Periodic Fasting (PF)	Time-Restricted Eating (TRE)	References
Principle	Compensatory Metabolic Responses	Compensatory Metabolic Responses	Circadian Rhythms	[25]
Frequency	Every other day	Two days weekly	Every day	[11]

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Table 1. *Cont.*

Features	Alternate-Day Fasting (ADF)	Periodic Fasting (PF)	Time-Restricted Eating (TRE)	References
Duration (fasting period)	24 h	24 h each fasting day	14–18 h	[26]
Eating window	24 h (ad libitum on feeding days)	24 h (ad libitum on feeding days)	4–12 h	[27]
Energy intake during fasting periods	Strictly controlled energy intake (≈25% of usual intake, ≈500 kcal)	Strictly controlled energy intake (500–800 kcal)	No energy intake (water allowed)	[23]

2. Literature Search Methods

A structured narrative search was conducted in the PubMed/MEDLINE database up to October 2025. The search strategy employed MeSH terms and free-text terms combined with the Boolean operators OR and AND, including "intermittent fasting", "time-restricted eating", "alternate-day fasting", "thermogenesis", "brown adipose tissue", "white adipose tissue", "CNS–BAT communication", and "browning". Original preclinical and clinical studies, recent systematic reviews and meta-analyses, and relevant in vitro and in vivo studies related to intermittent fasting, thermogenesis, and associated outcomes were included. Editorial letters, non-peer-reviewed sources, and studies not published in English were excluded. Reference lists of key articles were also hand-searched. Owing to the substantial heterogeneity in study designs and reported outcomes, a narrative synthesis was undertaken, focusing on the clinical outcomes and molecular mechanisms related to thermogenesis.

3. Intermittent Fasting

IF is an innovative approach to weight control, consisting of different timing schedules for temporary food avoidance in healthy individuals. Its particularity relies on producing favorable metabolic effects by intermittently shifting the metabolism from fatty acids to ketones. Three types of IF have received the most research attention: Alternate-day fasting (ADF), periodic fasting (5:2 diet) and time-restricted eating (TRE) (Figure 1).

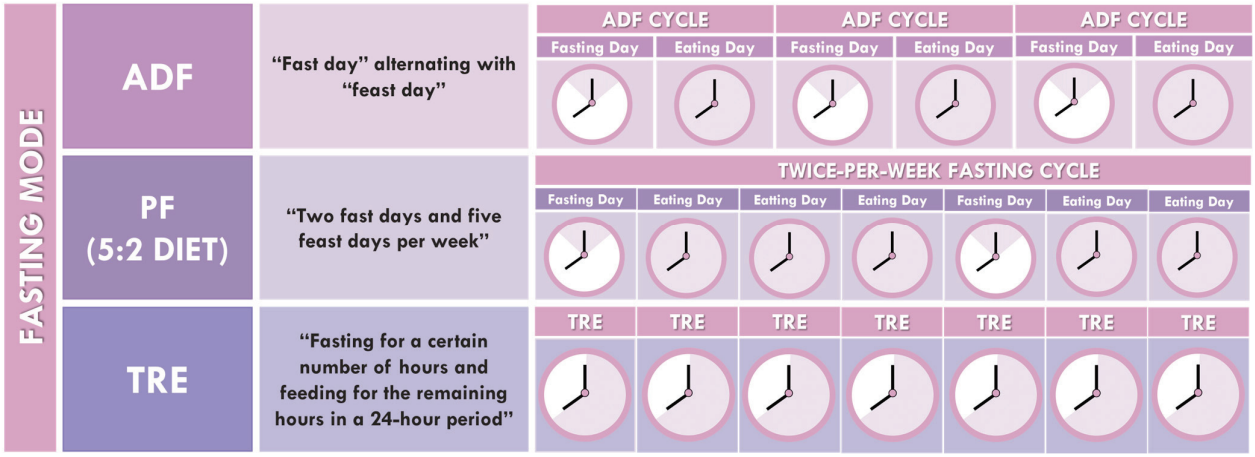


Figure 1. Types of intermittent fasting. Three commonly used IF protocols are illustrated: alternate-day fasting (ADF), periodic fasting (5:2), and time-restricted eating (TRE). The diagrams depict the fasting duration and corresponding eating windows for each approach. Darker shading indicates eating periods within the fasting regimen, facilitating visual comparisons of timing and duration across protocols.

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ADF involves a "fast day" alternating with a "feast day", with fasting for 24 h every other day [26]. On feast days, individuals are allowed to eat ad libitum, with no restrictions on the types or quantities of food consumed. During fasting days, individuals may choose to consume only water, which is referred to as "zero-calorie alternate-day fasting" [11]. Alternatively, individuals can consume 25% of their energy needs (500–800 kcal per day), which is known as "modified alternate-day fasting" [28]. Similarly, PF is a modified version of ADF that includes two fast days and five feast days per week (5:2 diet) [29]. Fasting can occur on consecutive or nonconsecutive days, and individuals are permitted to consume 25% of their energy needs (500–1000 kcal per day). Likewise, on feast days, individuals are allowed to eat ad libitum [30]. Finally, the TRE protocol involves a daily routine that requires fasting for a specific number of hours and feeding during the remaining hours of a 24-h period [17]. TRE typically includes an eating window of 4–8 h, with fasting supported by water or zero-calorie beverages for the rest of the day. The most commonly used protocol is 16/8 [24].

3.1. Effects of Intermittent Fasting

3.1.1. Corporal Weight and Body Composition

IF appears to be an effective method that may be beneficial for obesity [25,31]. Animal studies and human clinical trials have reported that the main outcome of IF is weight loss, but it also impacts visceral obesity, cardiometabolic health, metabolism, and glucose homeostasis [28,32,33]. Most human clinical trials have been conducted on normal-weight, overweight, and obese individuals [34–45]. In particular, ADF and the 5:2 diet produce similar degrees of weight loss (4–8% from baseline) in men and women with obesity over short intervention periods of 8–12 weeks [28,34,40–42,46–48]. Studies of longer duration (12–52 weeks) have not shown greater reductions in body weight, suggesting that weight loss efficacy may peak around 12 weeks, with reductions of 0.2–0.5 kg per week [36,37,43,44,49]. Compared with ADF and the 5:2 diet, the TRE regimen has shown a less marked degree of weight loss, approximately 3–4% from baseline [38,39,50]. Notably, both ADF and the 5:2 diet helped prevent weight regain during the 12–24 months of follow-up [34,35,37,43]. When ADF and 5:2 are implemented as maintenance diets, the energy intake allowed on fast days is increased (1000–1200 kcal per day instead of 500 kcal/day), which facilitates weight maintenance. Regarding body composition, during IF practices, most of the weight lost corresponds to a reduction in fat mass. No differences have been reported between IF and continuous calorie restriction in terms of the type of mass loss [51–54]. However, it has been observed that when the TRE regimen is combined with exercise, subcutaneous fat mass decreases significantly [17,55,56]. Remarkably, individuals following ADF or the 5:2 diet showed significant decreases in visceral fat mass compared with controls, whereas no changes were reported with the TRE regimen [34–37,40,43,49].

3.1.2. Metabolic Risk Factors

Blood pressure. The effect of IF on blood pressure is currently controversial. While several studies have shown reductions in systolic and diastolic blood pressure in humans [40,43,45,49,57,58], others have demonstrated the opposite effects [35,36,38,41,44,48,59–62]. Notably, most trials reporting decreased blood pressure involved participants with elevated blood pressure at baseline or individuals older than 45 years [63]. Additionally, IF has been observed to decrease blood pressure in overweight or obese populations, even without weight loss [30]. One potential explanation for this effect may be associated with the activation of the parasympathetic system, driven by increased activity of cholinergic neurons and elevated venous plasma levels of noradrenaline [64,65].

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Lipid profile. The relationship between plasma lipids and IF remains debatable [66,67]. A limitation of the current evidence is the lack of clarity regarding the timing of blood sample collection during IF protocols [26]. Several studies have shown that fasting plasma triglyceride concentrations are not affected after 4–10 h of TRE interventions [38,39,50,68,69]. Only one study reported that 9 h of TRE for 7 days led to a significant decrease in triglyceride levels, suggesting that these levels may depend on additional factors [70]. While some studies observed reductions in total cholesterol levels in overweight individuals [39,68], others found no significant effects of IF [38,68]. LDL cholesterol (LDL-c) concentrations decreased by 10–22% from baseline in three IF trials [34,37,49]. However, most studies indicate that TRE for 5–12 weeks does not affect LDL-c in individuals with excessive adiposity [38,39,50,57]. Similarly, HDL cholesterol (HDL-c) levels generally remain unchanged or slightly decrease with IF [34,37,43,49]. HDL-c concentrations may decline during the acute phase of weight loss but tend to rebound to baseline levels thereafter [71].

Glucose homeostasis and insulin sensitivity. Fasting plasma glucose levels do not change under IF alternatives, including ADF, the 5:2 diet, and TRE [35,41,46]. Almost all studies have been conducted in individuals without type 1 DM (T1DM) or T2DM at baseline, in whom fasting glucose is generally well controlled [72]. Additionally, fasting insulin levels were reduced from the baseline in several trials [35,37,38,48,49,57]. This pattern has been observed mostly in individuals with baseline hyperinsulinemia compared to those with normal insulin levels [37,38,40]. The modified 5:2 diet affects body weight in patients with T2DM [13,37]; however, adherence to this IF alternative is challenging. In contrast, TRE appears to be an appropriate alternative to decrease HbA1c (hemoglobin A1c) levels in individuals with diabetes, although this effect is observed only over long-term periods [13,73]. Patients with T1DM and obesity have shown changes in body weight but not in HbA1c levels [44]. IF diets do not appear to significantly affect glucose management, although the certainty of this evidence remains low [74].

Inflammation and oxidative stress. Evidence shows that IF decreases plasma levels of proinflammatory molecules such as tumor necrosis factor alpha (TNF-α), homocysteine, interleukin-6 (IL-6), and C-reactive protein (CRP), all of which contribute to atherosclerotic plaque formation [75]. Despite these findings, other studies suggest that IF generally does not affect these circulating biomarkers [38,50,55,57]. Moreover, IF has been shown to increase adiponectin secretion from adipocytes, which inhibits the release of the adhesion molecules VCAM and ICAM from the endothelium, resulting in reduced local and systemic inflammation [41,47,64]. In parallel, IF enhances the body's antioxidant defense systems, as evidenced by a reduction in malondialdehyde (MDA) levels and an increase in glutathione (GSH) during fasting periods [76]. Furthermore, the upregulation of enzymes such as superoxide dismutase-2 (SOD-2) and nuclear factor erythroid 2-related factor 2 (Nrf-2) during IF reinforces antioxidant activity [77]. Other plasma markers have also shown decreased levels under IF, including 8-isoprostane, nitrotyrosine, oxidized protein products, and carbonyls [38,49,57,77,78].

Given the physiological complexity and distinct metabolic features of IF, the next section examines how this dietary pattern influences thermogenesis and the molecular mechanisms that support its effects.

4. Intermittent Fasting and Thermogenesis: The Role of Adipose Tissue

The primary functions of white adipose tissue (WAT) and brown adipose tissue (BAT) are energy storage and thermogenesis, respectively [79,80]. Both tissues exert endocrine functions by secreting hormones and factors that regulate energy homeostasis, glucose and lipid metabolism, and cellular inflammatory processes [80–82]. Differences between WAT and BAT include gene expression patterns and the origin of their progenitor cells [79].

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The characteristic color of BAT results from its abundant mitochondria and multilocular lipid droplets, which support the inherent thermogenic properties of brown adipocytes in BAT depots. BAT adipocytes oxidize lipids in response to sympathetic nervous system activation and other hormonal stimuli to generate heat, thereby contributing to thermogenesis. In contrast, WAT is associated with macrophage infiltration and increased secretion of inflammatory cytokines in the context of obesity and metabolic disorders. In response to appropriate stimuli, WAT depots may transition into BAT in a process known as "browning", which gives rise to beige adipose tissue that displays characteristics of both depots. External stimuli, such as cold exposure, exercise, fasting, and pharmacological treatments, promote the development of beige adipocytes in WAT in mice. Animal studies have shown that daily caloric restriction and IF promote WAT browning [83–85], possibly mediated through alternative macrophage activation. In humans, however, WAT browning has been reported mainly in response to extreme adrenergic stimulation, such as burn injuries or the administration of β3-adrenergic receptor agonists. Therefore, BAT activation and browning are promising strategies for increasing energy expenditure and combating obesity [86]. Given the central role and excessive accumulation of WAT in obesity, stimulating WAT browning through different approaches or therapies may represent an alternative to improve metabolic health and reduce obesity [87–89].

BAT plays a crucial role in energy homeostasis through thermogenesis [86], which is mediated by uncoupling protein 1 (UCP1) or thermogenin [90], by uncoupling mitochondrial respiration from adenosine triphosphate (ATP) synthesis, ultimately generating heat. In addition to its thermogenic function, BAT is recognized for its endocrine capacity, suggesting that it may play a key role in the regulation of systemic energetic homeostasis. Experimental evidence supports that BAT secretes regulatory molecules and utilizes metabolic substrates during thermogenesis [91]. Brown and beige adipocytes secrete autocrine and paracrine factors that modulate their own expansion and activity, as well as the browning of WAT [92]. BAT releases endocrine factors that act on various peripheral tissues, including WAT, liver, pancreas, heart, and bone, and also influences systemic metabolism through interactions with the central nervous system. These endocrine factors, termed "batokines", include fibroblast growth factor 21 (FGF21), IL-6, and neuregulin 4. They exert beneficial metabolic effects and may be key components of therapeutic strategies to control obesity and its associated metabolic complications. BAT, as a primary site of adaptive thermogenesis, has been experimentally associated with protection against obesity and metabolic diseases such as T2DM and dyslipidemia. Although the ability of BAT to utilize glucose and lipids for thermogenesis is considered essential for heat production, its emerging endocrine role is increasingly recognized as fundamental to these protective effects.

To elucidate the link between thermogenesis and its molecular effects, it is essential to examine the underlying brain mechanisms. The following section details the central nervous system pathways and their role in regulating thermogenesis.

5. Brain–Brown Fat Crosstalk: Mechanisms of Thermogenic Regulation During Intermittent Fasting

The regulation of BAT thermogenesis by the central nervous system (CNS) represents a fundamental mechanism for maintaining energy balance and metabolic flexibility [93,94]. Classical studies have established that BAT activation depends on sympathetic outflow originating from the hypothalamic and brainstem circuits [95,96], whereas recent work has revealed a far more complex and bidirectional communication between the brain and peripheral metabolic signals [97–99]. In this context, IF provides a physiological framework to examine how the CNS dynamically recalibrates energy expenditure accord-

ing to nutrient availability, although the molecular and circuit-level mechanisms remain incompletely understood.

Thermogenic control originates in hypothalamic nuclei such as the preoptic area (POA), dorsomedial hypothalamus (DMH), and paraventricular nucleus (PVH), which integrate thermal, sensory, and hormonal cues to regulate sympathetic premotor neurons in the rostral raphe pallidus (rRPa) and intermediolateral spinal nucleus (IML) [100–102]. Activation of this axis triggers norepinephrine release onto β3-adrenergic receptors, driving lipolysis and mitochondrial uncoupling via UCP1, although alternative pathways, such as calcium cycling, creatine phosphate cycling, and futile lipid turnover, enable thermogenesis independently of UCP1 [103]. The DMH–rRPa pathway also mediates stress-induced thermogenesis [104], whereas hypothalamic orexigenic (NPY/AgRP) and anorexigenic (POMC, orexin, and MCH) neurons contribute to diet- and hormone-dependent regulation [105]. AgRP and POMC neurons in the arcuate nucleus (ARC) are particularly sensitive to energy status, integrating leptin, insulin, nutrient, and neural signals. During fasting, declining leptin and insulin levels reduce the excitability of ARC, DMH, and PVH neurons, transiently suppressing sympathetic outflow and BAT thermogenesis [106,107]. Leptin administration reverses this inhibition, confirming its role as a permissive signal. Hypothalamic nutrient sensors, including AMPK, mTOR, and SIRT1, detect shifts in cellular energy status and modulate sympathetic drive to BAT [108,109], linking energy deprivation to adaptive reductions in thermogenesis. Zagmutt et al. demonstrated that loss of CPT1A in AgRP neurons enhances BAT activation and reduces weight gain, with a stronger effect in females, highlighting the importance of central energetic status in thermogenic regulation [21].

IF imposes repeated cycles of energy deprivation and refeeding, which likely amplify these adaptive mechanisms. Repeated fasting–refeeding may increase the sensitivity of AgRP and POMC neurons to hormonal cues, promoting the efficient suppression of BAT thermogenesis during fasting and rapid rebound upon refeeding. Peripheral signals, including BMP8 [110], estrogens [111], GLP-1 [112], and fatty acids [113], further refine these responses. Together, these observations suggest that IF leverages hypothalamic energy-sensing circuits to dynamically recalibrate thermogenesis, optimizing energy expenditure and metabolic flexibility under oscillating nutrient states (Figure 2).

Beyond efferent control, BAT also communicates back to the CNS via batokines, including FGF21, IL-6, and IGF-1, as well as extracellular vesicles carrying microRNAs and lipids [114–116]. These signals influence appetite, glucose metabolism, and autonomic tone, highlighting BAT as an active participant in systemic energy sensing rather than a mere effector (Figure 2).

IF, cold exposure, and exercise converge on transcriptional programs involving UCP1, PGC-1α, PRDM16, and SIRT3, promoting mitochondrial biogenesis and oxidative metabolism in brown and beige adipocytes [84,117,118]. Yet, how the CNS integrates these peripheral adaptations to coordinate whole-body energy expenditure remains unclear, and repeated fasting–refeeding cycles may induce long-term "metabolic memory" by altering hypothalamic sensitivity to leptin, insulin, or incretins. At the same time, the role of BAT in mediating fasting-induced thermogenesis remains controversial. Studies using ADF models have shown that this intervention selectively induces beigeing of inguinal white adipose tissue (iWAT) rather than activation of BAT, with suppressed UCP1 expression and no upregulation of other classical thermogenic genes [83].

Complementing these observations, proteomic analyses revealed that ADF increases mitochondrial protein content in both subcutaneous (scWAT) and visceral white adipose tissue (vWAT), whereas BAT remains largely unaffected. In parallel, fatty acid synthesis enzymes are upregulated in WAT depots, but lipolysis is specifically downregulated in vWAT due to decreased ADRB3 abundance, which helps preserve visceral lipid stores during fast-

ing. Additionally, downregulation of inflammatory collagen IV in vWAT improves insulin sensitivity. Together, these findings indicate that intermittent fasting triggers depot-specific adaptations, favoring beige adipocyte recruitment and mitochondrial remodeling in WAT while classical BAT activation may be limited [119].

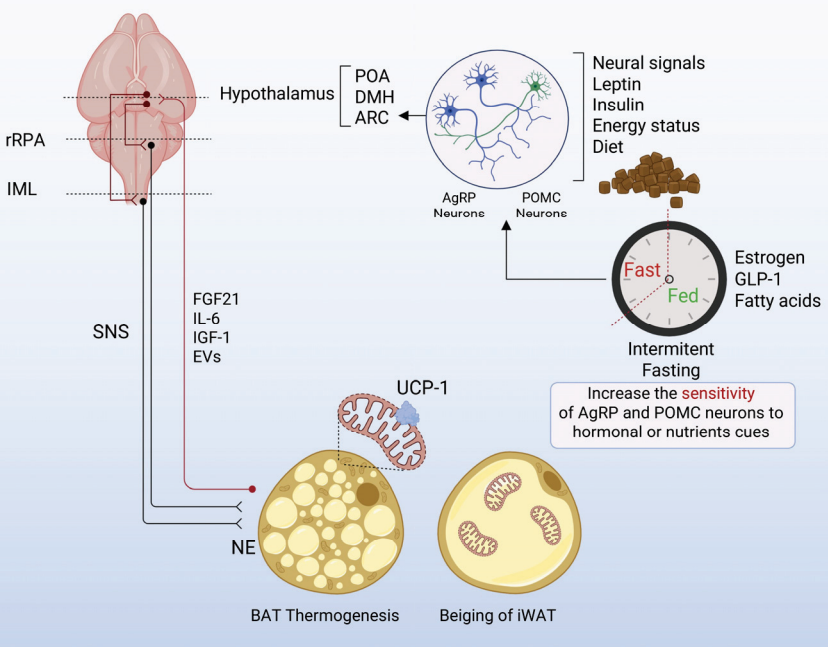


Figure 2. Central and peripheral mechanisms by which intermittent fasting (IF) modulates brown adipose tissue (BAT) thermogenesis. Intermittent fasting imposes recurrent cycles of fasting and refeeding, which alter the activity and hormonal sensitivity of hypothalamic circuits involved in energy balance. During fasting, reduced leptin and insulin levels suppress thermogenic output by decreasing ARC POMC neuron activity and enhancing AgRP neuronal signaling, ultimately lowering sympathetic drive to BAT. Refeeding restores hormonal cues and increases the responsiveness of these neurons, enabling rapid reinstatement of BAT thermogenesis. IF is proposed to heighten the sensitivity of AgRP and POMC neurons to metabolic and nutrient-derived signals, facilitating dynamic switching between low- and high-thermogenic states. Peripheral adaptations include shifts in lipid handling and mitochondrial remodeling in adipose depots, with potential recruitment of beige adipocytes. Together, these CNS–adipose interactions position IF as a modulator of thermogenesis and metabolic flexibility in adipose tissue. Figure was created using BioRender.com. Abbreviations: ARC, arcuate nucleus; AgRP, agouti-related peptide; POMC, proopiomelanocortin; POA, preoptic area; DMH, dorsomedial hypothalamus; BAT, brown adipose tissue; WAT, white adipose tissue; IF, intermittent fasting; NE, norepinephrine; UCP1, uncoupling protein 1; SNS, sympathetic nervous system.

In contrast, other IF protocols, such as TRE (16/8 regimen), appear to directly engage BAT thermogenesis. Mice subjected to 16/8 IF showed improved glucose homeostasis, reduced body weight, food intake, and overall adiposity, along with increased postprandial oxygen consumption (VO_2), heat production, BAT temperature, and ketone bodies. Crucially, postprandial thermogenesis is abolished after BAT denervation or Ucp1 deletion, and most metabolic effects are absent in leptin-deficient ob/ob mice, indicating that BAT activation under 16/8 IF is UCP1- and leptin-dependent. Additionally, 16/8 IF enhances leptin sensitivity, further linking hypothalamic energy-sensing circuits to BAT-mediated energy expenditure [120].

These observations collectively suggest that intermittent fasting triggers protocol-specific thermogenic adaptations: some regimens, like ADF, preferentially recruit beige adipocytes and β-adrenergic-independent pathways, whereas others, like TRE protocol,

robustly activate classical BAT via UCP1 and leptin signaling (Figure 2). This highlights the complexity of CNS–adipose communication and underscores the need for further studies to delineate how different IF strategies modulate energy balance under oscillating nutrient states.

Despite recent advances, major questions remain. It is unclear whether IF sensitizes or desensitizes hypothalamic neurons to metabolic hormones, how batokines influence central networks, and the contribution of UCP1-independent thermogenesis across species. Integration with microbiome-derived metabolites, exosomal communication, and hypothalamic inflammatory signaling also warrants investigation. Translating these findings to humans, particularly regarding melanocortin and catecholaminergic regulation of BAT, remains challenging. Future studies should use temporally resolved experimental designs, combining optogenetic or chemogenetic manipulation of fasting-responsive neurons with measures of sympathetic activity, UCP1 expression, and circulating batokines. Integrative omics, including ATAC-seq and single-cell RNA-seq in hypothalamic nuclei, will be essential to reveal transcriptional and epigenetic mechanisms of adaptive thermogenesis. In humans, multimodal PET-CT imaging with hormonal and inflammatory profiling may help translate these insights clinically.

From a therapeutic perspective, targeting the brain–BAT axis represents an attractive strategy to combat obesity. However, metabolic adaptation during caloric restriction often limits thermogenic output. Combining IF with GLP-1 receptor agonists, β3-adrenergic sensitizers, or batokine-based therapies could enhance BAT recruitment if leptin-permissive signaling is maintained. Understanding CNS–BAT communication during fasting will inform strategies to safely increase energy expenditure and metabolic resilience.

To effectively implement and optimize IF, it is important to recognize that its benefits depend on specific nutritional strategies. The following section discusses and highlights food groups that can support the outcomes of IF practices.

6. Nutritional Strategies During Refeeding

While fasting initiates beneficial metabolic adaptations, the refeeding period plays an equally critical role in determining long-term outcomes. The timing, duration, and composition of meals consumed after fasting can either reinforce or counteract these metabolic benefits, making nutritional strategies during refeeding a central focus for optimizing IF protocols.

6.1. Timing and Duration of Refeeding Windows

The timing and duration of the eating window play a decisive role in sustaining the benefits of the IF-induced metabolic switch. Narrower refeeding periods favor fat oxidation, improve insulin sensitivity, and reduce postprandial spikes in blood glucose levels compared with unrestricted feeding schedules [27,121]. In addition, synchronizing refeeding with circadian rhythms further enhances these effects. Early time-restricted feeding, in which meals are consumed earlier in the day, has been associated with improved glycemic control, blood pressure, and lipid metabolism [57]. In contrast, late-night eating tends to impair glucose tolerance and may counteract fasting-induced benefits [122]. Thus, optimizing the refeeding window not only supports metabolic flexibility but also synergizes with intrinsic circadian regulation of energy metabolism.

6.2. Thermogenic Ingredients and Diet-Induced Thermogenesis in Refeeding Windows

Refeeding after fasting represents a critical window during which dietary choices can either reinforce or counteract fasting-induced adaptations. A key mechanism is diet-induced thermogenesis (DIT), which contributes approximately 8–10% of the total daily

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energy expenditure, in addition to basal metabolic rate, physical activity, and adaptive thermogenesis [123–125]. Because diet-induced thermogenesis varies in magnitude according to macronutrient composition (highest for protein, intermediate for carbohydrate, and lowest for fat), DIT represents a modifiable factor that can be strategically leveraged during refeeding [124].

Protein and Fiber. Proteins have the strongest thermogenic effect, with 20–30% of the ingested energy dissipated as heat. Clinical evidence has shown that combining IF with protein pacing results in greater reductions in total and visceral fat than continuous caloric restriction [126]. Moreover, variations in protein content during fasting-mimicking diets modulate metabolic responses, underscoring the importance of protein quality and timing during refeeding [127]. Dietary fiber further supports this metabolic switch by improving glycemic control and insulin sensitivity [128]. Fermentable fibers generate short-chain fatty acids (SCFAs) that may modestly increase thermogenesis. In overweight adults, combining fiber with probiotic supplementation increased resting energy expenditure by approximately 84 kcal/day [129]. Still, the effect of fiber depends on its type and context, as some high-fiber meals have been shown to reduce postprandial thermogenesis while increasing satiety [130,131].

Bioactive Compounds. Several phytochemicals enhance thermogenesis through sympathetic activation or direct stimulation of BAT. Capsaicin from chili peppers increases fat oxidation and total energy expenditure [132], and additional findings confirm that both capsaicin and grains of paradise, a species of the ginger family, can activate BAT in humans [133]. Green tea catechins and caffeine exert synergistic effects, modestly raising resting energy expenditure and fat oxidation [134].

Lipid Quality. The type of fat consumed during refeeding strongly influences DIT. Medium-chain triglycerides (MCTs) are rapidly oxidized and stimulate ketone production, resulting in a higher thermogenic effect compared with long-chain fats [135]. Polyunsaturated fats, particularly omega-3 fatty acids, also support mitochondrial efficiency and fat oxidation, whereas excessive saturated fat intake is associated with lower thermogenesis and impaired insulin sensitivity [136,137].

Polyphenols and Anthocyanins. Polyphenol-rich foods may complement fasting adaptations by modulating energy expenditure and substrate utilization. In diet-induced obese mice, maqui berry (Aristotelia chilensis), rich in anthocyanins, induced browning of WAT, improved glucose tolerance, and reduced hepatic steatosis [138,139]. These findings suggest that anthocyanin-rich foods could enhance metabolic flexibility when included in refeeding meals.

Foods and Patterns to Limit. Not all foods support fasting-induced benefits. Highly processed and refined foods reduce the energy cost of digestion and blunt satiety [131]. High-glycemic foods produce exaggerated postprandial glucose and insulin responses, thereby suppressing fat oxidation [140,141]. Large, late-night meals misalign feeding with circadian rhythms, lowering glucose tolerance and thermogenesis [122]. Alcohol further contributes to calories with a minimal thermogenic effect and impairs fat oxidation, promoting hepatic fat accumulation [142].

DIT can potentially be enhanced by optimizing refeeding meals, emphasizing protein, fermentable fibers, thermogenic bioactives, and beneficial fats, while limiting refined carbohydrates, saturated fats, late-night eating, and alcohol. These strategies may help sustain the metabolic switch, promote metabolic flexibility, and reinforce the physiological benefits of intermittent fasting.

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7. Discussion

While substantial advancements have been achieved in pharmacological therapies, there has been a growing emphasis in recent years on optimizing dietary patterns through nutritional interventions [29]. Within this framework, IF has emerged as a prominent strategy for modulating caloric intake, with evidence indicating its beneficial effects on weight management, cardiovascular health, oxidative stress, and overall health and lifestyle outcomes [24].

Our results identified three primary IF protocols: alternate-day fasting (ADF), periodic fasting (PF), and time-restricted eating (TRE) [27]. Despite their documented benefits, the practicality of implementing such dietary approaches in daily life remains uncertain. One argument supporting IF is its greater adaptability compared to other restrictive dietary regimens, such as ketogenic, vegan, or daily caloric restriction diets [25]. Importantly, because IF aligns with circadian rhythms, it may represent a more physiologically compatible dietary strategy, potentially offering adaptive advantages over alternative approaches [143]. Tinsley et al. evaluated the feasibility of TRE in mitigating lifestyle-related diseases and found that TRE is not only practicable but also associated with improvements in quality of life [59]. Similarly, Wegman et al. reported that IF demonstrated high adherence rates, further supporting its feasibility [144].

However, it remains unclear whether IF affects appetite regulation. Beaulieu et al. reported that fasting did not result in a compensatory increase in appetite or appetite loss when weight loss did not exceed 5% [145]. In contrast, the Early Time-Restricted Feeding study found that TRE reduced mean ghrelin levels and decreased participants' desire to eat [146]. Furthermore, our findings suggest that the inclusion of specific food groups during the eating window may optimize the outcomes of IF. Such dietary strategies may help sustain the metabolic switch, enhance metabolic flexibility, and reinforce the physiological benefits associated with IF [57]. In this context, diet-induced thermogenesis (DIT) is primarily driven by the intake of proteins, dietary fiber, bioactive compounds, and essential fatty acids, which are strongly recommended for breaking a fast because of their pronounced capacity to stimulate thermogenesis and modulate metabolic adaptations during the fasting state [123,124].

Thermogenesis represents a central molecular mechanism activated during fasting. Brown adipose tissue (BAT) oxidizes lipids in response to sympathetic nervous system activation and hormonal stimuli, thereby generating heat and contributing to whole-body thermogenesis [80]. Our findings indicate that, under appropriate stimuli, white adipose tissue (WAT) depots can undergo a phenotypic transition toward BAT in a process known as 'browning,' resulting in the formation of beige adipose tissue that exhibits characteristics of both depots [79]. In addition, BAT functions as an endocrine organ by releasing signaling molecules that act on multiple peripheral tissues, including WAT, liver, pancreas, heart, and bone, and modulates systemic metabolism through interactions with the central nervous system. These endocrine factors, collectively referred to as 'batokines,' include fibroblast growth factor 21 (FGF21), interleukin-6 (IL-6), and neuregulin 4, which exert beneficial metabolic effects and may represent key targets for therapeutic strategies aimed at controlling obesity and its associated metabolic complications [81]. In particular, the interaction between the central nervous system (CNS) and BAT is of considerable relevance, as targeting the brain–BAT axis represents an attractive strategy for combating obesity [94]. However, metabolic adaptations during caloric restriction often attenuate thermogenic output, underscoring the need for further research in this field.

Despite the promising outcomes of IF, it is not devoid of side effects. Limited evidence has documented adverse effects of IF regimens, including hypoglycemia, dizziness, and weakness, with hypoglycemia appearing to be the most severe [147]. Beshyah et al.

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highlighted a significant risk of hypoglycemia during fasting, particularly in individuals using antidiabetic medications [148]. Accordingly, individuals with diabetes are advised to exercise heightened caution when undertaking IF, as this practice may entail certain risks despite its metabolic benefits. These risks may also extend to individuals with hormonal imbalances, pregnant or breastfeeding women, young children, older adults, and those with immunodeficiencies [64]. Therefore, while IF offers potential benefits, its effects should not be overestimated, and further research is required to confirm its long-term safety in humans.

The limitations of this study include potential publication bias and variability in the quality of evidence across studies. Additionally, most of the clinical trials reviewed involved small sample sizes, and the duration of IF interventions was relatively short, ranging from weeks to months. Further research is warranted regarding molecular mechanisms, ideally focusing on comparative IF regimens in both human and animal models with extended follow-up periods. These studies should also include measurements of batokines and related signaling molecules to directly assess the thermogenic capacity of IF. In the future, large-scale cohort studies are needed to confirm the long-term benefits and potential risks of IF, particularly in individuals with impaired adipose tissue browning or insulin resistance, such as older adults and patients with diabetes. Data generated from these studies could provide deeper insights into the mechanisms underlying the metabolic improvements observed during fasting.

8. Conclusions

IF has emerged as a non-pharmacological nutritional strategy with promising effects on body weight regulation, glucose homeostasis, and insulin sensitivity. Current evidence supports its feasibility and metabolic benefits in individuals with obesity or impaired metabolic health; however, its efficacy is closely linked to the physiological mechanisms engaged during fasting and refeeding cycles.

This review highlights that thermogenesis, particularly BAT activation, beige adipocyte recruitment, and CNS–BAT crosstalk, constitutes a central component of the metabolic response to IF. The integration of autonomic, hormonal, nutrient-derived, and circadian signals appears to be essential for coordinating energy expenditure, although important mechanistic gaps remain, including the contribution of UCP1-independent pathways and fasting-induced adaptations in hypothalamic circuits.

The refeeding window also represents a critical opportunity to enhance fasting-induced benefits. Diet-induced thermogenesis, protein quality and timing, fermentable fibers, thermogenic bioactive compounds, and lipid composition emerge as nutritional tools capable of enhancing metabolic flexibility when strategically incorporated into post-fast meals.

IF provides a biologically robust framework to study energy balance and represents a promising avenue for the development of targeted nutritional interventions. Additional research integrating neurobiology, adipose tissue physiology, chrono-nutrition, and multi-omics approaches will be essential to establish evidence-based guidelines and to translate IF into optimized, personalized metabolic therapies.

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Abbreviations

The following abbreviations are used in this manuscript:

IF	Intermittent Fasting
T2DM	Diabetes Mellitus Type 2
T1DM	Diabetes Mellitus Type 1
DIT	Diet-Induced Thermogenesis
CR	Caloric Restriction
ADF	Alternative Day Fasting
PF	Periodic Fasting
TRE	Time-Restricted Eating
CRP	C Reactive Protein
WAT	White Adipose Tissue
BAT	Brown Adipose Tissue
CNS	Central Nervous System
POA	Preoptic Area
DMH	Dorsomedial Hypothalamus
PVH	Paraventricular Nucleus
ARC	Arcuate Nucleus
UCP1	Uncoupling Protein One

References

1. Sørensen, T.I.A.; Martinez, A.R.; Jørgensen, T.S.H. Epidemiology of Obesity. In *From Obesity to Diabetes. Handbook of Experimental Pharmacology*; Springer: Cham, Switzerland, 2022; Volume 274, pp. 3–27. [CrossRef]
2. Theodorakis, N.; Nikolaou, M. The Human Energy Balance: Uncovering the Hidden Variables of Obesity. *Diseases* **2025**, *13*, 55. [CrossRef]
3. McPake, B. Overweight, Obesity and Diabetes: Global Trends and a Better Future? *Health Syst. Reform* **2025**, *11*, 2518797. [CrossRef]
4. Higgins, V.; Asgari, S.; Hamilton, J.K.; Wolska, A.; Remaley, A.T.; Hartmann, B.; Holst, J.J.; Adeli, K. Postprandial Dyslipidemia, Hyperinsulinemia, and Impaired Gut Peptides/Bile Acids in Adolescents with Obesity. *J. Clin. Endocrinol. Metab.* **2020**, *105*, 1228–1241. [CrossRef]
5. Avgerinos, K.I.; Spyrou, N.; Mantzoros, C.S.; Dalamaga, M. Obesity and Cancer Risk: Emerging Biological Mechanisms and Perspectives. *Metabolism* **2019**, *92*, 121–135. [CrossRef] [PubMed]
6. Yazıcı, D.; Demir, S.Ç.; Sezer, H. Insulin Resistance, Obesity, and Lipotoxicity. *Adv. Exp. Med. Biol.* **2024**, *1460*, 391–430. [CrossRef] [PubMed]
7. Mori, Y.; Terasaki, M.; Hiromura, M.; Saito, T.; Kushima, H.; Koshibu, M.; Osaka, N.; Ohara, M.; Fukui, T.; Ohtaki, H.; et al. Luseogliflozin Attenuates Neointimal Hyperplasia after Wire Injury in High-Fat Diet-Fed Mice via Inhibition of Perivascular Adipose Tissue Remodeling. *Cardiovasc. Diabetol.* **2019**, *18*, 143. [CrossRef] [PubMed]

8. Gonçalves, C.G.; Glade, M.J.; Meguid, M.M. Metabolically Healthy Obese Individuals: Key Protective Factors. *Nutrition* **2016**, *32*, 14–20. [CrossRef]
9. Perdomo, C.M.; Cohen, R.V.; Sumithran, P.; Clément, K.; Frühbeck, G. Contemporary Medical, Device, and Surgical Therapies for Obesity in Adults. *Lancet* **2023**, *401*, 1116–1130. [CrossRef]
10. Chao, A.M.; Quigley, K.M.; Wadden, T.A. Dietary Interventions for Obesity: Clinical and Mechanistic Findings. *J. Clin. Investig.* **2021**, *131*, e140065. [CrossRef] [PubMed]
11. Vasim, I.; Majeed, C.N.; DeBoer, M.D. Intermittent Fasting and Metabolic Health. *Nutrients* **2022**, *14*, 631. [CrossRef]
12. de Cabo, R.; Mattson, M.P. Effects of Intermittent Fasting on Health, Aging, and Disease. *N. Engl. J. Med.* **2019**, *381*, 2541–2551. [CrossRef] [PubMed]
13. Longo, V.D. Intermittent and Periodic Fasting in the Treatment of Obesity and Type 2 Diabetes Mellitus: Fasting Interventions in 2024. *Nat. Rev. Endocrinol.* **2025**, *21*, 73–74. [CrossRef] [PubMed]
14. Mattson, M.P.; Longo, V.D.; Harvie, M. Impact of Intermittent Fasting on Health and Disease Processes. *Ageing Res. Rev.* **2017**, *39*, 46–58. [CrossRef] [PubMed]
15. Longo, V.D.; Mattson, M.P. Fasting: Molecular Mechanisms and Clinical Applications. *Cell Metab.* **2014**, *19*, 181–192. [CrossRef]
16. Paoli, A.; Tinsley, G.; Bianco, A.; Moro, T. The Influence of Meal Frequency and Timing on Health in Humans: The Role of Fasting. *Nutrients* **2019**, *11*, 719. [CrossRef]
17. Tinsley, G.M.; La Bounty, P.M. Effects of Intermittent Fasting on Body Composition and Clinical Health Markers in Humans. *Nutr. Rev.* **2015**, *73*, 661–674. [CrossRef]
18. Stekovic, S.; Hofer, S.J.; Tripolt, N.; Aon, M.A.; Royer, P.; Pein, L.; Stadler, J.T.; Pendl, T.; Prietl, B.; Url, J.; et al. Alternate Day Fasting Improves Physiological and Molecular Markers of Aging in Healthy, Non-Obese Humans. *Cell Metab.* **2019**, *30*, 462–476.e5. [CrossRef]
19. Fernández, M.R.; Vilca, C.C.; Batista, L.O.; Figueiredo, L.S.; Ribeiro, R.A.; Do Carmo, M.D.G.T.; Albuquerque, K.T. Fasting and Refeeding Cycles Alter Subcutaneous White Depot Growth Dynamics and the Morphology of Brown Adipose Tissue in Female Rats. *Br. J. Nutr.* **2021**, *126*, 460–469. [CrossRef]
20. Varghese, M.; Griffin, C.; Abrishami, S.; Eter, L.; Lanzetta, N.; Hak, L.; Clemente, J.; Agarwal, D.; Lerner, A.; Westerhoff, M.; et al. Sex Hormones Regulate Metainflammation in Diet-Induced Obesity in Mice. *J. Biol. Chem.* **2021**, *297*, 101229. [CrossRef]
21. Zagmutt, S.; Mera, P.; González-García, I.; Ibeas, K.; del Mar Romero, M.; Obri, A.; Martin, B.; Esteve-Codina, A.; Soler-Vázquez, M.C.; Bastias-Pérez, M.; et al. CPT1A in AgRP Neurons Is Required for Sex-Dependent Regulation of Feeding and Thirst. *Biol. Sex Differ.* **2023**, *14*, 14. [CrossRef]
22. Madeo, F.; Carmona-Gutierrez, D.; Hofer, S.J.; Kroemer, G. Caloric Restriction Mimetics against Age-Associated Disease: Targets, Mechanisms, and Therapeutic Potential. *Cell Metab.* **2019**, *29*, 592–610. [CrossRef] [PubMed]
23. Sainsbury, A.; Wood, R.E.; Seimon, R.V.; Hills, A.P.; King, N.A.; Gibson, A.A.; Byrne, N.M. Rationale for Novel Intermittent Dieting Strategies to Attenuate Adaptive Responses to Energy Restriction. *Obes. Rev.* **2018**, *19*, 47–60. [CrossRef]
24. Zang, B.Y.; He, L.X.; Xue, L. Intermittent Fasting: Potential Bridge of Obesity and Diabetes to Health? *Nutrients* **2022**, *14*, 981. [CrossRef]
25. Mishra, A.; Sobha, D.; Patel, D.; Suresh, P.S. Intermittent Fasting in Health and Disease. *Arch. Physiol. Biochem.* **2024**, *130*, 755–767. [CrossRef]
26. Varady, K.A.; Cienfuegos, S.; Ezpeleta, M.; Gabel, K. Cardiometabolic Benefits of Intermittent Fasting. *Annu. Rev. Nutr.* **2021**, *41*, 333–361. [CrossRef] [PubMed]
27. Patterson, R.E.; Sears, D.D. Metabolic Effects of Intermittent Fasting. *Annu. Rev. Nutr.* **2017**, *37*, 371–393. [CrossRef] [PubMed]
28. Varady, K.A.; Cienfuegos, S.; Ezpeleta, M.; Gabel, K. Clinical Application of Intermittent Fasting for Weight Loss: Progress and Future Directions. *Nat. Rev. Endocrinol.* **2022**, *18*, 309–321. [CrossRef] [PubMed]
29. Brogi, S.; Tabanelli, R.; Puca, S.; Calderone, V. Intermittent Fasting: Myths, Fakes and Truth on This Dietary Regimen Approach. *Foods* **2024**, *13*, 1960. [CrossRef] [PubMed]
30. Zhu, S.; Surampudi, P.; Rosharavan, B.; Chondronikola, M. Intermittent Fasting as a Nutrition Approach against Obesity and Metabolic Disease. *Curr. Opin. Clin. Nutr. Metab. Care* **2020**, *23*, 387–394. [CrossRef] [PubMed]
31. Khalafi, M.; Maleki, A.H.; Ehsanifar, M.; Symonds, M.E.; Rosenkranz, S.K. Longer-Term Effects of Intermittent Fasting on Body Composition and Cardiometabolic Health in Adults with Overweight and Obesity: A Systematic Review and Meta-Analysis. *Obes. Rev.* **2025**, *26*, e13855. [CrossRef]
32. Kim, R.Y.; Lee, J.H.; Oh, Y.; Sung, H.K.; Kim, K.H. Assessment of the Metabolic Effects of Isocaloric 2:1 Intermittent Fasting in Mice. *J. Vis. Exp.* **2019**, *153*, e60174. [CrossRef]
33. Olsen, M.K.; Choi, M.H.; Kulseng, B.; Zhao, C.M.; Chen, D. Time-Restricted Feeding on Weekdays Restricts Weight Gain: A Study Using Rat Models of High-Fat Diet-Induced Obesity. *Physiol. Behav.* **2017**, *173*, 298–304. [CrossRef]

34. Catenacci, V.A.; Pan, Z.; Ostendorf, D.; Brannon, S.; Gozansky, W.S.; Mattson, M.P.; Martin, B.; MacLean, P.S.; Melanson, E.L.; Troy Donahoo, W. A Randomized Pilot Study Comparing Zero-Calorie Alternate-Day Fasting to Daily Caloric Restriction in Adults with Obesity. *Obesity* **2016**, *24*, 1874–1883. [CrossRef]
35. Trepanowski, J.F.; Kroeger, C.M.; Barnosky, A.; Klempel, M.C.; Bhutani, S.; Hoddy, K.K.; Gabel, K.; Freels, S.; Rigdon, J.; Rood, J.; et al. Effect of Alternate-Day Fasting on Weight Loss, Weight Maintenance, and Cardioprotection among Metabolically Healthy Obese Adults: A Randomized Clinical Trial. *JAMA Intern. Med.* **2017**, *177*, 930–938. [CrossRef]
36. Schübel, R.; Nattenmüller, J.; Sookthai, D.; Nonnenmacher, T.; Graf, M.E.; Riedl, L.; Schlett, C.L.; Von Stackelberg, O.; Johnson, T.; Nabers, D.; et al. Effects of Intermittent and Continuous Calorie Restriction on Body Weight and Metabolism over 50 Wk: A Randomized Controlled Trial. *Am. J. Clin. Nutr.* **2018**, *108*, 933–945. [CrossRef] [PubMed]
37. Carter, S.; Clifton, P.M.; Keogh, J.B. Effect of Intermittent Compared with Continuous Energy Restricted Diet on Glycemic Control in Patients With Type 2 Diabetes: A Randomized Noninferiority Trial. *JAMA Netw. Open* **2018**, *1*, e180756. [CrossRef] [PubMed]
38. Cienfuegos, S.; Gabel, K.; Kalam, F.; Ezpeleta, M.; Wiseman, E.; Pavlou, V.; Lin, S.; Oliveira, M.L.; Varady, K.A. Effects of 4- and 6-h Time-Restricted Feeding on Weight and Cardiometabolic Health: A Randomized Controlled Trial in Adults with Obesity. *Cell Metab.* **2020**, *32*, 366–378.e3. [CrossRef] [PubMed]
39. Chow, L.S.; Manoogian, E.N.C.; Alvear, A.; Fleischer, J.G.; Thor, H.; Dietsche, K.; Wang, Q.; Hodges, J.S.; Esch, N.; Malaeb, S.; et al. Time-Restricted Eating Effects on Body Composition and Metabolic Measures in Humans Who Are Overweight: A Feasibility Study. *Obesity* **2020**, *28*, 860–869. [CrossRef] [PubMed]
40. Parvaresh, A.; Razavi, R.; Abbasi, B.; Yaghoobloo, K.; Hassanzadeh, A.; Mohammadifard, N.; Safavi, S.M.; Hadi, A.; Clark, C.C.T. Modified Alternate-Day Fasting vs. Calorie Restriction in the Treatment of Patients with Metabolic Syndrome: A Randomized Clinical Trial. *Complement. Ther. Med.* **2019**, *47*, 102187. [CrossRef]
41. Bhutani, S.; Klempel, M.C.; Kroeger, C.M.; Trepanowski, J.F.; Varady, K.A. Alternate Day Fasting and Endurance Exercise Combine to Reduce Body Weight and Favorably Alter Plasma Lipids in Obese Humans. *Obesity* **2013**, *21*, 1370–1379. [CrossRef]
42. Fitzgerald, K.C.; Vizthum, D.; Henry-Barron, B.; Schweitzer, A.; Cassard, S.D.; Kossoff, E.; Hartman, A.L.; Kapogiannis, D.; Sullivan, P.; Baer, D.J.; et al. Effect of Intermittent vs. Daily Calorie Restriction on Changes in Weight and Patient-Reported Outcomes in People with Multiple Sclerosis. *Mult. Scler. Relat. Disord.* **2018**, *23*, 33–39. [CrossRef]
43. Sundfør, T.M.; Svendsen, M.; Tonstad, S. Effect of Intermittent versus Continuous Energy Restriction on Weight Loss, Maintenance and Cardiometabolic Risk: A Randomized 1-Year Trial. *Nutr. Metab. Cardiovasc. Dis.* **2018**, *28*, 698–706. [CrossRef] [PubMed]
44. Overland, J.; Toth, K.; Gibson, A.A.; Sainsbury, A.; Franklin, J.; Gauld, A.; Wong, J. The Safety and Efficacy of Weight Loss via Intermittent Fasting or Standard Daily Energy Restriction in Adults with Type 1 Diabetes and Overweight or Obesity: A Pilot Study. *Obes. Med.* **2018**, *12*, 13–17. [CrossRef]
45. Gabel, K.; Hoddy, K.K.; Haggerty, N.; Song, J.; Kroeger, C.M.; Trepanowski, J.F.; Panda, S.; Varady, K.A. Effects of 8-Hour Time Restricted Feeding on Body Weight and Metabolic Disease Risk Factors in Obese Adults: A Pilot Study. *Nutr. Healthy Aging* **2018**, *4*, 345–353. [CrossRef]
46. Cho, A.R.; Moon, J.Y.; Kim, S.; An, K.Y.; Oh, M.; Jeon, J.Y.; Jung, D.H.; Choi, M.H.; Lee, J.W. Effects of Alternate Day Fasting and Exercise on Cholesterol Metabolism in Overweight or Obese Adults: A Pilot Randomized Controlled Trial. *Metabolism* **2019**, *93*, 52–60. [CrossRef]
47. Bhutani, S.; Klempel, M.C.; Berger, R.A.; Varady, K.A. Improvements in Coronary Heart Disease Risk Indicators by Alternate-Day Fasting Involve Adipose Tissue Modulations. *Obesity* **2010**, *18*, 2152–2159. [CrossRef]
48. Harvie, M.; Wright, C.; Pegington, M.; McMullan, D.; Mitchell, E.; Martin, B.; Cutler, R.G.; Evans, G.; Whiteside, S.; Maudsley, S.; et al. The Effect of Intermittent Energy and Carbohydrate Restriction *v.* Daily Energy Restriction on Weight Loss and Metabolic Disease Risk Markers in Overweight Women. *Br. J. Nutr.* **2013**, *110*, 1534–1547. [CrossRef] [PubMed]
49. Harvie, M.N.; Pegington, M.; Mattson, M.P.; Frystyk, J.; Dillon, B.; Evans, G.; Cuzick, J.; Jebb, S.A.; Martin, B.; Cutler, R.G.; et al. The Effects of Intermittent or Continuous Energy Restriction on Weight Loss and Metabolic Disease Risk Markers: A Randomized Trial in Young Overweight Women. *Int. J. Obes.* **2011**, *35*, 714–727. [CrossRef] [PubMed]
50. Gabel, K.; Kroeger, C.M.; Trepanowski, J.F.; Hoddy, K.K.; Cienfuegos, S.; Kalam, F.; Varady, K.A. Differential Effects of Alternate-Day Fasting Versus Daily Calorie Restriction on Insulin Resistance. *Obesity* **2019**, *27*, 1443–1450. [CrossRef]
51. Willoughby, D.; Hewlings, S.; Kalman, D. Body Composition Changes in Weight Loss: Strategies and Supplementation for Maintaining Lean Body Mass, a Brief Review. *Nutrients* **2018**, *10*, 1876. [CrossRef]
52. Heymsfield, S.B.; Gonzalez, M.C.C.; Shen, W.; Redman, L.; Thomas, D. Weight Loss Composition Is One-Fourth Fat-Free Mass: A Critical Review and Critique of This Widely Cited Rule. *Obes. Rev.* **2014**, *15*, 310–321. [CrossRef]
53. Pownall, H.J.; Bray, G.A.; Wagenknecht, L.E.; Walkup, M.P.; Heshka, S.; Hubbard, V.S.; Hill, J.; Kahn, S.E.; Nathan, D.M.; Schwartz, A.V.; et al. Changes in Body Composition over 8 Years in a Randomized Trial of a Lifestyle Intervention: The Look AHEAD Study. *Obesity* **2015**, *23*, 565–572. [CrossRef]

54. Ravussin, E.; Redman, L.M.; Rochon, J.; Das, S.K.; Fontana, L.; Kraus, W.E.; Romashkan, S.; Williamson, D.A.; Meydani, S.N.; Villareal, D.T.; et al. A 2-Year Randomized Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity. *J. Gerontol.-Ser. A Biol. Sci. Med. Sci.* **2015**, *70*, 1097–1104. [CrossRef] [PubMed]
55. Moro, T.; Tinsley, G.; Bianco, A.; Marcolin, G.; Pacelli, Q.F.; Battaglia, G.; Palma, A.; Gentil, P.; Neri, M.; Paoli, A. Effects of Eight Weeks of Time-Restricted Feeding (16/8) on Basal Metabolism, Maximal Strength, Body Composition, Inflammation, and Cardiovascular Risk Factors in Resistance-Trained Males. *J. Transl. Med.* **2016**, *14*, 290. [CrossRef]
56. Khalafi, M.; Symonds, M.E.; Maleki, A.H.; Sakhaei, M.H.; Ehsanifar, M.; Rosenkranz, S.K. Combined versus Independent Effects of Exercise Training and Intermittent Fasting on Body Composition and Cardiometabolic Health in Adults: A Systematic Review and Meta-Analysis. *Nutr. J.* **2024**, *23*, 7. [CrossRef]
57. Sutton, E.F.; Beyl, R.; Early, K.S.; Cefalu, W.T.; Ravussin, E.; Peterson, C.M. Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes. *Cell Metab.* **2018**, *27*, 1212–1221.e3. [CrossRef]
58. De Toledo, F.W.; Grundler, F.; Bergouignan, A.; Drinda, S.; Michalsen, A. Safety, Health Improvement and Well-Being during a 4 to 21-Day Fasting Period in an Observational Study Including 1422 Subjects. *PLoS ONE* **2019**, *14*, e0209353. [CrossRef]
59. Tinsley, G.M.; Forsse, J.S.; Butler, N.K.; Paoli, A.; Bane, A.A.; La Bounty, P.M.; Morgan, G.B.; Grandjean, P.W. Time-Restricted Feeding in Young Men Performing Resistance Training: A Randomized Controlled Trial. *Eur. J. Sport Sci.* **2017**, *17*, 200–207. [CrossRef] [PubMed]
60. Tinsley, G.M.; Moore, M.L.; Graybeal, A.J.; Paoli, A.; Kim, Y.; Gonzales, J.U.; Harry, J.R.; Vandusseldorp, T.A.; Kennedy, D.N.; Cruz, M.R. Time-Restricted Feeding plus Resistance Training in Active Females: A Randomized Trial. *Am. J. Clin. Nutr.* **2019**, *110*, 628–640. [CrossRef]
61. Varady, K.A.; Bhutani, S.; Klempel, M.C.; Kroeger, C.M.; Trepanowski, J.F.; Haus, J.M.; Hoddy, K.K.; Calvo, Y. Alternate Day Fasting for Weight Loss in Normal Weight and Overweight Subjects: A Randomized Controlled Trial. *Nutr. J.* **2013**, *12*, 146. [CrossRef]
62. Lowe, D.A.; Wu, N.; Rohdin-Bibby, L.; Moore, A.H.; Kelly, N.; Liu, Y.E.; Philip, E.; Vittinghoff, E.; Heymsfield, S.B.; Olgin, J.E.; et al. Effects of Time-Restricted Eating on Weight Loss and Other Metabolic Parameters in Women and Men with Overweight and Obesity: The TREAT Randomized Clinical Trial. *JAMA Intern. Med.* **2020**, *180*, 1491–1499. [CrossRef]
63. Guo, Y.; Lu, L.; Chen, L.; Wang, Y.; Zhao, F.; Jiang, Y. Effectiveness and Safety of Intermittent Fasting on Blood Pressure in Adults with Overweight or Obesity: A Systematic Review. *Int. J. Obes.* **2025**, *49*, 1240–1251. [CrossRef] [PubMed]
64. Malinowski, B.; Zalewska, K.; Węsierska, A.; Sokołowska, M.M.; Socha, M.; Liczner, G.; Pawlak-Osińska, K.; Wiciński, M. Intermittent Fasting in Cardiovascular Disorders—An Overview. *Nutrients* **2019**, *11*, 673. [CrossRef]
65. Andersson, B.; Wallin, G.; Hedner, T.; Ahlberg, A.-C.; Andersson, O.K. Acute Effects of Short-term Fasting on Blood Pressure, Circulating Noradrenaline and Efferent Sympathetic Nerve Activity. *Acta Med. Scand.* **1988**, *223*, 485–490. [CrossRef] [PubMed]
66. Diab, R.; Dimachkie, L.; Zein, O.; Dakroub, A.; Eid, A.H. Intermittent Fasting Regulates Metabolic Homeostasis and Improves Cardiovascular Health. *Cell Biochem. Biophys.* **2024**, *82*, 1583–1597. [CrossRef]
67. Zhang, S.; Sun, B.; Sun, L.; Zou, S.; Chen, Q. Effect of Intermittent Fasting on Obesity and Metabolic Indices in Patients with Metabolic Syndrome: A Systematic Review and Meta Analysis. *BMC Endocr. Disord.* **2025**, *25*, 130. [CrossRef]
68. Antoni, R.; Robertson, T.M.; Robertson, M.D.; Johnston, J.D. A Pilot Feasibility Study Exploring the Effects of a Moderate Time-Restricted Feeding Intervention on Energy Intake, Adiposity and Metabolic Physiology in Free-Living Human Subjects. *J. Nutr. Sci.* **2018**, *7*, e22. [CrossRef]
69. Wilkinson, M.J.; Manoogian, E.N.C.; Zadourian, A.; Lo, H.; Fakhouri, S.; Shoghi, A.; Wang, X.; Fleischer, J.G.; Navlakha, S.; Panda, S.; et al. Ten-Hour Time-Restricted Eating Reduces Weight, Blood Pressure, and Atherogenic Lipids in Patients with Metabolic Syndrome. *Cell Metab.* **2020**, *31*, 92–104.e5. [CrossRef]
70. Hutchison, A.T.; Regmi, P.; Manoogian, E.N.C.; Fleischer, J.G.; Wittert, G.A.; Panda, S.; Heilbronn, L.K. Time-Restricted Feeding Improves Glucose Tolerance in Men at Risk for Type 2 Diabetes: A Randomized Crossover Trial. *Obesity* **2019**, *27*, 724–732. [CrossRef]
71. Rolland, C.; Broom, I. The Effects of Very-Low-Calorie Diets on HDL: A Review. *Cholesterol* **2011**, *2011*, 306278. [CrossRef] [PubMed]
72. Freckmann, G.; Hagenlocher, S.; Baumstark, A.; Jendrike, N.; Gillen, R.C.; Rössner, K.; Haug, C. Continuous Glucose Profiles in Healthy Subjects under Everyday Life Conditions and after Different Meals. *J. Diabetes Sci. Technol.* **2007**, *1*, 695–703. [CrossRef]
73. Lakhani, H.A.; Biswas, D.; Kuruvila, M.; Chava, M.S.; Raj, K.; Varghese, J.T.; Swathi, N.L. Intermittent Fasting versus Continuous Caloric Restriction for Glycemic Control and Weight Loss in Type 2 Diabetes: A Traditional Review. *Prim. Care Diabetes* **2025**, *19*, 203–213. [CrossRef]
74. Zeng, J.; Beck, M.; Barouti, A.A.; Löfvenborg, J.E.; Carlsson, S.; Lampousi, A.M. Effects of Different Dietary Patterns on Glucose Management in Type 1 Diabetes: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *eClinicalMedicine* **2025**, *83*, 103222. [CrossRef] [PubMed]

75. Aksungar, F.B.; Topkaya, A.E.; Akyildiz, M. Interleukin-6, C-Reactive Protein and Biochemical Parameters during Prolonged Intermittent Fasting. *Ann. Nutr. Metab.* **2007**, *51*, 88–95. [CrossRef]
76. Ensminger, D.C.; Salvador-Pascual, A.; Arango, B.G.; Allen, K.N.; Vázquez-Medina, J.P. Fasting Ameliorates Oxidative Stress: A Review of Physiological Strategies across Life History Events in Wild Vertebrates. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2021**, *256*, 110929. [CrossRef] [PubMed]
77. Sharsher, S.I.; Ahmed, A.I.; Metwally, M.; Arisha, A.H.; Ahmed, K.E.-D. Intermittent Fasting Decreases Oxidative Stress Parameters and Increases Total Antioxidant Capacity. *Biointerface Res. Appl. Chem.* **2022**, *12*, 6763–6775. [CrossRef]
78. Johnson, J.B.; Summer, W.; Cutler, R.G.; Martin, B.; Hyun, D.H.; Dixit, V.D.; Pearson, M.; Nassar, M.; Tellejohan, R.; Maudsley, S.; et al. Alternate Day Calorie Restriction Improves Clinical Findings and Reduces Markers of Oxidative Stress and Inflammation in Overweight Adults with Moderate Asthma. *Free Radic. Biol. Med.* **2007**, *42*, 665–674. [CrossRef]
79. Chait, A.; den Hartigh, L.J. Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. *Front. Cardiovasc. Med.* **2020**, *7*, 22. [CrossRef]
80. Ghesmati, Z.; Rashid, M.; Fayezi, S.; Gieseler, F.; Alizadeh, E.; Darabi, M. An Update on the Secretory Functions of Brown, White, and Beige Adipose Tissue: Towards Therapeutic Applications. *Rev. Endocr. Metab. Disord.* **2024**, *25*, 279–308. [CrossRef]
81. Ahmad, B.; Vohra, M.S.; Saleemi, M.A.; Serpell, C.J.; Fong, I.L.; Wong, E.H. Brown/Beige Adipose Tissues and the Emerging Role of Their Secretory Factors in Improving Metabolic Health: The Batokines. *Biochimie* **2021**, *184*, 26–39. [CrossRef]
82. Villarroya, J.; Cereijo, R.; Gavald-Navarro, A.; Peyrou, M.; Giralt, M.; Villarroya, F. New Insights into the Secretory Functions of Brown Adipose Tissue. *J. Endocrinol.* **2019**, *243*, R19–R27. [CrossRef]
83. Li, G.; Xie, C.; Lu, S.; Nichols, R.G.; Tian, Y.; Li, L.; Patel, D.; Ma, Y.; Brocker, C.N.; Yan, T.; et al. Intermittent Fasting Promotes White Adipose Browning and Decreases Obesity by Shaping the Gut Microbiota. *Cell Metab.* **2017**, *26*, 672–685.e4, https://doi.org/10.1016/j.cmet.2017.08.019. Erratum in *Cell Metab.***2017**, *26*, 801. [CrossRef] [PubMed]
84. Kim, K.H.; Kim, Y.H.; Son, J.E.; Lee, J.H.; Kim, S.; Choe, M.S.; Moon, J.H.; Zhong, J.; Fu, K.; Lenglin, F.; et al. Intermittent Fasting Promotes Adipose Thermogenesis and Metabolic Homeostasis via VEGF-Mediated Alternative Activation of Macrophage. *Cell Res.* **2017**, *27*, 1309–1326. [CrossRef]
85. Fabbiano, S.; Suárez-Zamorano, N.; Rigo, D.; Veyrat-Durebex, C.; Stevanovic Dokic, A.; Colin, D.J.; Trajkovski, M. Caloric Restriction Leads to Browning of White Adipose Tissue through Type 2 Immune Signaling. *Cell Metab.* **2016**, *24*, 434–446. [CrossRef] [PubMed]
86. Sidossis, L.S.; Porter, C.; Saraf, M.K.; Børsheim, E.; Radhakrishnan, R.S.; Chao, T.; Ali, A.; Chondronikola, M.; Mlcak, R.; Finnerty, C.C.; et al. Browning of Subcutaneous White Adipose Tissue in Humans after Severe Adrenergic Stress. *Cell Metab.* **2015**, *22*, 219–227. [CrossRef]
87. Rachid, T.L.; Penna-de-Carvalho, A.; Bringhenti, I.; Aguila, M.B.; Mandarim-de-Lacerda, C.A.; Souza-Mello, V. Fenofibrate (PPAR*alpha* Agonist) Induces Beige Cell Formation in Subcutaneous White Adipose Tissue from Diet-Induced Male Obese Mice. *Mol. Cell. Endocrinol.* **2015**, *402*, 86–94. [CrossRef]
88. de Oliveira Correia, B.R.; Rachid, T.L.; de Oliveira Glauser, J.S.; Martins, F.F.; Mandarim-de-Lacerda, C.A.; Souza-Mello, V. High Dose of Linagliptin Induces Thermogenic Beige Adipocytes in the Subcutaneous White Adipose Tissue in Diet-Induced Obese C57BL/6 Mice. *Endocrine* **2019**, *65*, 252–262. [CrossRef]
89. Liu, B.; Page, A.J.; Hutchison, A.T.; Wittert, G.A.; Heilbronn, L.K. Intermittent Fasting Increases Energy Expenditure and Promotes Adipose Tissue Browning in Mice. *Nutrition* **2019**, *66*, 38–43. [CrossRef]
90. Martins, F.F.; Bargut, T.C.L.; Aguila, M.B.; Mandarim-de-Lacerda, C.A. Thermogenesis, Fatty Acid Synthesis with Oxidation, and Inflammation in the Brown Adipose Tissue of *Ob/Ob* (−/−) Mice. *Ann. Anat.* **2017**, *210*, 44–51. [CrossRef]
91. Nedergaard, J.; Cannon, B. The Browning of White Adipose Tissue: Some Burning Issues. *Cell Metab.* **2014**, *20*, 396–407. [CrossRef] [PubMed]
92. Gaspar, R.C.; Pauli, J.R.; Shulman, G.I.; Muñoz, V.R. An Update on Brown Adipose Tissue Biology: A Discussion of Recent Findings. *Am. J. Physiol. Endocrinol. Metab.* **2021**, *320*, 488–495. [CrossRef]
93. Dey, P.; Lal, H.; Saha, P.; Ramanathan, A. Molecular Mediators of Cold Adaptation in Mammalian Cells. *Commun. Biol.* **2025**, *8*, 1441. [CrossRef]
94. Contreras, C.; Gonzalez, F.; Fernø, J.; Diéguez, C.; Rahmouni, K.; Nogueiras, R.; López, M. The Brain and Brown Fat. *Ann. Med.* **2015**, *47*, 150–168. [CrossRef]
95. Halanobis, A.B.B.I.; Yang, J.H.; Byeon, E.H.; Park, S.W.; Kim, H.J.; Kang, D.; Kim, D.R.; Yang, J.; Kim, W.; Kim, D.H.; et al. Regulation of BAT Thermogenesis via TRPA1-Expressing Hypothalamic POMC Neurons. *Anim. Cells Syst.* **2025**, *29*, 584–597. [CrossRef]
96. Wei, Z.; Cha, J.; Lee, S. Dax1 in AgRP Neurons Regulates Thermogenesis via GR-HDAC3-Mediated CRFR1 Suppression. *Mol. Metab.* **2025**, *102*, 102258. [CrossRef]
97. Huang, X.; Wang, Y.J.; Xiang, Y. Bidirectional Communication between Brain and Visceral White Adipose Tissue: Its Potential Impact on Alzheimer's Disease. *eBioMedicine* **2022**, *84*, 104263. [CrossRef] [PubMed]

98. Yan, C.; Burley, G.; Gao, H.; Shi, Y.C. Emerging Insights into Brown Adipose Tissue Crosstalk With Pancreatic β-Cells in Metabolic Regulation. *Endocrinology* **2025**, *166*, bqaf118. [CrossRef] [PubMed]
99. Bastías-Pérez, M.; Zagmutt, S.; Soler-Vázquez, M.C.; Serra, D.; Mera, P.; Herrero, L. Impact of Adaptive Thermogenesis in Mice on the Treatment of Obesity. *Cells* **2020**, *9*, 316. [CrossRef] [PubMed]
100. Cano, G.; Passerin, A.M.; Schiltz, J.C.; Card, J.P.; Morrison, S.F.; Sved, A.F. Anatomical Substrates for the Central Control of Sympathetic Outflow to Interscapular Adipose Tissue during Cold Exposure. *J. Comp. Neurol.* **2003**, *460*, 303–326. [CrossRef]
101. Lindberg, D.; Chen, P.; Li, C. Conditional Viral Tracing Reveals That Steroidogenic Factor 1-Positive Neurons of the Dorsomedial Subdivision of the Ventromedial Hypothalamus Project to Autonomic Centers of the Hypothalamus and Hindbrain. *J. Comp. Neurol.* **2013**, *521*, 3167–3190. [CrossRef]
102. Asghari Alashti, F.; Goliaei, B. Rethinking Fat Browning: Uncovering New Molecular Insights into the Synergistic Roles of Fasting, Exercise, and Cold Exposure. *Eur. J. Pharmacol.* **2025**, *998*, 177651. [CrossRef]
103. Roesler, A.; Kazak, L. UCP1-Independent Thermogenesis. *Biochem. J.* **2020**, *477*, 709–725. [CrossRef] [PubMed]
104. Zhang, W.; Sunanaga, J.; Takahashi, Y.; Mori, T.; Sakurai, T.; Kanmura, Y.; Kuwaki, T. Orexin Neurons Are Indispensable for Stress-Induced Thermogenesis in Mice. *J. Physiol.* **2010**, *588*, 4117–4129. [CrossRef] [PubMed]
105. Zhang, W.; Bi, S. Hypothalamic Regulation of Brown Adipose Tissue Thermogenesis and Energy Homeostasis. *Front. Endocrinol.* **2015**, *6*, 136. [CrossRef] [PubMed]
106. Caron, A.; Lee, S.; Elmquist, J.K.; Gautron, L. Leptin and Brain-Adipose Crosstalks. *Nat. Rev. Neurosci.* **2018**, *19*, 153–165. [CrossRef]
107. Dodd, G.T.; Decherf, S.; Loh, K.; Simonds, S.E.; Wiede, F.; Balland, E.; Merry, T.L.; Münzberg, H.; Zhang, Z.Y.; Kahn, B.B.; et al. Leptin and Insulin Act on POMC Neurons to Promote the Browning of White Fat. *Cell* **2015**, *160*, 88–104. [CrossRef]
108. van der Vaart, J.I.; Boon, M.R.; Houtkooper, R.H. The Role of AMPK Signaling in Brown Adipose Tissue Activation. *Cells* **2021**, *10*, 1122. [CrossRef]
109. López, M. Hypothalamic AMPK and Energy Balance. *Eur. J. Clin. Investig.* **2018**, *48*, e12996. [CrossRef]
110. Urisarri, A.; González-García, I.; Estévez-Salguero, Á.; Pata, M.P.; Milbank, E.; López, N.; Mandiá, N.; Grijota-Martinez, C.; Salgado, C.A.; Nogueiras, R.; et al. BMP8 and Activated Brown Adipose Tissue in Human Newborns. *Nat. Commun.* **2021**, *12*, 5274. [CrossRef]
111. Martínez De Morentin, P.B.; González-García, I.; Martins, L.; Lage, R.; Fernández-Mallo, D.; Martínez-Sánchez, N.; Ruíz-Pino, F.; Liu, J.; Morgan, D.A.; Pinilla, L.; et al. Estradiol Regulates Brown Adipose Tissue Thermogenesis via Hypothalamic AMPK. *Cell Metab.* **2014**, *20*, 41–53. [CrossRef]
112. López, M.; Diéguez, C.; Nogueiras, R. Hypothalamic GLP-1: The Control of BAT Thermogenesis and Browning of White Fat. *Adipocyte* **2015**, *4*, 141–145. [CrossRef]
113. Fosch, A.; Rodriguez-Garcia, M.; Miralpeix, C.; Zagmutt, S.; Larrañaga, M.; Reguera, A.C.; Garcia-Chica, J.; Herrero, L.; Serra, D.; Casals, N.; et al. Central Regulation of Brown Fat Thermogenesis in Response to Saturated or Unsaturated Long-Chain Fatty Acids. *Int. J. Mol. Sci.* **2023**, *24*, 1697. [CrossRef]
114. Camino, T.; Lago-Baameiro, N.; Sueiro, A.; Bravo, S.B.; Couto, I.; Santos, F.F.; Baltar, J.; Casanueva, F.F.; Pardo, M. Brown Adipose Tissue Sheds Extracellular Vesicles That Carry Potential Biomarkers of Metabolic and Thermogenesis Activity Which Are Affected by High Fat Diet Intervention. *Int. J. Mol. Sci.* **2022**, *23*, 10826. [CrossRef] [PubMed]
115. Ding, Y.; Su, J.; Shan, B.; Fu, X.; Zheng, G.; Wang, J.; Wu, L.; Wang, F.; Chai, X.; Sun, H.; et al. Brown Adipose Tissue-Derived FGF21 Mediates the Cardioprotection of Dexmedetomidine in Myocardial Ischemia/Reperfusion Injury. *Sci. Rep.* **2024**, *14*, 18292. [CrossRef]
116. Kristóf, E.; Klusóczki, Á.; Veress, R.; Shaw, A.; Combi, Z.S.; Varga, K.; Győry, F.; Balajthy, Z.; Bai, P.; Bacso, Z.; et al. Interleukin-6 Released from Differentiating Human Beige Adipocytes Improves Browning. *Exp. Cell Res.* **2019**, *377*, 47–55. [CrossRef] [PubMed]
117. Brychta, R.J.; Chen, K.Y. Cold-Induced Thermogenesis in Humans. *Eur. J. Clin. Nutr.* **2017**, *71*, 345–352. [CrossRef] [PubMed]
118. Zhu, Y.; Qi, Z.; Ding, S. Exercise-Induced Adipose Tissue Thermogenesis and Browning: How to Explain the Conflicting Findings? *Int. J. Mol. Sci.* **2022**, *23*, 13142. [CrossRef]
119. Harney, D.J.; Cielesh, M.; Chu, R.; Cooke, K.C.; James, D.E.; Stöckli, J.; Larance, M. Proteomics Analysis of Adipose Depots after Intermittent Fasting Reveals Visceral Fat Preservation Mechanisms. *Cell Rep.* **2021**, *34*, 108804. [CrossRef]
120. Arruda, A.C.; Santos, R.B.; Freitas-Lima, L.C.; Budu, A.; Perilhão, M.S.; Wasinski, F.; Arthur, G.M.; Guzmán, R.R.; Gomes, G.; Pesquero, J.B.; et al. 16/8 Intermittent Fasting in Mice Protects from Diet-Induced Obesity by Increasing Leptin Sensitivity and Postprandial Thermogenesis. *Acta Physiol.* **2025**, *241*, e70036. [CrossRef]
121. Manoogian, E.N.C.; Panda, S. Circadian Rhythms, Time-Restricted Feeding, and Healthy Aging. *Ageing Res. Rev.* **2017**, *39*, 59–67. [CrossRef]
122. Gu, C.; Brereton, N.; Schweitzer, A.; Cotter, M.; Duan, D.; Børsheim, E.; Wolfe, R.R.; Pham, L.V.; Polotsky, V.Y.; Jun, J.C. Metabolic Effects of Late Dinner in Healthy Volunteers-A Randomized Crossover Clinical Trial. *J. Clin. Endocrinol. Metab.* **2020**, *105*, 2789–2802. [CrossRef] [PubMed]

123. Westerterp, K.R. Diet Induced Thermogenesis. *Nutr. Metab.* **2004**, *1*, 5. [CrossRef]
124. Hall, K.D.; Guo, J. Obesity Energetics: Body Weight Regulation and the Effects of Diet Composition. *Gastroenterology* **2017**, *152*, 1718–1727.e3. [CrossRef]
125. Bastías-Pérez, M.; Serra, D.; Herrero, L. Dietary Options for Rodents in the Study of Obesity. *Nutrients* **2020**, *12*, 3234. [CrossRef]
126. Arciero, P.J.; Poe, M.; Mohr, A.E.; Ives, S.J.; Arciero, A.; Sweazea, K.L.; Gumpricht, E.; Arciero, K.M. Intermittent Fasting and Protein Pacing Are Superior to Caloric Restriction for Weight and Visceral Fat Loss. *Obesity* **2023**, *31*, 139–149. [CrossRef]
127. Burns, L.; Cooper, S.; Sarmad, S.; Funke, G.; Di Mauro, A.; Gaitanos, G.C.; Tsintzas, K. Effects of Fasting-Mimicking Diets with Low and High Protein Content on Cardiometabolic Health and Autophagy: A Randomized, Parallel Group Study. *Clin. Nutr.* **2025**, *52*, 299–312. [CrossRef]
128. Tsitsou, S.; Athanasaki, C.; Dimitriadis, G.; Papakonstantinou, E. Acute Effects of Dietary Fiber in Starchy Foods on Glycemic and Insulinemic Responses: A Systematic Review of Randomized Controlled Crossover Trials. *Nutrients* **2023**, *15*, 2383. [CrossRef]
129. Baba, Y.; Azuma, N.; Saito, Y.; Takahashi, K.; Matsui, R.; Takara, T. Effect of Intake of Bifidobacteria and Dietary Fiber on Resting Energy Expenditure: A Randomized, Placebo-Controlled, Double-Blind, Parallel-Group Comparison Study. *Nutrients* **2024**, *16*, 2345. [CrossRef]
130. Raben, A.; Christensen, N.J.; Madsen, J.; Holst, J.J.; Astrup, A. Decreased Postprandial Thermogenesis and Fat Oxidation but Increased Fullness after a High-Fiber Meal Compared with a Low-Fiber Meal. *Am. J. Clin. Nutr.* **1994**, *59*, 1386–1394. [CrossRef]
131. Barr, S.B.; Wright, J.C. Postprandial Energy Expenditure in Whole-Food and Processed-Food Meals: Implications for Daily Energy Expenditure. *Food Nutr. Res.* **2010**, *54*, 5144. [CrossRef] [PubMed]
132. Whiting, S.; Derbyshire, E.J.; Tiwari, B. Could Capsaicinoids Help to Support Weight Management? A Systematic Review and Meta-Analysis of Energy Intake Data. *Appetite* **2014**, *73*, 183–188. [CrossRef]
133. Sugita, J.; Yoneshiro, T.; Hatano, T.; Aita, S.; Ikemoto, T.; Uchiwa, H.; Iwanaga, T.; Kameya, T.; Kawai, Y.; Saito, M. Grains of Paradise (*Aframomum melegueta*) Extract Activates Brown Adipose Tissue and Increases Whole-Body Energy Expenditure in Men. *Br. J. Nutr.* **2013**, *110*, 733–738. [CrossRef]
134. Hursel, R.; Viechtbauer, W.; Dulloo, A.G.; Tremblay, A.; Tappy, L.; Rumpler, W.; Westerterp-Plantenga, M.S. The Effects of Catechin Rich Teas and Caffeine on Energy Expenditure and Fat Oxidation: A Meta-Analysis. *Obes. Rev.* **2011**, *12*, e573–e581. [CrossRef]
135. St-Onge, M.P.; Ross, R.; Parsons, W.D.; Jones, P.J.H. Medium-Chain Triglycerides Increase Energy Expenditure and Decrease Adiposity in Overweight Men. *Obes. Res.* **2003**, *11*, 395–402. [CrossRef]
136. Flachs, P.; Rossmeisl, M.; Kopecky, J. The Effect of *n-3* Fatty Acids on Glucose Homeostasis and Insulin Sensitivity. *Physiol. Res.* **2014**, *63*, S93–S118. [CrossRef]
137. Lepretti, M.; Martucciello, S.; Aceves, M.A.B.; Putti, R.; Lionetti, L. Omega-3 Fatty Acids and Insulin Resistance: Focus on the Regulation of Mitochondria and Endoplasmic Reticulum Stress. *Nutrients* **2018**, *10*, 350. [CrossRef] [PubMed]
138. Sandoval, V.; Femenias, A.; Martínez-Garza, Ú.; Sanz-Lamora, H.; Castagnini, J.M.; Quifer-Rada, P.; Lamuela-Raventós, R.M.; Marrero, P.F.; Haro, D.; Relat, J. Lyophilized Maqui (*Aristotelia chilensis*) Berry Induces Browning in the Subcutaneous White Adipose Tissue and Ameliorates the Insulin Resistance in High Fat Diet-Induced Obese Mice. *Antioxidants* **2019**, *8*, 360. [CrossRef] [PubMed]
139. Sandoval, V.; Sanz-Lamora, H.; Marrero, P.F.; Relat, J.; Haro, D. Lyophilized Maqui (*Aristotelia chilensis*) Berry Administration Suppresses High-Fat Diet-Induced Liver Lipogenesis through the Induction of the Nuclear Corepressor SMILE. *Antioxidants* **2021**, *10*, 637. [CrossRef]
140. Ludwig, D.S. The Glycemic Index: Physiological Mechanisms Relating to Obesity, Diabetes, and Cardiovascular Disease. *JAMA* **2002**, *287*, 2414–2423. [CrossRef]
141. Augustin, L.S.A.; Kendall, C.W.C.; Jenkins, D.J.A.; Willett, W.C.; Astrup, A.; Barclay, A.W.; Björck, I.; Brand-Miller, J.C.; Brighenti, F.; Buyken, A.E.; et al. Glycemic Index, Glycemic Load and Glycemic Response: An International Scientific Consensus Summit from the International Carbohydrate Quality Consortium (ICQC). *Nutr. Metab. Cardiovasc. Dis.* **2015**, *25*, 795–815. [CrossRef]
142. Suter, P.M.; Häsler, E.; Vetter, W. Effects of Alcohol on Energy Metabolism and Body Weight Regulation: Is Alcohol a Risk Factor for Obesity? *Nutr. Rev.* **1997**, *55*, 157–171. [CrossRef]
143. Marko, D.M.; Conn, M.O.; Schertzer, J.D. Intermittent Fasting Influences Immunity and Metabolism. *Trends Endocrinol. Metab.* **2024**, *35*, 821–833. [CrossRef]
144. Wegman, M.P.; Guo, M.H.; Bennion, D.M.; Shankar, M.N.; Chrzanowski, S.M.; Goldberg, L.A.; Xu, J.; Williams, T.A.; Lu, X.; Hsu, S.I.; et al. Practicality of Intermittent Fasting in Humans and Its Effect on Oxidative Stress and Genes Related to Aging and Metabolism. *Rejuvenation Res.* **2015**, *18*, 162. [CrossRef] [PubMed]
145. Beaulieu, K.; Casanova, N.; Oustric, P.; Turicchi, J.; Gibbons, C.; Hopkins, M.; Varady, K.; Blundell, J.; Finlayson, G. Matched Weight Loss Through Intermittent or Continuous Energy Restriction Does Not Lead to Compensatory Increases in Appetite and Eating Behavior in a Randomized Controlled Trial in Women with Overweight and Obesity. *J. Nutr.* **2020**, *150*, 623–633. [CrossRef] [PubMed]

146. Ravussin, E.; Beyl, R.A.; Poggiogalle, E.; Hsia, D.S.; Peterson, C.M. Early Time-Restricted Feeding Reduces Appetite and Increases Fat Oxidation but Does Not Affect Energy Expenditure in Humans. *Obesity* **2019**, *27*, 1244–1254. [CrossRef] [PubMed]
147. Corley, B.T.; Carroll, R.W.; Hall, R.M.; Weatherall, M.; Parry-Strong, A.; Krebs, J.D. Intermittent Fasting in Type 2 Diabetes Mellitus and the Risk of Hypoglycaemia: A Randomized Controlled Trial. *Diabet. Med.* **2018**, *35*, 588–594. [CrossRef]
148. Beshyah, S.A.; Hassanein, M.; Ahmedani, M.Y.; Shaikh, S.; Ba-Essa, E.M.; Megallaa, M.H.; Afandi, B.; Ibrahim, F.; Al-Muzaffar, T. Diabetic Hypoglycaemia during Ramadan Fasting: A Trans-National Observational Real-World Study. *Diabetes Res. Clin. Pract.* **2019**, *150*, 315–321. [CrossRef]

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Review

Beyond Green: The Therapeutic Potential of Chlorophyll and Its Derivatives in Diabetes Control

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Abstract

Chlorophyll, the green pigment essential for photosynthesis, abundantly found in green vegetables and algae, has attracted growing scientific interest for its potential therapeutic effects, particularly in diabetes management. Recent research highlighted that chlorophyll and its derivatives may beneficially influence glucose metabolism and oxidative stress, key factors in diabetes. This review examines current knowledge on how chlorophyll compounds could aid diabetes control. Chlorophyll and its derivatives appear to support glucose regulation primarily through actions in the gastrointestinal tract. They modulate gut microbiota, improve glucose tolerance, reduce inflammation, and alleviate obesity-related markers. While chlorophyll itself does not directly inhibit digestive enzymes like α-glucosidase, its derivatives such as pheophorbide *a*, pheophytin *a*, and pyropheophytin *a* may slow carbohydrate digestion, acting as α-amylase and α-glucosidase inhibitors, reducing postprandial glucose spikes. Additionally, chlorophyll enhances resistant starch content, further controlling glucose absorption. Beyond digestion, chlorophyll derivatives show promise in inhibiting glycation processes, improving insulin sensitivity through nuclear receptor modulation, and lowering oxidative stress. However, some compounds pose risks due to photosensitizing effects and toxicity, warranting careful consideration. Chlorophyllin, a stable semi-synthetic derivative, also shows potential in improving glucose and lipid metabolism. Notably, pheophorbide *a* demonstrates insulin-mimetic activity by stimulating glucose uptake via glucose transporters, offering a novel therapeutic avenue. Overall, the antioxidant, anti-inflammatory, and insulin-mimicking properties of chlorophyll derivatives suggest a multifaceted approach to diabetes management. While promising, these findings require further clinical validation to establish effective therapeutic applications.

Keywords: chlorophyll; pheophorbide *a*; pheophytin *a*; chlorophyllin; diabetes; α-glucosidase inhibition

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. It primarily exists in two major forms: type 1 diabetes (T1D), an autoimmune condition leading to the destruction of pancreatic β-cells, which are responsible for the production and secretion of insulin; and type 2 diabetes (T2D), which involves insulin resistance and progressive β-cell dysfunction [1]. The global burden of diabetes continues to grow, with 589 million adults

aged 20–79 affected worldwide—a number projected to rise significantly in the coming decades [2].

The therapeutic approaches to T1D and T2D differ significantly due to their distinct pathophysiological mechanisms [1]. Insulin replacement therapy remains the cornerstone of T1D management. Modern regimens often include multiple daily injections using basal-bolus strategies or continuous subcutaneous insulin infusion via insulin pumps. Recent advancements include ultra-rapid insulin analogues, smart pens, and hybrid closed-loop systems that automate insulin delivery based on continuous glucose monitoring data [3,4]. Also, promising new immunotherapies have emerged and are being developed [5].

Treatment of T2D typically begins with lifestyle modification—including diet, physical activity, and weight management—and progresses to pharmacologic therapy if glycaemic goals are not achieved. First-line pharmacotherapy commonly involves metformin, due to its efficacy, safety, and low cost [6–8]. In patients with established cardiovascular disease or high-risk features, sodium-glucose transport protein 2 (SGLT2) inhibitors and glucagon-like peptide 1 (GLP-1) receptor agonists are increasingly recommended for their dual glycaemic and cardiovascular benefits [6]. Other options include dipeptidyl peptidase 4 (DPP-4) inhibitors, thiazolidinediones, sulfonylureas, and, in some cases, insulin. While insulin is essential in T1D, it may only be introduced later in the treatment of T2D, particularly when oral agents are no longer sufficient to maintain adequate glycaemic control [6,7].

Alternative and complementary therapies have been proposed in the management of diabetes by lowering blood glucose [9]. There is great interest in the use of plant and plant-derived substances for effective treatment of diabetes and its complications [10–14], including cardiac disorders [15]. For instance, several herbs have been demonstrated to reduce glucose absorption by inhibiting α-glucosidase [16], or by improving sensitivity for endogenous insulin, as well as protecting pancreatic β-cells from damage [10], and providing useful antioxidant nutraceuticals [17] to counteract the deleterious effect of advanced glycation end-products.

In this context, it is well known that an adequate daily assumption of fruits and vegetables can reduce risk factors for many communicable and non-communicable diseases [18], including diabetes [19]. A useful tip to assure adequate intake of fruits and vegetables and optimize the assumption of useful protective substances is the colour: "eating a rainbow" of fruits and vegetables is a common suggestion to ensure a wide range of healthy nutraceuticals, as documented by a recent umbrella review that provided evidence of healthy effects of colour-associated bioactive pigments [18]. Although the health benefits of variously coloured pigments—such as lycopene (red), alpha- and beta-carotene (yellow and orange), anthocyanins (red, purple, and blue), flavonols (pale yellow), and flavones (white)—are well-documented [18], the role of the common and ubiquitous chlorophyll (green) remains comparatively underexplored. Even after more than two centuries of research, chlorophyll continues to be a subject of active investigation and discussion, as evidenced by recent studies and publications [20]. Chlorophylls are green pigments of plants that play a central role in photosynthesis and have recently gained attention for their potential therapeutic properties, including antioxidant, anti-inflammatory, anti-obesogenic and antidiabetic effects [21]. Anecdotal reports also refer to the use of chlorophyll in treating chronic ulcers as a complication of diabetes [22,23]. More recently, chlorophyll catabolites have been proposed as potent potential candidates for antiviral therapy against SARS-CoV-2 [24]. In the context of diabetes management, emerging studies suggest that chlorophylls and their derivatives may influence glucose metabolism and oxidative stress pathways.

Interestingly, a historical and complementary perspective from chromotherapy associates the colour green with balance and healing. According to Pandey et al. [9], chromotherapy considers diabetes to be linked to a deficiency of orange, yellow, and green colours in

the body's energy system, and green light is believed to support internal harmony. While this concept is anecdotal and lacks scientific validation, the alignment between the green colour of chlorophyll and its potential biological effects offers a curious, though speculative, intersection of traditional colour-based theories and modern biomedical research.

In light of the above considerations, the aim of the present review is to evaluate the current knowledge on the potential effects of chlorophyll and its derivatives in the context of diabetes, including their mechanisms of action, preclinical and clinical evidence, and directions for future research.

2. Literature Search Strategy

Although this is a narrative review, a structured approach was adopted for article selection. A comprehensive literature search was conducted using PubMed, Scopus and Google Scholar to identify relevant studies on chlorophyll and its derivatives in the context of diabetes. The search in the PubMed and Scopus databases employed the terms: (chlorophyll AND diabetes) OR (chlorophyll derivatives AND diabetes) OR (chlorophyllin AND diabetes) OR (pheophorbide AND diabetes) OR (pheophytin AND diabetes), with no time restrictions applied. Similarly, Google Scholar searches used key phrases like "chlorophyll AND diabetes", "chlorophyllin AND diabetes", and "chlorophyll derivatives AND diabetes". Only articles published in English were considered. Studies were selected based on their relevance to the biological roles, mechanisms of action, and therapeutic potential of chlorophyll compounds in diabetes. Selected articles were then grouped thematically to highlight emerging trends and research gaps.

3. Chlorophylls and Related Compounds

Chlorophyll (from Greek χλωρός, green and φύλλον, leaf) is the most typical pigment present in the chloroplast grains of plant cells [25,26]. Chlorophyll *a*, *b*, *d*, and *f* have a chlorin nucleus (17,18-dihydroporphyrin) with a magnesium atom at the centre and a propionate residue with a phytyl ester. Porphyrin is a heterocycle with 22 conjugated π-electrons as shown in Figure 1, where the 18 π-electrons involved in the aromatic system are highlighted in red. The other four π-electrons possess more double-bond characteristics and are not involved in the aromatic system (Figure 1).

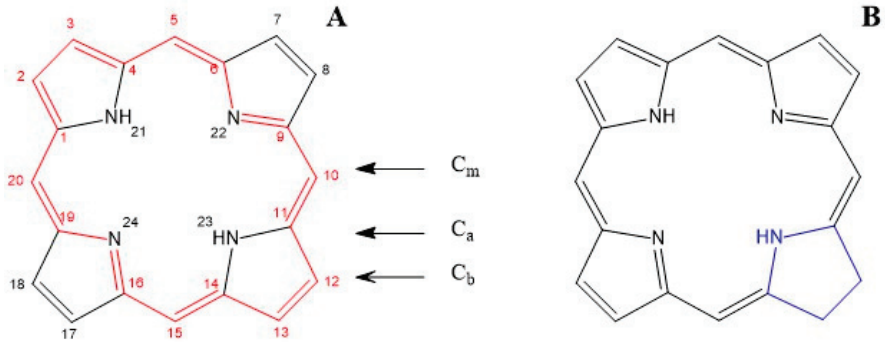


Figure 1. Porphyrin (**A**) with the IUPAC atom numbering and carbon types, and the chlorin ring (2,3-dihydroporphyrin, (**B**)) with the hydrogenated site in blue.

This conjugated macrocycle, with or without the metal atom, is primarily responsible for the colours of many biological molecules containing the porphyrin ring. Different substituents lead to variations in the electronic distribution and energy, resulting in different absorption spectra in the visible range and, consequently, different colours (e.g., haemoglobin/myoglobin and chlorophyll). Carbon atoms of the porphyrin nucleus can be chemically and structurally classified as C_a, C_b and C_m. Among these, only the C_m and C_b

carbon atoms can undergo various addition and substitution reactions. Chlorophylls are magnesium (rarely zinc) complexes, where the magnesium atom is tetra-coordinated with the four porphyrinic nitrogens and has a fifth axial ligand in solution within a polypeptide matrix (Figure 2). By contrast, haeme consists of the tetrapyrrole protoporphyrin IX (Figure 3) with a single iron atom. In haemoglobin, the iron is coordinated to the four porphyrin nitrogen atoms, while one axial site is ligated to an amino acid residue that links the haeme to the protein, and the other axial site binds oxygen, carbon dioxide, or a protein amino acid.

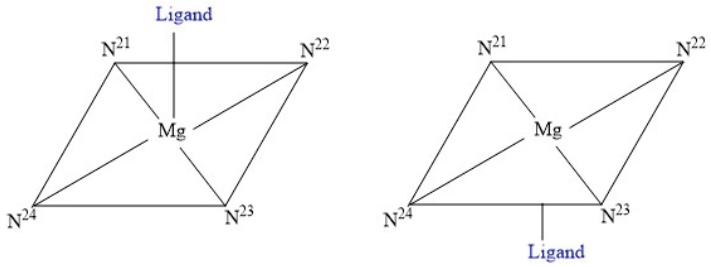


Figure 2. Schematic drawing for single axial coordination of chlorophylls to form five-coordinated species with chirality at the central magnesium.

Electrons in the porphyrin system can move freely within the ring structure, and energized electrons, generated by sunlight photons, transfer chemical energy to other substrates, enabling the synthesis of carbohydrates. Chlorophyll also presents a hydrophobic tail that permits insertion into the chloroplast thylakoid membrane, which is a specialized membrane system found inside the chloroplasts of plant cells, as well as in cyanobacteria, playing a fundamental role in photosynthesis.

Several kinds of chlorophyll exist, but the most relevant ones are chlorophyll *a* and *b* (Figure 3). Their structures differ by a single substituent on the chlorin ring: chlorophyll *a* possesses a methyl group, whereas in chlorophyll *b*, this methyl is oxidized to a formyl group. As previously mentioned, these different substituents result in distinct light absorption properties, enabling a broader range of the visible spectrum to be absorbed, primarily in the blue and red wavelengths. The absorption peaks of chlorophyll *a* are at 372 nm and 642 nm, while for chlorophyll *b* they are 392 nm and 626 nm, as determined free of perturbing solvent interactions [27].

Pheophytin is a dark bluish pigment obtained by treating chlorophyll with a weak acid, or after thermal treatment [28]; the molecule lacks the central magnesium ion (Figure 3). As with chlorophyll, two main types of pheophytin exist: pheophytin *a* and pheophytin *b*, corresponding to chlorophyll *a* and *b*, respectively.

Pheophorbide is obtained by chlorophyll breakdown [28] and consists, like pheophytin, of a chlorin ring lacking the central magnesium ion, but also without the phytyl tail (Figure 3). As with pheophytin, two main types of pheophorbide exist: pheophorbide *a* and pheophorbide *b*, corresponding to chlorophyll *a* and *b*. Interestingly, pheophorbide is structurally similar to animal protoporphyrin IX (Figure 3, inset), highlighting a conserved molecular framework that links the plant and animal kingdoms through shared tetrapyrrolic scaffolds.

Although pheophytin and pheophorbide are chlorophyll breakdown products found in plants [29], pheophytin is the predominant derivative formed during chlorophyll digestion in animals, accounting for approximately 75–77% of total chlorophyll pigments after gastrointestinal processing [30] and it also arises during thermal treatment of foods [28].

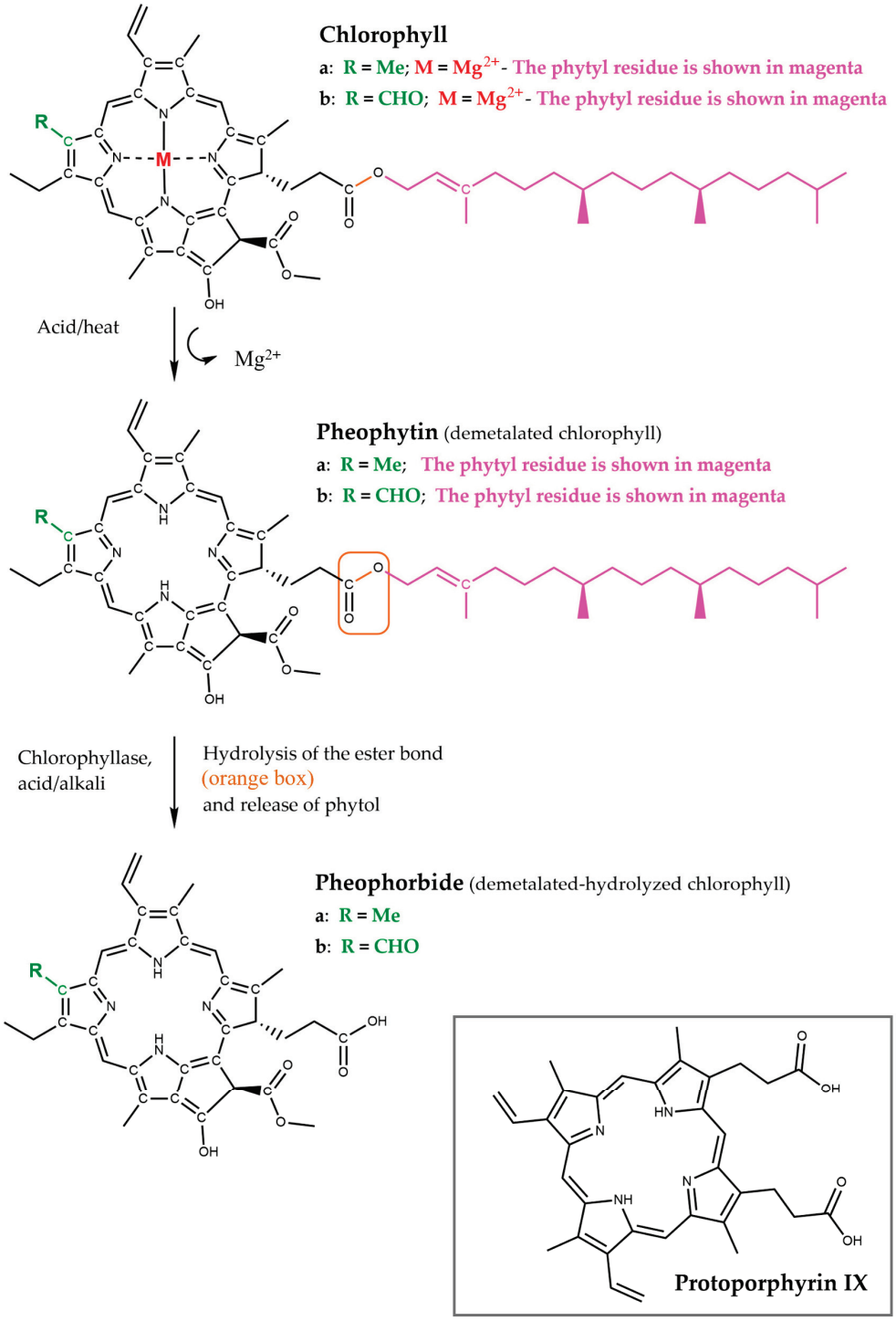


Figure 3. Chemical structure of the most abundant chlorophylls—chlorophyll *a* and chlorophyll *b*—and of the degradation products phaeophytin and pheophorbide. Inset: protoporphyrin IX.

4. Natural Sources of Chlorophyll and Dietary Intake

Chlorophylls are widely present in many dietary sources, particularly those with dark green coloration, including leafy green vegetables, green beans, seaweed and algae [28]. Recent research underscores the nutritional value of the "Couve-Manteiga" collard green (*Brassica oleracea* var. *viridis*), particularly its high chlorophyll content and suitability for convenience food applications aimed at enhancing daily nutrient intake [31]. Average intake of chlorophylls varies based on dietary habits, especially consumption of green vegetables. By using modern mathematical techniques, a recent study has evaluated the

chlorophyll intake in different European countries [32], obtaining a mean intake of 207 mg of green chlorophylls/day per adult person. Assuming all green vegetables consumed were spinach (containing 39 mg chlorophyll per gram of dry weight and 12.5% dry matter), the estimated daily intake of chlorophyll would be around 375 mg per person [28]. Chlorophyll supplements containing for instance powered chlorella algae, or chlorophyll derivatives (such as chlorophyllin, see below) are also available and used for their proposed antioxidant, anti-inflammatory, and detoxifying effects [28].

5. Nutritional and Functional Role of Chlorophyll

Mammalian studies reveal that the absorption of dietary chlorophyll ranges from 1–4%, while the primary route of elimination is faecal excretion of its metabolites [28,33]. Recent studies have demonstrated that natural chlorophylls are absorbed and metabolized within the body, with a tendency for pheophorbide derivatives to accumulate in the liver [21,34,35]. The liver also plays a role in converting these compounds into metabolites like phytyl-chlorin e6 [36] (Figure 4), highlighting the ongoing exploration of new chlorophyll-related compounds [28,35].

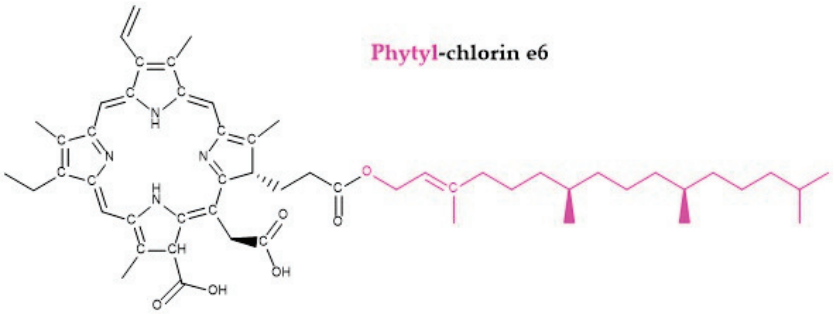


Figure 4. Chemical structure of the phytyl-chlorin e6, according to [36]. The phytyl residue is shown in magenta.

The health benefits of chlorophyll are also related to its ability to resist digestion and its absorption. Viera et al. [37] explored whether food composition influences chlorophyll bioaccessibility. Their findings showed that chlorophyll stability during digestion varies widely (15–85%) and may be influenced by salt content. Most absorbable chlorophylls in mixed micelles were pheophytins due to their high digestive stability, except in foods rich in pheophorbides. While food composition can affect digestive outcomes to some extent, chlorophyll bioaccessibility is primarily determined by its chemical structure, especially pheophorbide content. Experiments using human Caco-2 intestinal cell lines demonstrated that chlorophyll is taken up primarily in the form of its digestion-derived derivatives, including pheophytins and allomerized pheophytins, highlighting their potential bioavailability and physiological relevance [30].

6. Effects of Chlorophyll and Its Derivatives on Glucose Metabolism

Emerging research indicates that chlorophyll and its derivatives can influence glucose metabolism through various mechanisms in the gastrointestinal tract.

Chlorophyll supplementation has been shown to positively affect the gut microbiota composition, which is closely linked to glucose metabolism. In a study involving high-fat diet-induced obese mice [38], early-life chlorophyll supplementation (0.18 mg per 10 g body weight each day for 13 weeks) resulted in improved glucose tolerance and reduced low-grade inflammation, as well as reduced adipose tissue accumulation, counteracting obesity. This effect was associated with a significant reversal of gut dysbiosis, notably a decreased Firmicutes-to-Bacteroidetes ratio, with an increase in *Blautia* and *Bacteroidales*

S24-7 group members and a reduction in *Lactococcus* and *Lactobacillus*, suggesting that chlorophyll's impact on gut microbiota may play a role in modulating glucose absorption and metabolism, as well as favourable effects on obesity-related parameters [38].

Although chlorophyll itself has not been shown to directly inhibit human α-glucosidase enzymes, its derivatives have attracted interest for their potential metabolic effects. Alpha-glucosidase enzymes, in particular maltase-glucoamylase and sucrase-isomaltase, are located in the brush border of the small intestine and play a critical role in carbohydrate digestion by breaking down oligosaccharides into glucose [39]. The *N*-terminal (near the membrane-bound end) and *C*-terminal (luminal) subunits of both enzymes possess catalytic activity, with inhibitors—like acarbose, an oral α-glucosidase inhibitor approved by the U.S. Food and Drug Administration (FDA) for use in the management of T2D [40]—effectively binding to their highly specific, hydrophilic active sites by mimicking carbohydrate structures [39]. In contrast, chlorophyll molecules are large, lipophilic, and structurally dissimilar to native substrates, making direct interaction with α-glucosidase unlikely. However, although definitive evidence is lacking, computational docking and preliminary bioactivity studies suggest that chlorophyll derivatives warrant further investigation as modulators of carbohydrate metabolism [41].

Wang et al. [42] investigated the effects of chlorophylls and of the Mg^{2+}-free derivative, pheophytin *a*, on starch digestion in vitro. The findings revealed that both chlorophylls and pheophytin *a* significantly decreased starch hydrolysis while increasing the content of resistant starch. Scanning electron microscopy demonstrated that chlorophylls either existed in free form or were absorbed and embedded on the surface of starch granules, suggesting a direct interaction that impedes enzymatic activity. By means of spectrometry and molecular docking, the authors were able to demonstrate that the phytol chain of chlorophyll forms a double helix structure with starch, hindering the accessibility of digestive enzymes such as α-amylase and α-glucosidase, thereby slowing down carbohydrate breakdown. Also, the porphyrin ring can interact with specific sites of the enzymes. By inhibiting these enzymes in two different ways, chlorophyll may lead to a more gradual glucose absorption in the gut, potentially aiding in blood glucose regulation. This mechanism makes chlorophyll and its derivatives potential natural agents for controlling postprandial blood glucose levels, offering a dietary strategy for diabetes management.

Turkiewicz et al. [43] explored the use of polysaccharide- and protein-based microencapsulation carriers to enhance the stability and bioavailability of chlorophyll-rich extracts, aiming to improve their antidiabetic potential through inhibition of digestive enzymes. This innovative encapsulation approach protected chlorophyll compounds from degradation, enabling controlled release and improved interactions with biological targets. Functionally, the microencapsulated chlorophyll extracts demonstrated significant in vitro inhibitory activity against α-amylase and α-glucosidase. While these enzymes are involved in carbohydrate digestion and glucose release, their inhibition primarily targets postprandial hyperglycaemia control. However, DPP-4 plays a distinct role by degrading incretin hormones, which regulate insulin secretion, making its inhibition a key strategy for enhancing the insulin response and prolonging glucose homeostasis [44]. In this context, Turkiewicz et al. [43] demonstrated that chlorophyll-rich microcapsules exhibited statistically significant DPP-4 inhibitory activity, with IC_{50} values ranging from 20.38 mg/mL for pea protein isolate (PPI)-based capsules to 434.63 mg/mL for maltodextrin-based capsules. Notably, soy protein isolate (SPI)-based capsules showed exceptional potency, with IC_{50} values below 0.01 mg/mL, indicating that minimal extract concentrations were sufficient to effectively suppress DPP-4 activity. The highest inhibitory potential was generally associated with formulations dried at lower temperatures and those rich in carotenoids, pheophorbides, and chlorophylls, as evidenced by strong correlations (r = 0.88, 0.87, and

0.79, respectively). The enhanced bioactivity of SPI- and PPI-based microcapsules was attributed to a potential synergistic effect between chlorophyll derivatives and peptides known to inhibit DPP-4. Despite these promising findings, further mechanistic studies, such as molecular docking or structural analyses, are needed to clarify the precise binding interactions of chlorophyll-related compounds with the DPP-4 active site. The combined inhibition of these enzymes suggests a multifaceted mechanism by which microencapsulated chlorophyll extracts may support glycaemic control. This research underscores the potential of advanced biopolymer carriers not only to preserve chlorophyll integrity but also to potentiate its enzymatic inhibition effects, offering a promising strategy for natural, plant-based antidiabetic therapies.

Further in vitro studies have recently provided compelling evidence that chlorophyll derivatives can directly inhibit carbohydrate-digesting enzymes, specifically α-amylase and α-glucosidase [41]. Molecular docking studies revealed that pheophytin *a* and pyropheophytin *a*, both Mg^{2+}-free chlorophyll derivatives formed during digestion or heating, can effectively bind to the active site region of α-glucosidase. The docking scores indicated strong binding affinities, with pheophytin *a* and pyropheophytin *a* exhibiting binding energies of approximately −9.5 and −9.2 kcal/mol, respectively. Both compounds occupy a hydrophobic pocket near the enzyme's catalytic site, primarily stabilized through hydrophobic interactions and hydrogen bonds. Notably, pyropheophytin *a* formed more hydrogen bonds (four compared to the two of pyropheophytin *a*) with residues adjacent to the catalytic triad, which likely underpins its relatively higher inhibitory potency. Although neither molecule directly interacted with the catalytic triad residues critical for enzymatic activity, their binding induced conformational changes in the enzyme, suggesting a non-competitive inhibition mechanism. These docking insights, corroborated by fluorescence quenching and spectroscopic analyses, highlight the potential of pheophytin *a* and pyropheophytin *a* as natural α-glucosidase inhibitors that could contribute to blood glucose regulation.

Chlorophyll derivatives such as pheophorbide *a*, which lack both the phytyl tail and central magnesium ion, exhibit increased polarity and structural flexibility. These modifications may enhance their ability to interact with metabolic enzymes, either at allosteric sites or through nonspecific binding. Supporting this hypothesis, Kim et al. [45] demonstrated in streptozotocin-induced diabetic mice that pheophorbide *a*—isolated from the edible red seaweed *Gelidium amansii*, commonly found along the coasts of Japan and Korea—showed potent inhibitory activity against α-glucosidase and α-amylase. This activity effectively reduced hyperglycaemia following a controlled feeding, better than acarbose, which was used as a positive control. In this context, acarbose, currently used clinically to manage diabetes, is widely recognized as a classical reference compound in studies on intestinal α-glucosidase inhibition [46]. The crystal structure of the *C*-terminal domain of a typical α-glucosidase, the human maltase-glucoamylase (MGAM-C), available under PDB ID as 3TOP (https://doi.org/10.2210/pdb3TOP/pdb, accessed on 12 July 2025) and 3TON (https://doi.org/10.2210/pdb3TON/pdb, accessed on 12 July 2025), can provide insights into the molecular interactions between the enzyme and various inhibitors, including acarbose [47]. Structural analysis reveals that acarbose forms multiple hydrogen bonds with key residues in the MGAM-C active catalytic domain, such as Asp1157, Asp1279, Arg1510, Asp1526, Thr1528, and His1584 [47]. These interactions play essential roles in substrate stabilization, ligand positioning, and catalysis. Additionally, Trp1355 and Phe1559 contribute through hydrophobic interactions, further anchoring the inhibitor within the binding pocket. Collectively, these contacts mimic natural substrate engagement and effectively block enzymatic activity.

As a complement to the present review, a molecular docking exploration was performed using CB-Dock2 web-based software (https://cadd.labshare.cn/cb-dock2/index.php, accessed on 12 July 2025), indicating that the chlorophyll *a* degradation product pheophorbide *a* also exhibits affinity for MGAM-C (PDB ID: 3TOP). Two potential binding cavities can be identified on chains A and B of the enzyme, with volumes of 1083 Å^3 and 683 Å^3, respectively, and corresponding Vina scores of −8.8 and −8.4 kcal/mol. Notably, the 683 Å^3 pocket on chain B includes ten residues—Asp1157, Pro1159, Tyr1251,Trp1355, Trp1369, Arg1510, Asp1526, Thr1528, Phe1559 and Phe1560—that are also involved in acarbose binding (according to Ren et al. [47]), suggesting a shared interaction profile between the two ligands (Figure 5).

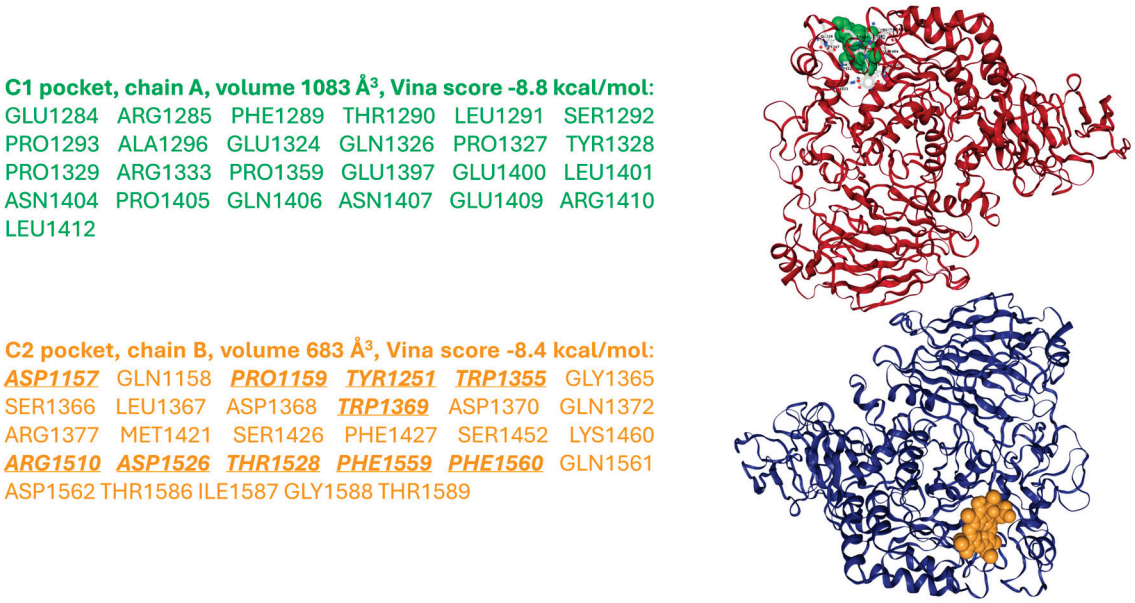


Figure 5. Molecular docking obtained with CB-Dock2 web-based software showing the most relevant interactions of pheophorbide *a* (shown as green and orange space filling molecules) with the human maltase-glucoamylase enzyme (PDB ID: 3TOP). In the C2 pocket, the residues of the active site, identified also for acarbose interactions [47], are in bold and underlined. Protein structures of chains A and B are shown as ribbon diagrams in red and blue, respectively.

In addition to its inhibitory effects on digestive enzymes, pheophorbide *a*, isolated from *Capsosiphon fulvescens*, a green alga growing along the coast of Korea, Japan, North Atlantic and Pacific, and used as functional food [48], has shown promising anti-glycation activity relevant to diabetes-related complications [49]. Advanced glycation end-products (AGEs), which accumulate under hyperglycaemic conditions, contribute to oxidative stress and tissue damage in diabetic patients [50]; the study by Hong et al. [49] demonstrated that pheophorbide *a* effectively suppressed AGE formation in vitro, suggesting a protective role against glycation-mediated cellular dysfunction. This scavenging ability positions chlorophyll derivatives like pheophorbide *a* as potential multifunctional agents for mitigating both glycaemic and oxidative stress in diabetes management. Due to the known photosensitizing activity of pheophorbide *a* and pyropheophorbide *a* [51], caution is warranted in their use. Pheophorbide *a*, in particular, has been identified as a potent photosensitizer, with its accumulation linked to phototoxic skin reactions resembling pseudoporphyria, especially in individuals consuming commercial chlorophyll supplements or laver products [51–54]. Case reports have described severe photosensitivity reactions and bullous skin lesions following self-administration of chlorophyll-containing supplements [52], and a case series by Zhao et al. [53] highlighted prolonged photosensitivity persisting even after cessation of chlorophyll intake. These reactions may be due to accumulation of pheophorbide a in the

skin and subsequent activation by sunlight, leading to porphyria-like symptoms. In one study, Hwang et al. [51] quantified high levels of pheophorbide *a* and pyropheophorbide *a* in dried laver implicated in food-related phototoxicity cases, underscoring the importance of analytical monitoring of commercial products. Moreover, Cinčárová et al. [54] have emphasized the need for quantitative assessment and regulatory control of pheophorbide content in algae-based supplements, as even trace amounts may pose a clinical risk when accumulated. This adverse effect may represent a significant limitation to the clinical application of these compounds, particularly in long-term or high-dose contexts. Therefore, thorough safety evaluations, including controlled dosage, proper formulation, and patient education on sun exposure, is essential before considering possible pheophorbide-based interventions in diabetes therapy. Phytanic acid, a metabolite of chlorophyll-derived phytol (Figure 6) produced by rumen microbiota and marine organisms [55–57], may influence metabolic regulation relevant to diabetes by acting as a potent natural agonist of retinoic X receptor (RXR) at physiological concentrations [58,59]. RXR agonists act as insulin sensitizers by activating heterodimers formed between RXR and peroxisome proliferator-activated receptor gamma (PPARγ), thereby improving glucose and lipid metabolism in models of T2D [60]. Based on this evidence, Elmazar et al. [61] conducted molecular docking simulations, which permitted the suggestion that phytanic acid is able to fit within the PPARγ ligand binding domain via four hydrogen bonds with affinities comparable to those of the insulin sensitizer thiazolidinediones. These data support the finding by Heim et al. [62], who found in rat primary hepatocytes that phytanic acid can activate PPARγ, RXR and PPARα as well, suggesting its potential role in improving insulin sensitivity and managing T2D.

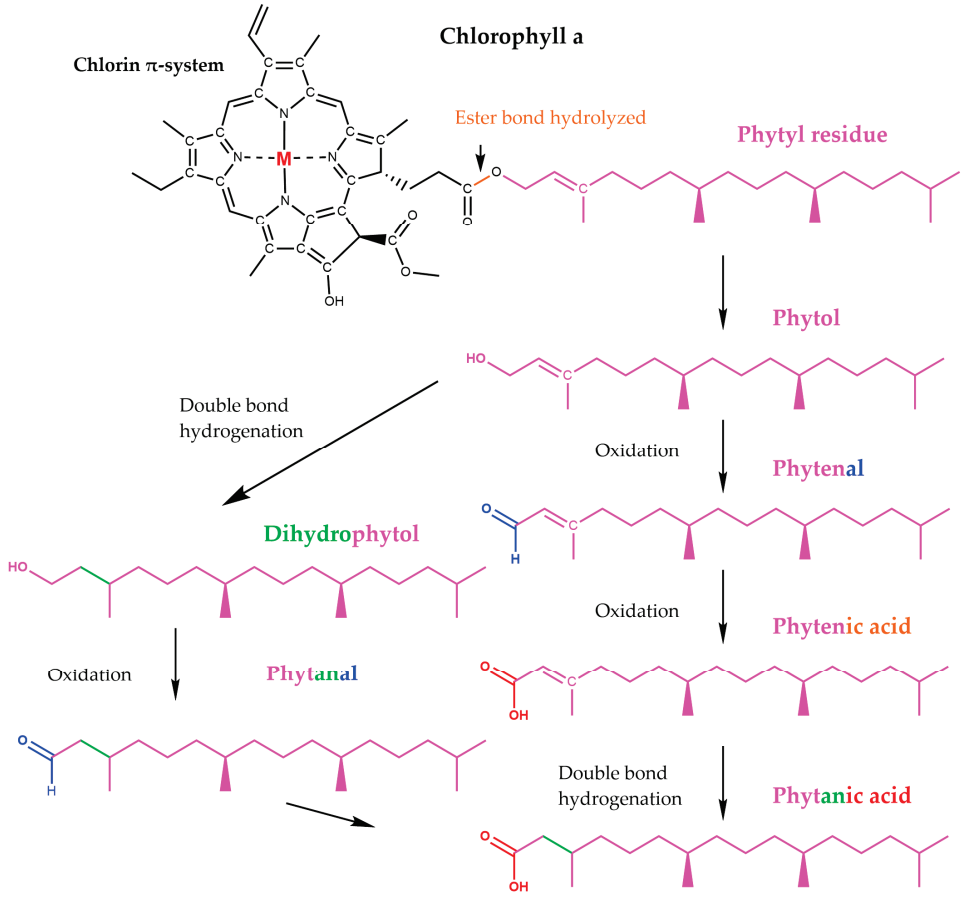


Figure 6. Phytanic acid from chlorophyll. Drawn based on chemical structures presented in reference [56]. Colour coding is used for visual distinction between different functional groups and molecular modifications.

Phytanic acid promotes adipocyte differentiation in both 3T3-L1 cells and human pre-adipocytes, likely by acting as a natural rexinoid (a ligand that activates RXRs), suggesting potential therapeutic value for T2D and obesity [63]. However, a 2023 review [64] questioned these findings, highlighting the limited and inconclusive evidence regarding phytanic acid's antidiabetic effects and emphasizing the need for further research to confirm its efficacy and safety. High levels of phytanic acid can cause health risks, including neuronal damage through mitochondrial dysfunction and disrupted calcium balance, oxidative stress, and inflammation. Phytanic acid can cause vascular and skin cell toxicity and has been suggested to be linked to Refsum's disease and other peroxisomal enzyme deficiency diseases, as well as to Alzheimer's disease and some lymphomas. Overall, excessive phytanic acid accumulation is harmful, especially to the nervous system and through oxidative mechanisms [56].

Based on the fact that chlorophyll metabolites may act as RXR agonists, Wunderlich et al. [65] considered another, more direct, potential anti-diabetic activity. The authors investigated the effects of chlorophyll *a*, incorporated into P123 micellar copolymer, combined with photostimulation on in vitro hepatocytes from a rat model of T1D. The findings, confirmed by post-analysis in situ perfusion of intact liver, demonstrated that chlorophyll *a* decreased hepatic glucose release, stimulated glycolysis, and reduced oxidative stress markers in liver cells, indicating its potential in modulating glucose metabolism and oxidative stress pathways in diabetes management [65].

While these findings are promising, further research, particularly in human subjects, is necessary to fully understand the extent and mechanisms by which chlorophyll and derivatives can influence glucose absorption and metabolism.

7. The Role of Chlorophyllin

Chlorophyllin is a semi-synthetic water-soluble derivative of chlorophyll, often used in research and supplements due to its stability and solubility [66,67]. Chlorophyllin is produced through alkaline hydrolysis of chlorophyll, with replacement of the magnesium ion, most commonly with copper or zinc, absence of the phytyl tail, and presenting carboxyl groups—from hydrolysis of chlorophyll's fifth ring as in chlorin e6—on the chlorin ring [28,66,68] (Figure 7). Chlorophyllin can interact with environmental toxins and has a history of safe medical use [66,67]. Approved by the FDA for internal deodorant use at doses up to 300 mg/day, it also helps manage odours in elderly patients and supports wound healing. Additionally, chlorophyllin serves as a food additive and colouring agent. Research has shown that it has antioxidant and antigenotoxic effects, and it has recently gained attention for its potential anticancer properties [66,67].

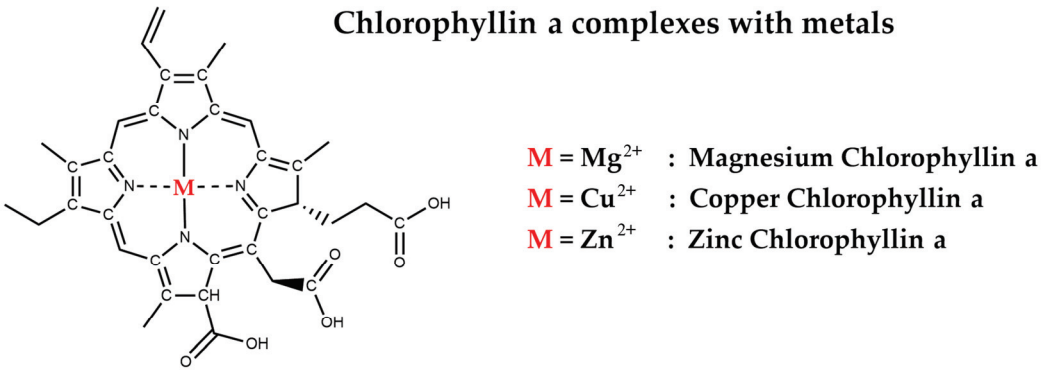


Figure 7. Magnesium, copper and zinc chlorophyllin *a*.

Chlorophyllin has been shown to acutely lower postprandial blood glucose levels in humans, as observed in a small-scale glucose tolerance test [69]. While the precise mecha-

nism remains unclear, it has been hypothesized that this effect may involve modulation of intestinal glucose transporters or incretin signalling, possibly acting as a GLP-1 secretagogue [69]. Supporting this hypothesis, several clinical studies have demonstrated that daily supplementation with green plant membranes (thylakoids), which are naturally rich in chlorophyll, can enhance GLP-1 secretion, reduce hunger, and reduce obesity-related risk factors. For example, Montelius et al. [70] reported that a three-month supplementation with green plant membranes in overweight women led to increased GLP-1 levels, body weight loss, and reduced cravings for palatable food. Similarly, Stenblom et al. [71] demonstrated that thylakoid intake prior to a carbohydrate-rich meal significantly increased GLP-1 and cholecystokinin (CCK) levels while reducing postprandial hypoglycaemia and hunger. These findings were supported by a systematic review by Amirinejad et al. [72], which concluded that thylakoids enhance satiety and support weight loss, likely through incretin-related mechanisms. This effect of chlorophyll and its derivatives may be attributed to their proposed DPP-4 inhibitory activity, resulting in elevated incretin levels, including GLP-1, as above discussed referring to the work by Turkiewicz et al. [43]. Together, these findings support a broader role for chlorophyll-rich plant components in appetite regulation and glycaemic control, although further studies are needed to isolate the specific contribution of chlorophyll or derivatives and clarify its direct mechanistic role in GLP-1 signalling.

Chlorophyllin has demonstrated significant potential in mitigating diabetes-induced hepatic damage. In a study involving streptozotocin-induced diabetic mice [73], sodium–copper chlorophyllin supplementation resulted in a marked reduction of oxidative stress markers, including reactive oxygen species (ROS), malondialdehyde (MDA), and protein carbonyl levels in the liver. Concurrently, there was an upregulation of the activity and expression of key antioxidant enzymes such as copper–zinc superoxide dismutase (CuZn-SOD), manganese superoxide dismutase (MnSOD), and catalase, indicating an enhanced antioxidant defence mechanism. Furthermore, chlorophyllin exhibited hepatoprotective effects, as evidenced by decreased activities of liver enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). Histological analyses corroborated these findings, revealing that chlorophyllin ameliorated morphological and cellular alterations in the liver tissue of diabetic mice. At the molecular level, chlorophyllin modulated apoptotic pathways by downregulating pro-apoptotic proteins such as caspase-3 and caspase-9, while upregulating the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2). These multifaceted actions suggest that chlorophyllin exerts its protective effects through the modulation of hyperglycaemia-induced oxidative stress and apoptosis, highlighting its potential as a therapeutic agent in the management of diabetes-related hepatic complications. The same authors [74] had also demonstrated the therapeutic potential of chlorophyllin in managing diabetes, specifically its effects on hyperglycaemia and hyperlipidaemia, using the same streptozotocin-induced diabetic mouse model as in the previous study. Chlorophyllin, at the optimum dose of 50 mg/kg body weight, significantly reduced blood glucose levels in both acute and sub-acute treatment periods. In an intraperitoneal glucose tolerance test (IPGTT), diabetic mice treated with chlorophyllin showed marked reductions in glucose levels at both 2 and 4 h compared to untreated diabetic controls. Furthermore, chlorophyllin treatment led to a significant reduction in glycosylated haemoglobin levels and normalized lipid profiles, indicating not only antihyperglycaemic but also antihyperlipidaemic effects.

Zheng et al. [75] investigated the effects of chlorophyllin on liver fibrosis induced by treatment with carbon tetrachloride in BALB/c mice. While the study does not directly address diabetes, its findings are relevant to metabolic conditions often associated with diabetes. The researchers found that oral administration of chlorophyllin attenuated both intestinal and hepatic inflammation and ameliorated liver fibrosis. Importantly, chloro-

phyllin treatment strengthened intestinal barrier integrity, reducing intestinal permeability and restoring tight junction proteins in the ileum; additionally, the treatment rebalanced the gut microbiota, characterized by a decrease in the phylum Firmicutes and an increase in Bacteroidetes. Additionally, in vitro experiments demonstrated that chlorophyllin inhibited the NF-κB pathway via suppression of IKK phosphorylation, suggesting a mechanism for its anti-inflammatory effects. These findings suggest that chlorophyllin may have potential applications in regulating intestinal microbiota and reducing inflammation, which are factors relevant to metabolic diseases such as diabetes. A more robust intestinal barrier can prevent systemic inflammation and endotoxaemia, factors that are known to impair insulin sensitivity and glucose metabolism [75].

A study by Samadder et al. [76] investigated the protective effects of chlorophyllin on glucose metabolism disrupted by alloxan, which induces diabetes and mitochondrial dysfunction. In a mouse model, pre-treatment with chlorophyllin significantly delayed the onset of diabetes and mitigated the alloxan-induced rise in blood glucose levels. Chlorophyllin helped restore glucose metabolism by optimizing key metabolic and enzymatic markers (such as glucokinase, pyruvate, and insulin), while also modulating the expression of insulin signalling proteins (IRS1, IRS2, and GLUT2). Additionally, chlorophyllin stabilized mitochondrial function by maintaining the membrane potential, ATP/ADP ratio, and ATPase activity, thus preserving energy production and reducing cellular damage.

8. Chlorophyll-Based Compounds with Antidiabetic Potential

There is growing scientific interest in chlorophyll-based compounds as promising candidates for future development in diabetes treatment strategies. In this context, as discussed above, pheophorbide *a* has demonstrated notable antidiabetic potential. In a study by Ribeiro et al. [77], the authors conducted a phenotypic screening of 182 fractions derived from 19 cyanobacterial strains using zebrafish larvae, aiming to identify compounds with insulin-mimetic activity. Two fractions, named 06104_D and 03283_B, were found to significantly enhance glucose uptake, as demonstrated by the 2-NBDG fluorescent glucose analogue assay. Further analysis revealed that fraction 06104_D not only lowered free glucose levels but also upregulated the expression of the insulin gene *insa* in zebrafish larvae. Metabolomic profiling of this active fraction identified several chlorophyll derivatives, with pheophorbide *a* emerging as a major constituent. Subsequent testing of commercially available pheophorbide *a* confirmed its ability to stimulate glucose uptake in vivo, highlighting its potential as a novel insulin-mimetic compound.

These findings align with previous reports supporting the antidiabetic potential of chlorophyll derivatives, also derived from sources of ethnobotanical interest. Attention has been drawn to *Clerodendrum infortunatum* L., a shrub from the Verbenaceae family that is widely distributed across tropical and subtropical regions and is traditionally known as "Hill Glory Bower" in English and "Bhant" in Bangla; it is described to hold ethnomedicinal importance among tribal communities in Bangladesh [78]. Recent studies have indicated that the leaves of *Clerodendrum infortunatum* exhibit anti-diabetic activity [79]. However, the specific bioactive compound (s) responsible for this effect remained to be identified, until research was directed to evaluate the anti-diabetic potential of its ethanolic leaf extracts and to isolate and characterize the active constituents responsible for this activity. From the leaves of *Clerodendrum infortunatum*, pheophytin *a* was isolated as fraction-1 and has been shown to reduce both blood glucose and cholesterol levels in rats, accompanied by increased insulin secretion following oral administration (1.25 g/kg body weight), suggesting a significant anti-diabetic action [78].

Moreover, pheophorbide *a* has demonstrated significant inhibitory activity against the receptor for advanced glycation end products (RAGE), a key mediator in the development

of diabetic complications. In a study by Matsumoto et al. [80], ethanol extracts from the leaves of *Mallotus japonicus*, a plant that is used in the Japanese pharmacopoeia against gastric diseases, were found to inhibit the binding of AGEs to RAGE. Activity-guided fractionation and LC/MS analysis identified pheophorbide *a* as a principal component responsible for this activity. Pheophorbide *a* demonstrated strong inhibition of the AGE–RAGE interaction, with an IC_{50} of 0.102 μM, closely matching the efficacy of the established RAGE inhibitor dalteparin, which has an IC_{50} of 0.084 μM. The authors proposed that pheophorbide *a* holds potential as a natural compound for development as a therapeutic or nutraceutical agent targeting health issues associated with AGE–RAGE axis activation.

Paul et al. [81] investigated the role of pheophorbide *a* as an insulin mimetic, focusing on its interaction with glucose transporters (GLUTs), in particular GLUT1, which is expressed in many cell types, including pancreatic β-cells, and permits basal glucose uptake, and GLUT4, the insulin-responsive transporter of glucose in peripheral tissues [82,83]. GLUT1 is required for the activity of pancreatic β-cells leading to insulin secretion [83]. Using in silico molecular docking, pheophorbide *a* demonstrated a strong binding affinity to GLUT1 and GLUT4 transporters, surpassing that of metformin and glucose [81]. Molecular docking and 100 ns molecular dynamics simulations revealed that pheophorbide *a* forms a highly stable complex with GLUT1, consistent hydrogen bonding, minimal structural fluctuations and sustained structural compactness, highlighting its strong binding affinity and potential as a GLUT1-targeted therapeutic agent in diabetes [81]. Complementing these computational findings, in vitro experiments with INS-1 cells (rat insulinoma cell line secreting insulin) showed that pheophorbide *a* treatment increased GLUT1 density at the plasma membrane under high glucose conditions, enhanced glucose uptake, and reduced GLUT1 mobility, suggesting a stabilized presence of the transporter [81]. These findings indicate that pheophorbide *a* could facilitate glucose-stimulated insulin secretion by modulating GLUT1 trafficking and function, positioning it as a potential candidate for diabetes management strategies. Paul et al. [81] also evaluated the potential of pheophorbide *a* as a therapeutic agent in diabetes, with particular attention to its pharmacokinetic and drug-likeness profiles. Computational ADMET (absorption, distribution, metabolism, excretion, and toxicity) predictions suggested that the compound is generally non-toxic, exhibiting no predicted mutagenic, carcinogenic, immunotoxic, or hepatotoxic effects, and a relatively safe toxicity classification [81]. Its physicochemical properties, including a moderate lipophilicity (cLogP 3.82) and a topological polar surface area (133 $Å^2$), are consistent with acceptable membrane permeability, supporting its suitability for drug development. Although the molecular weight of 592.7 slightly exceeds the conventional limit of 500 Da, it maintains compliance with several criteria in Lipinski's Rule of Five. Pheophorbide *a* shows limited gastrointestinal absorption and poor blood–brain barrier permeability, which may be advantageous for targeting peripheral tissues without central nervous system effects. Moreover, it is important to note that the inhibitory activity of pheophorbide *a* on intestinal α-glucosidase and α-amylase, as discussed above, does not require systemic absorption; like acarbose, this action is exerted locally within the intestinal lumen, which may mitigate concerns regarding its bioavailability for this specific mechanism of action. However, to direct the compound into specific sites of cellular action, such as pancreatic β-cells, it might be interesting to find strategies to overcome pheophorbide *a*'s pharmacokinetic limitations, particularly its high molecular weight and poor gastrointestinal absorption. Recent research has focused on innovative delivery systems that can improve its solubility, targeting, and bioefficacy. One approach includes PLGA-based nanoparticles modified with polyethylene glycol and folate for active tumour targeting; these folate–poly(lactic-co-glycolic acid) nanoparticles demonstrated enhanced pheophorbide *a* uptake and retention in gastric cancer models (MKN28) in vivo, suggesting improved delivery efficiency [84].

Another promising strategy utilizes amphiphilic copolymer nanoparticles formed from N-vinylpyrrolidone, which encapsulate pheophorbide (or its chlorin derivative) and significantly enhance its photophysical activity upon interaction with biomembranes, indicating better bioavailability in situ [85]. Additionally, casein micelles, naturally evolved carriers for hydrophobic compounds, have been shown to stably encapsulate pheophorbide *a* (final micelle size was about 220 nm) and facilitate efficient cellular uptake and photodynamic activity in tumour cells [86]. Finally, poloxamer F127 (a non-ionic copolymer surfactant) micelles co-loaded with doxorubicin and pheophorbide *a* have been developed for combined chemo–photodynamic therapy; these micelles maintained structural integrity and demonstrated strong tumour growth inhibition in vivo upon light activation [87]. Although originally developed for cancer applications, these diverse delivery platforms collectively show promise for enhancing the clinical translation of pheophorbide *a* by addressing its solubility, bioavailability, and targeting challenges. This suggests that pheophorbide *a* may also hold potential as an orally deliverable agent for modulating metabolic dysfunctions in diabetes.

Interestingly, Paul et al. [88], in another investigation, had earlier reported that the activity of a commercial antidiabetic polyherbal formulation from Indian markets (containing *Mangifera indica*, *Momordica charantia, Gymnema sylvestre* and *Syzygium cumini*), can be attributed to the presence of pheophorbide *a* and pyropheophorbide *a* (decarbomethoxylated pheophorbide *a*), identified in a hydro-alcoholic extract by using HPLC–ESI–MS/MS. The previously discussed risk of phototoxic skin reactions resembling pseudoporphyria, associated with pheophorbide *a* exposure, must always be carefully considered [51–54]. While the therapeutic potential of pheophorbide *a* in metabolic disorders such as diabetes is an emerging area of interest, it is essential to also acknowledge its well-documented phototoxic properties, particularly from studies in oncology [89–93]. Upon light activation, pheophorbide *a* undergoes photochemical reactions that produce reactive oxygen species, including singlet oxygen, hydroxyl radicals, and superoxide anions, which damage cellular components and lead to cell death [89]. As a potent photosensitizer, pheophorbide *a* has demonstrated significant efficacy in preclinical cancer models through photodynamic mechanisms; however, this same activity raises concerns regarding photosensitivity and unintended tissue damage upon light exposure. Several in vivo and in vitro cancer studies have reported cutaneous phototoxicity and systemic oxidative stress following light activation of pheophorbide *a*, even at low doses [94]. Although such effects are context-dependent and typically arise under photoactivating conditions, this inherent characteristic underscores the need for caution when translating pheophorbide *a* to non-oncological settings. In the context of diabetes management, where chronic administration may be considered, minimizing exposure to light or modifying the molecular structure to reduce its photosensitizing potential should be carefully evaluated in future preclinical and clinical investigations.

Taken together, these data indicate the therapeutic potential of chlorophyll-derived compounds as possible candidates for the development of antidiabetic agents targeting multiple aspects of glucose regulation, in particular glucose transport pathways, besides the aforementioned activity on carbohydrate digestion.

9. Chlorophylls and Pheophytins as Antioxidants

Chlorophylls and their derivatives have attracted increasing interest due to their complex biological roles, which include both antioxidant and pro-oxidant activities. These pigments may contribute to cancer prevention not only by neutralizing reactive species and modulating detoxification pathways but also by promoting selective oxidative stress and apoptosis in tumour cells [95]. The complete loss of cytotoxic activity in dechlorophyllized

olive leaf and green tea extracts highlights the essential contribution of chlorophylls to their anticancer effects, suggesting that these pigments are key bioactive components likely responsible for inducing tumour cell death, independent of their phenolic content [95].

The properties linked to their antioxidant effects are attracting increasing attention from scientific and industrial communities [21,34,96]. As discussed in the previous sections, recent findings highlight the potential of chlorophyll-derived compounds to modulate glucose homeostasis. Notably, pheophorbide *a*, identified through phenotypic screening in zebrafish larvae, demonstrated insulin-mimetic activity by enhancing glucose uptake and reducing glucose levels in vivo [77]. While this metabolic activity is central to their therapeutic promise, these compounds also exhibit well-documented antioxidant properties, which may offer complementary benefits. For instance, chlorophyll *a* and *b*, as well as pheophytin *a* and *b*, which are abundant in avocado oil [97], have been recognized for their antioxidative capacity [98], potentially contributing to broader cellular protection and metabolic regulation. These compounds may mitigate hyperglycaemia-induced oxidative stress, a major factor in the deterioration of β-cell function and the development of insulin resistance. Moreover, degradation products of chlorophyll *a*, such as pheophorbide *a* and chlorophyllin, have been shown to inhibit NF-κB activation, a key transcription factor regulating pro-inflammatory cytokine expression, suggesting an additional anti-inflammatory mechanism relevant to diabetes pathophysiology [75,99]. By reducing oxidative stress and inflammatory signalling, chlorophyll derivatives could help preserve insulin signalling pathways and promote metabolic homeostasis, thereby offering a multifaceted therapeutic approach for diabetes management.

10. Conclusions

In conclusion, the current body of evidence highlights the promising role of chlorophyll and its derivatives as multifaceted antidiabetic agents (Figure 8). As illustrated, these mechanisms include digestive enzyme inhibition, incretin modulation, nuclear receptor activation, glucose transporter regulation, and attenuation of oxidative stress pathways. While preclinical studies using zebrafish, rodent, and in silico models provide valuable mechanistic insights, they provide limited predictive value for human efficacy and safety. On average, only about 5% of therapies tested in animal models ultimately gain regulatory approval for human use, underscoring the limitations of preclinical approaches [100], a consideration that applies directly to the present context. Moreover, the pharmacokinetic profiles of many chlorophyll derivatives remain poorly characterized, complicating assessments of their bioavailability, metabolism, and dose optimization in human systems. Safety concerns also warrant closer attention. For example, the phototoxic potential of pheophorbide *a*, a known chlorophyll degradation product, has been documented under light exposure and may pose a significant risk in therapeutic applications. These factors underscore the need for caution when evaluating the clinical readiness of these compounds. To repurpose pheophorbide *a* as an intestinal α-glucosidase inhibitor for diabetes, strategies such as chemical derivatization to reduce singlet-oxygen yield, conjugation to non-absorbable carriers, or formulation in gut-targeted delivery systems could reduce the undesired photochemical reactivity and systemic distribution to minimize the phototoxic risk while preserving local enzymatic inhibition.

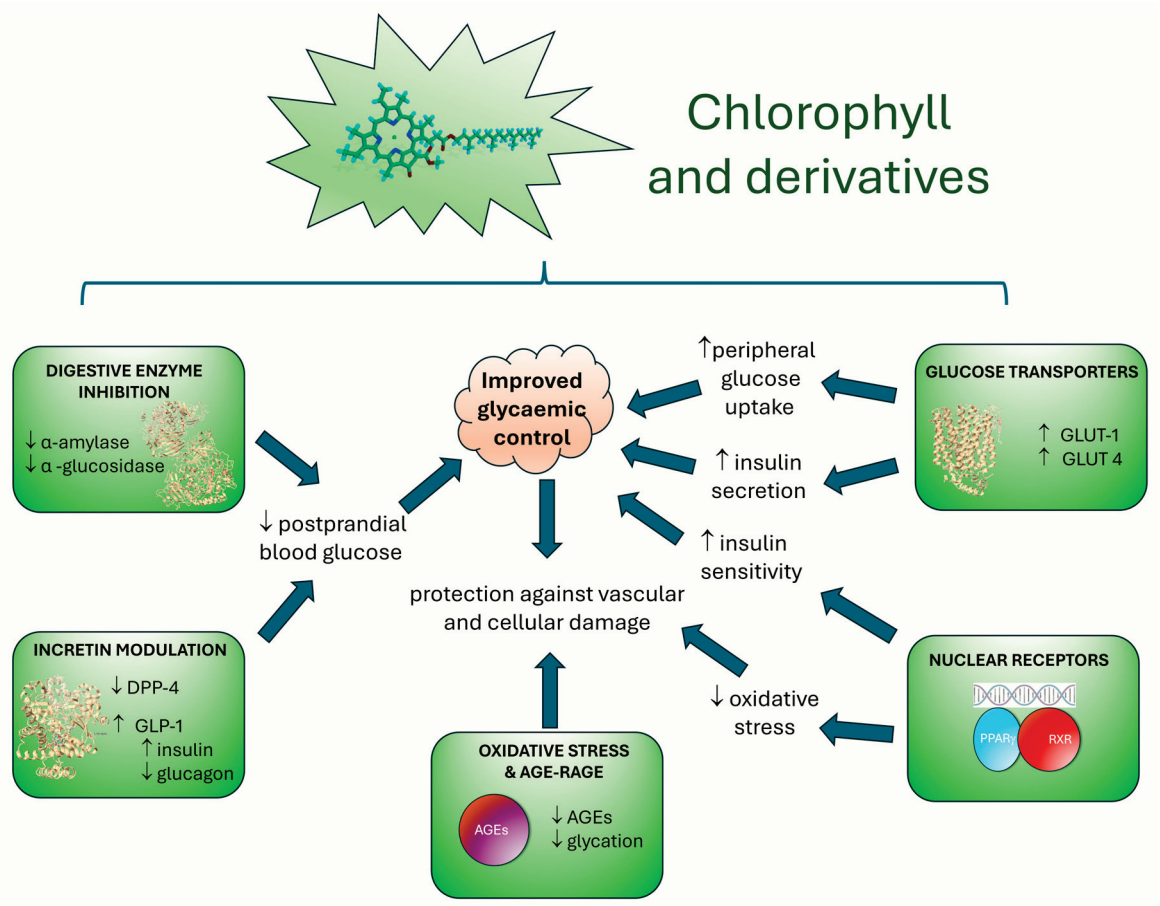


Figure 8. Proposed mechanistic pathways by which chlorophyll and its derivatives may exert antidiabetic effects. This schematic summarizes the multifaceted biological actions identified across preclinical studies.

Recent analyses further highlight these translational gaps and advocate for more human-relevant methodologies to improve predictive accuracy [101]. Our literature search did not identify any published human clinical trials specifically evaluating chlorophyll or its derivatives for diabetes management, highlighting a critical unmet need. To help address this, innovative strategies such as organ-on-a-chip platforms, human tissue-based assays, and in silico clinical trial simulations could be explored as more predictive alternatives [100]. Additionally, systematic reviews and meta-analyses of animal studies may help identify the most promising therapeutic candidates and guide the design of future clinical trials. Specific next steps could include target validation in human-relevant systems, formulation optimization, and dose–response studies to support safe and effective translation to clinical contexts. Continued research, particularly in advanced preclinical and clinical settings, is essential to determine the true therapeutic potential and safety of chlorophyll-based interventions in diabetes management.

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Abbreviations

The following abbreviations are used in this manuscript:

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
ADMET	Absorption, distribution, metabolism, excretion, and toxicity
AGEs	Advanced glycation end-products
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
Bcl-2	B-cell lymphoma-2
CCK	Cholecystokinin
CuZnSOD	Copper–zinc superoxide dismutase
DPP-4	Dipeptidyl peptidase 4
FDA	U.S. Food and Drug Administration
GLP-1	Glucagon-like peptide 1
GLUTs	Glucose transporters
HPLC–ESI–MS/MS	High-Performance liquid chromatography–electrospray ionization–tandem mass spectrometry
IKK	IκB kinase
IPGTT	Intraperitoneal glucose tolerance test
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
IUPAC	International Union of Pure and Applied Chemistry
LC/MS	Liquid chromatography–mass spectrometry
MDA	Malondialdehyde
MGAM-C	Maltase-glucoamylase *C*-terminal
MnSOD	Manganese superoxide dismutase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PPI	Pea protein isolate
RAGE	Advanced glycation end products
ROS	Reactive oxygen species
RXR	Retinoic X receptor
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SGLT2	Sodium-glucose transport protein 2
SPI	Soy protein isolate
T1D	Type 1 diabetes
T2D	Type 2 diabetes

References

1. American Diabetes Association. Classification and diagnosis of diabetes: Standards of Care in Diabetes—2024. *Diabetes Care* **2024**, *47* (Suppl. S1), S19–S38. [CrossRef]
2. International Diabetes Federation. *IDF Diabetes Atlas*, 11th ed.; International Diabetes Federation: Brussels, Belgium, 2025. Available online: https://diabetesatlas.org (accessed on 12 August 2025).
3. Di Molfetta, S.; Di Gioia, L.; Caruso, I.; Cignarelli, A.; Green, S.C.; Natale, P.; Strippoli, G.F.M.; Sorice, G.P.; Perrini, S.; Natalicchio, A.; et al. Efficacy and Safety of Different Hybrid Closed Loop Systems for Automated Insulin Delivery in People with Type 1 Diabetes: A Systematic Review and Network Meta-Analysis. *Diabetes Metab. Res. Rev.* **2024**, *40*, e3842. [CrossRef]
4. Singh, A.; Afshan, N.; Singh, A.; Singh, S.K.; Yadav, S.; Kumar, M.; Sarma, D.K.; Verma, V. Recent trends and advances in type 1 diabetes therapeutics: A comprehensive review. *Eur. J. Cell Biol.* **2023**, *102*, 151329. [CrossRef]
5. Foster, T.P.; Bruggeman, B.S.; Haller, M.J. Emerging Immunotherapies for Disease Modification of Type 1 Diabetes. *Drugs* **2025**, *85*, 457–473. [CrossRef] [PubMed]

6. Davies, M.J.; Aroda, V.R.; Collins, B.S.; Gabbay, R.A.; Green, J.; Maruthur, N.M.; Rosas, S.E.; Del Prato, S.; Mathieu, C.; Mingrone, G.; et al. Management of hyperglycemia in type 2 diabetes, 2022. A Consensus Report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care* **2022**, *45*, 2753–2786. [CrossRef] [PubMed]
7. Gieroba, B.; Kryska, A.; Sroka-Bartnicka, A. Type 2 diabetes mellitus—Conventional therapies and future perspectives in innovative treatment. *Biochem. Biophys. Rep.* **2025**, *42*, 102037. [CrossRef]
8. Di Magno, L.; Di Pastena, F.; Bordone, R.; Coni, S.; Canettieri, G. The Mechanism of Action of Biguanides: New Answers to a Complex Question. *Cancers* **2022**, *14*, 3220. [CrossRef]
9. Pandey, A.; Tripathi, P.; Pandey, R.; Srivatava, R.; Goswami, S. Alternative therapies useful in the management of diabetes: A systematic review. *J. Pharm. Bioallied Sci.* **2011**, *3*, 504–512. [CrossRef]
10. Choudhury, H.; Pandey, M.; Hua, C.K.; Mun, C.S.; Jing, J.K.; Kong, L.; Ern, L.Y.; Ashraf, N.A.; Kit, S.W.; Yee, T.S.; et al. An update on natural compounds in the remedy of diabetes mellitus: A systematic review. *J. Tradit. Complement. Med.* **2018**, *8*, 361–376. [CrossRef] [PubMed]
11. Moradi, B.; Abbaszadeh, S.; Shahsavari, S.; Alizadeh, M.; Beyranvand, F. The most useful medicinal herbs to treat diabetes. *Biomed. Res. Ther.* **2018**, *5*, 2538–2551. [CrossRef]
12. Jugran, A.K.; Rawat, S.; Devkota, H.P.; Bhatt, I.D.; Rawal, R.S. Diabetes and plant-derived natural products: From ethnopharmacological approaches to their potential for modern drug discovery and development. *Phytother. Res.* **2021**, *35*, 223–245. [CrossRef] [PubMed]
13. Mahankali, S.; Kalava, J.; Garapati, Y.; Domathoti, B.; Maddumala, V.R.; Sundramurty, V.P. A Treatment to Cure Diabetes Using Plant-Based Drug Discovery. *Evid. Based Complement. Alternat. Med.* **2022**, *2022*, 8621665. [CrossRef] [PubMed]
14. Siddiqui, S.A.; Khan, S.; Wani, S.A. Controlling diabetes with the aid of medicinal herbs: A critical compilation of a decade of research. *Crit. Rev. Food Sci. Nutr.* **2023**, *63*, 12552–12566. [CrossRef]
15. Shabab, S.; Gholamnezhad, Z.; Mahmoudabady, M. Protective effects of medicinal plant against diabetes induced cardiac disorder: A review. *J. Ethnopharmacol.* **2021**, *265*, 113328. [CrossRef] [PubMed]
16. Djeujo, F.M.; Ragazzi, E.; Urettini, M.; Sauro, B.; Cichero, E.; Tonelli, M.; Froldi, G. Magnolol and Luteolin Inhibition of α-Glucosidase Activity: Kinetics and Type of Interaction Detected by In Vitro and In Silico Studies. *Pharmaceuticals* **2022**, *15*, 205. [CrossRef]
17. Nistor, M.; Pop, R.; Daescu, A.; Pintea, A.; Socaciu, C.; Rugina, D. Anthocyanins as Key Phytochemicals Acting for the Prevention of Metabolic Diseases: An Overview. *Molecules* **2022**, *27*, 4254. [CrossRef]
18. Blumfield, M.; Mayr, H.; De Vlieger, N.; Abbott, K.; Starck, C.; Fayet-Moore, F.; Marshall, S. Should We 'Eat a Rainbow'? An Umbrella Review of the Health Effects of Colorful Bioactive Pigments in Fruits and Vegetables. *Molecules* **2022**, *27*, 4061. [CrossRef]
19. Cooper, A.J.; Sharp, S.J.; Lentjes, M.A.; Luben, R.N.; Khaw, K.T.; Wareham, N.J.; Forouhi, N.G. A prospective study of the association between quantity and variety of fruit and vegetable intake and incident type 2 diabetes. *Diabetes Care* **2012**, *35*, 1293–1300. [CrossRef]
20. Scheer, H. Chlorophylls: A Personal Snapshot. *Molecules* **2022**, *27*, 1093. [CrossRef]
21. Martins, T.; Barros, A.N.; Rosa, E.; Antunes, L. Enhancing Health Benefits through Chlorophylls and Chlorophyll-Rich Agro-Food: A Comprehensive Review. *Molecules* **2023**, *28*, 5344. [CrossRef]
22. Cady, J.B.; Morgan, W.S. Treatment of chronic ulcers with chlorophyll: Review of a series of fifty cases. *Am. J. Surg.* **1948**, *75*, 562–569. [CrossRef]
23. Edwards, B.J. Treatment of chronic leg ulcers with ointment containing soluble chlorophyll. *Physiotherapy* **1954**, *40*, 177–179.
24. Jimenez-Aleman, G.H.; Castro, V.; Londaitsbehere, A.; Gutierrez-Rodríguez, M.; Garaigorta, U.; Solano, R.; Gastaminza, P. SARS-CoV-2 Fears Green: The Chlorophyll Catabolite Pheophorbide A Is a Potent Antiviral. *Pharmaceuticals* **2021**, *14*, 1048. [CrossRef]
25. Tamiaki, H. Chlorophylls. In *Fundamentals of Porphyrin Chemistry: A 21st Century Approach*; Brothers, P.J., Senge, M.O., Eds.; John Wiley & Sons: Hoboken, NJ, USA, 2022; Volume 2, pp. 743–776. [CrossRef]
26. Mishra, G.; Dash, S.P.; Mahapatra, S.K.; Swain, D.; Rout, G.R. Deeper insights into the physiological and metabolic functions of the pigments in plants and their applications: Beyond natural colorants. *Physiol. Plant.* **2025**, *177*, e70168. [CrossRef]
27. Milne, B.F.; Toker, Y.; Rubio, A.; Nielsen, S.B. Unraveling the intrinsic color of chlorophyll. *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 2170–2173. [CrossRef] [PubMed]
28. Zhong, S.; Bird, A.; Kopec, R.E. The Metabolism and Potential Bioactivity of Chlorophyll and Metallo-chlorophyll Derivatives in the Gastrointestinal Tract. *Mol. Nutr. Food Res.* **2021**, *65*, e2000761. [CrossRef]
29. Takamiya, K.I.; Tsuchiya, T.; Ohta, H. Degradation Pathway(s) of Chlorophyll: What Has Gene Cloning Revealed? *Trends Plant Sci.* **2000**, *5*, 426–431. [CrossRef]
30. Ferruzzi, M.G.; Failla, M.L.; Schwartz, S.J. Assessment of Degradation and Intestinal Cell Uptake of Carotenoids and Chlorophyll Derivatives from Spinach Puree Using an In Vitro Digestion and Caco-2 Human Cell Model. *J. Agric. Food Chem.* **2001**, *49*, 2082–2089. [CrossRef] [PubMed]

31. Canazza, E.; Tessari, P.; Mayr Marangon, C.; Lante, A. Nutritional Profile and Chlorophyll Intake of Collard Green as a Convenience Food. *Nutrients* **2024**, *16*, 4015. [CrossRef]
32. Roca, M.; Pérez-Gálvez, A. Application of EFSA EU menu database and R computing language to calculate the green chlorophyll intake in the European population. *Food Chem.* **2024**, *461*, 140912. [CrossRef] [PubMed]
33. Fernandes, T.M.; Gomes, B.B.; Lanfer-Marquez, U.M. Apparent absorption of chlorophyll from spinach in an assay with dogs. *Innov. Food Sci. Emerg. Technol.* **2007**, *8*, 426–432. [CrossRef]
34. Pérez-Gálvez, A.; Viera, I.; Roca, M. Carotenoids and Chlorophylls as Antioxidants. *Antioxidants* **2020**, *9*, 505. [CrossRef] [PubMed]
35. Viera, I.; Chen, K.; Ríos, J.J.; Benito, I.; Perez-Galvez, A.; Roca, M. First-pass metabolism of chlorophylls in mice. *Mol. Nutr. Food Res.* **2018**, *62*, e1800562. [CrossRef] [PubMed]
36. Isakau, H.A.; Trukhacheva, T.V.; Zhebentyaev, A.I.; Petrov, P.T. HPLC study of chlorin e6 and its molecular complex with polyvinylpyrrolidone. *Biomed. Chromatogr.* **2007**, *21*, 318–325. [CrossRef] [PubMed]
37. Viera, I.; Herrera, M.; Roca, M. Influence of food composition on chlorophyll bioaccessibility. *Food Chem.* **2022**, *386*, 132805. [CrossRef]
38. Li, Y.; Cui, Y.; Hu, X.; Liao, X.; Zhang, Y. Chlorophyll Supplementation in Early Life Prevents Diet-Induced Obesity and Modulates Gut Microbiota in Mice. *Mol. Nutr. Food Res.* **2019**, *63*, e1801219. [CrossRef]
39. Sim, L.; Quezada-Calvillo, R.; Sterchi, E.E.; Nichols, B.L.; Rose, D.R. Human intestinal maltase-glucoamylase: Crystal structure of the N-terminal catalytic subunit and basis of inhibition and substrate specificity. *J. Mol. Biol.* **2008**, *375*, 782–792. [CrossRef]
40. Yee, H.S.; Fong, N.T. A review of the safety and efficacy of acarbose in diabetes mellitus. *Pharmacotherapy* **1996**, *16*, 792–805. [CrossRef]
41. Liu, X.; Gu, Y.; Xu, W.; Zhang, R.; Koyama, T. Analysis for inhibition mechanisms of pheophytin a and pyropheophytin a against alpha-glucosidase by multi-spectroscopy; molecular docking, and molecular dynamics simulation. *LWT—Food Sci. Technol.* **2025**, *224*, 117828. [CrossRef]
42. Wang, X.; Yang, Z.; Shen, S.; Ji, X.; Chen, F.; Liao, X.; Zhang, H.; Zhang, Y. Inhibitory effects of chlorophylls and its derivative on starch digestion in vitro. *Food Chem.* **2023**, *413*, 135377. [CrossRef]
43. Turkiewicz, I.P.; Brzezowska, J.; Tkacz, K.; Wojdyło, A. Polysaccharide- and protein-based carriers as a key strategy for obtaining microencapsulated chlorophyll-rich extracts: UPLC-PDA/ESI-QToF-MS characterization and evaluation of antidiabetic potential. *Food Chem.* **2025**, *486*, 144627. [CrossRef]
44. Deacon, C.F. Dipeptidyl peptidase 4 inhibitors in the treatment of type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* **2020**, *16*, 642–653. [CrossRef]
45. Kim, M.J.; Kim, H.J.; Han, J.S. Pheophorbide A from *Gelidium amansii* improves postprandial hyperglycemia in diabetic mice through α-glucosidase inhibition. *Phytother. Res.* **2019**, *33*, 702–707. [CrossRef]
46. Martin, A.E.; Montgomery, P.A. Acarbose: An α-glucosidase inhibitor. *Am. J. Health Syst. Pharm.* **1996**, *53*, 2277–2337. [CrossRef]
47. Ren, L.; Qin, X.; Cao, X.; Wang, L.; Bai, F.; Bai, G.; Shen, Y. Structural insight into substrate specificity of human intestinal maltase-glucoamylase. *Protein Cell* **2011**, *2*, 827–836. [CrossRef]
48. Sun, S.M.; Yang, S.H.; Golokhvast, K.S.; Le, B.; Chung, G. Reconstructing the Phylogeny of *Capsosiphon fulvescens* (Ulotrichales, Chlorophyta) from Korea Based on *rbcL* and 18S rDNA Sequences. *BioMed Res. Int.* **2016**, *2016*, 1462916. [CrossRef] [PubMed]
49. Hong, C.O.; Nam, M.H.; Oh, J.S.; Lee, J.W.; Kim, C.T.; Park, K.W.; Lee, D.H.; Lee, K.W. Pheophorbide a from *Capsosiphon fulvescens* Inhibits Advanced Glycation End Products Mediated Endothelial Dysfunction. *Planta Medica* **2016**, *82*, 46–57. [CrossRef] [PubMed]
50. Khalid, M.; Petroianu, G.; Adem, A. Advanced Glycation End Products and Diabetes Mellitus: Mechanisms and Perspectives. *Biomolecules* **2022**, *12*, 542. [CrossRef] [PubMed]
51. Hwang, D.F.; Tsai, Y.S.; Chou, S.S.; Liu, S.M.; Wu, J.T.; Lin, S.J.; Tu, W.C. HPLC determination of pheophorbide a and pyropheophorbide a in dried laver product implicated in food poisoning. *J. Food Hyg. Soc. Jpn.* **2005**, *46*, 45–48. [CrossRef]
52. Rossi, E.; Borchard, K.; Cole, J.M. Pseudoporphyria following self-medication with chlorophyll. *Australas. J. Dermatol.* **2015**, *56*, 47–48. [CrossRef]
53. Zhao, C.Y.; Frew, J.W.; Muhaidat, J.; Cheung, K.; Lee, P.; Poulos, V.; McCrossin, I.; Cachia, A.R.; Tefany, F.; Murrell, D.F. Chlorophyll-induced pseudoporphyria with ongoing photosensitivity after cessation—A case series of four patients. *J. Eur. Acad. Dermatol. Venereol.* **2016**, *30*, 1239–1242. [CrossRef]
54. Cinčárová, D.; Hájek, J.; Dobřichovský, M.; Lukeš, M.; Hrouzek, P. Recommendations on the quantitative analysis of pheophorbides, photosensitizers present in algal biomass intended as food supplement. *Algal Res.* **2021**, *56*, 102298. [CrossRef]
55. Hellgren, L.I. Phytanic acid—An overlooked bioactive fatty acid in dairy fat? *Ann. N. Y. Acad. Sci.* **2010**, *1190*, 42–49. [CrossRef]
56. Roca-Saavedra, P.; Mariño-Lorenzo, P.; Miranda, J.M.; Porto-Arias, J.J.; Lamas, A.; Vazquez, B.I.; Franco, C.M.; Cepeda, A. Phytanic acid consumption and human health, risks, benefits and future trends: A review. *Food Chem.* **2017**, *221*, 237–247. [CrossRef]

57. Krauß, S.; Vetter, W. Phytol and phytyl fatty acid esters: Occurrence, concentrations, and relevance. *Eur. J. Lipid Sci. Technol.* **2018**, *120*, 1700387. [CrossRef]
58. Kitareewan, S.; Burka, L.T.; Tomer, K.B.; Parker, C.E.; Deterding, L.J.; Stevens, R.D.; Forman, B.M.; Mais, D.E.; Heyman, R.A.; McMorris, T.; et al. Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Mol. Biol. Cell* **1996**, *7*, 1153–1166. [CrossRef]
59. McCarty, M.F. The chlorophyll metabolite phytanic acid is a natural rexinoid--potential for treatment and prevention of diabetes. *Med. Hypotheses* **2001**, *56*, 217–219. [CrossRef]
60. Mukherjee, R.; Davies, P.J.; Crombie, D.L.; Bischoff, E.D.; Cesario, R.M.; Jow, L.; Hamann, L.G.; Boehm, M.F.; Mondon, C.E.; Nadzan, A.M.; et al. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* **1997**, *386*, 407–410. [CrossRef] [PubMed]
61. Elmazar, M.M.; El-Abhar, H.S.; Schaalan, M.F.; Farag, N.A. Phytol/Phytanic acid and insulin resistance: Potential role of phytanic acid proven by docking simulation and modulation of biochemical alterations. *PLoS ONE* **2013**, *8*, e45638. [CrossRef] [PubMed]
62. Heim, M.; Johnson, J.; Boess, F.; Bendik, I.; Weber, P.; Hunziker, W.; Fluhmann, B. Phytanic acid, a natural peroxisome proliferator-activated receptor agonist, regulates glucose metabolism in rat primary hepatocytes. *FASEB J.* **2002**, *16*, 718–720. [CrossRef]
63. Schlüter, A.; Yubero, P.; Iglesias, R.; Giralt, M.; Villarroya, F. The chlorophyll-derived metabolite phytanic acid induces white adipocyte differentiation. *Int. J. Obes.* **2002**, *26*, 1277–1280. [CrossRef]
64. Islam, M.T.; Bhuia, M.S.; de Lima, J.P.M.; Maia, F.P.A.; Ducati, A.B.H.; Coutinho, H.D.M. Phytanic acid, an inconclusive phytol metabolite: A review. *Curr. Res. Toxicol.* **2023**, *5*, 100120. [CrossRef]
65. Wunderlich, A.L.M.; Azevedo, S.C.S.F.; Yamada, L.A.; Bataglini, C.; Previate, C.; Campanholi, K.S.S.; Pereira, P.C.S.; Caetano, W.; Kaplum, V.; Nakamura, C.V.; et al. Chlorophyll treatment combined with photostimulation increases glycolysis and decreases oxidative stress in the liver of type 1 diabetic rats. *Braz. J. Med. Biol. Res.* **2020**, *53*, e8389. [CrossRef] [PubMed]
66. Tumolo, T.; Lanfer-Marquez, U.M. Copper chlorophyllin: A food colorant with bioactive properties? *Food Res. Int.* **2012**, *46*, 451–459. [CrossRef]
67. Nagini, S.; Palitti, F.; Natarajan, A.T. Chemopreventive Potential of Chlorophyllin: A Review of the Mechanisms of Action and Molecular Targets. *Nutr. Cancer* **2015**, *67*, 203–211. [CrossRef] [PubMed]
68. Ferruzzi, M.G.; Failla, M.L.; Schwartz, S.J. Sodium copper chlorophyllin: In vitro digestive stability and accumulation by Caco-2 human intestinal cells. *J. Agric. Food Chem.* **2002**, *50*, 2173–2179. [CrossRef]
69. Gertsch, J. The Metabolic Plant Feedback Hypothesis: How Plant Secondary Metabolites Nonspecifically Impact Human Health. *Planta Medica* **2016**, *82*, 920–929. [CrossRef]
70. Montelius, C.; Erlandsson, D.; Vitija, E.; Stenblom, E.L.; Egecioglu, E.; Erlanson-Albertsson, C. Body weight loss, reduced urge for palatable food and increased release of GLP-1 through daily supplementation with green-plant membranes for three months in overweight women. *Appetite* **2014**, *81*, 295–304. [CrossRef]
71. Stenblom, E.L.; Montelius, C.; Östbring, K.; Håkansson, M.; Nilsson, S.; Rehfeld, J.F.; Erlanson-Albertsson, C. Supplementation by thylakoids to a high carbohydrate meal decreases feelings of hunger, elevates CCK levels and prevents postprandial hypoglycaemia in overweight women. *Appetite* **2013**, *68*, 118–123. [CrossRef]
72. Amirinejad, A.; Heshmati, J.; Shidfar, F. Effects of thylakoid intake on appetite and weight loss: A systematic review. *J. Diabetes Metab. Disord.* **2019**, *19*, 565–573. [CrossRef] [PubMed]
73. Patar, A.K.; Sharma, A.; Syiem, D.; Bhan, S. Chlorophyllin supplementation modulates hyperglycemia-induced oxidative stress and apoptosis in liver of streptozotocin-administered mice. *BioFactors* **2018**, *44*, 418–430. [CrossRef] [PubMed]
74. Patar, A.K.; Bhan, S.; Syiem, D. Effect of chlorophyllin, a semi-synthetic chlorophyll molecule on hyperglycemia and hyperlipidemia in streptozotocin induced diabetic mice. *Int. J. Pharm. Pharm. Sci.* **2016**, *8*, 293–296.
75. Zheng, H.; You, Y.; Hua, M.; Wu, P.; Liu, Y.; Chen, Z.; Zhang, L.; Wei, H.; Li, Y.; Luo, M.; et al. Chlorophyllin Modulates Gut Microbiota and Inhibits Intestinal Inflammation to Ameliorate Hepatic Fibrosis in Mice. *Front. Physiol.* **2018**, *9*, 1671. [CrossRef]
76. Samadder, A.; Dey, S.; Sow, P.; Das, R.; Nandi, S.; Das, J.; Bhattacharjee, B.; Chakrovorty, A.; Biswas, M.; Guptaroy, P. Phytochlorophyllin Prevents Food Additive Induced Genotoxicity and Mitochondrial Dysfunction via Cytochrome c Mediated Pathway in Mice Model. *Comb. Chem. High Throughput Screen.* **2021**, *24*, 1618–1627. [CrossRef]
77. Ribeiro, T.; Reis, M.; Vasconcelos, V.; Urbatzka, R. Phenotypic screening in zebrafish larvae identifies promising cyanobacterial strains and pheophorbide a as insulin mimetics. *Sci. Rep.* **2024**, *14*, 32142. [CrossRef]
78. Sarkar, P.K.; Sarkerb, U.K.; Farhanab, F.; Alib, M.M.; Islamb, M.A.; Haquec, M.A.; Ishigamid, K.; Rokeyae, B.; Royb, B. Isolation and characterization of anti-diabetic compound from *Clerodendrum infortunatum* L. leaves. *S. Afr. J. Bot.* **2021**, *142*, 380–390. [CrossRef]
79. Pillai, J.S.; Ratheesh, R.; Nair, K.P.; Sanalkumar, M.G.; Thomson, R.J. Evaluation of the anti-diabetic potential of aqueous extract of *Clerodendrum infortunatum* L. in vivo in streptozotocin-induced diabetic Wistar rats. *Plant Sci. Today* **2019**, *6*, 1–7. [CrossRef]

80. Matsumoto, T.; Matsuno, M.; Ikui, N.; Mizushina, Y.; Omiya, Y.; Ishibashi, R.; Ueda, T.; Mizukami, H. Identification of pheophorbide a as an inhibitor of receptor for advanced glycation end products in *Mallotus japonicus*. *J. Nat. Med.* **2021**, *75*, 675–681. [CrossRef]
81. Paul, S.; Pallavi, A.; Gandasi, N.R. Exploring the potential of pheophorbide A, a chlorophyll-derived compound in modulating GLUT for maintaining glucose homeostasis. *Front. Endocrinol.* **2024**, *15*, 1330058. [CrossRef]
82. Holman, G.D. Structure, function and regulation of mammalian glucose transporters of the *SLC2* family. *Pflügers Arch.-Eur. J. Physiol.* **2020**, *472*, 1155–1175. [CrossRef]
83. Caspi, I.; Tremmel, D.M.; Pulecio, J.; Yang, D.; Liu, D.; Yan, J.; Odorico, J.S.; Huangfu, D. Glucose Transporters Are Key Components of the Human Glucostat. *Diabetes* **2024**, *73*, 1336–1351. [CrossRef] [PubMed]
84. Son, J.; Yang, S.M.; Yi, G.; Roh, Y.J.; Park, H.; Park, J.M.; Choi, M.G.; Koo, H. Folate-modified PLGA nanoparticles for tumor-targeted delivery of pheophorbide a in vivo. *Biochem. Biophys. Res. Commun.* **2018**, *498*, 523–528. [CrossRef]
85. Rybkin, A.Y.; Kurmaz, S.V.; Urakova, E.A.; Filatova, N.V.; Sizov, L.R.; Kozlov, A.V.; Koifman, M.O.; Goryachev, N.S. Nanoparticles of N-Vinylpyrrolidone Amphiphilic Copolymers and Pheophorbide a as Promising Photosensitizers for Photodynamic Therapy: Design, Properties and In Vitro Phototoxic Activity. *Pharmaceutics* **2023**, *15*, 273. [CrossRef] [PubMed]
86. Kim, S.H.; Lee, Y.; Lim, S.G.; Lee, C.; Park, J.S.; Koo, H. Pheophorbide a-loaded casein micelle for in vivo drug delivery and efficient photodynamic therapy. *J. Drug Deliv. Sci. Technol.* **2024**, *95*, 105598. [CrossRef]
87. Zhang, C.; Zhang, J.; Qin, Y.; Song, H.; Huang, P.; Wang, W.; Wang, C.; Li, C.; Wang, Y.; Kong, D. Co-delivery of doxorubicin and pheophorbide A by pluronic F127 micelles for chemo-photodynamic combination therapy of melanoma. *J. Mater. Chem. B* **2018**, *6*, 3305–3314. [CrossRef]
88. Paul, S.; Majumdar, M. Comparative study of six antidiabetic polyherbal formulation for its multimodal approaches in diabetes management. *3Biotech* **2022**, *12*, 114. [CrossRef]
89. Nyman, E.S.; Hynninen, P.H. Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. *J. Photochem. Photobiol. B Biol.* **2004**, *73*, 1–28. [CrossRef]
90. Brandis, A.S.; Salomon, Y.; Scherz, A. Chlorophyll sensitizers in photodynamic therapy. In *Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications*; Grimm, B., Porra, R.J., Rüdiger, W., Scheer, H., Eds.; Springer: Berlin/Heidelberg, Germany, 2006; pp. 461–483. [CrossRef]
91. Juzeniene, A.; Nielsen, K.P.; Moan, J. Biophysical aspects of photodynamic therapy. *J. Environ. Pathol. Toxicol. Oncol.* **2006**, *25*, 7–28. [CrossRef]
92. Ahn, M.Y.; Kwon, S.M.; Kim, Y.C.; Ahn, S.G.; Yoon, J.H. Pheophorbide a-mediated photodynamic therapy induces apoptotic cell death in murine oral squamous cell carcinoma in vitro and in vivo. *Oncol. Rep.* **2012**, *27*, 1772–1778. [CrossRef]
93. Mansoori, B.; Mohammadi, A.; Doustvandi, M.A.; Mohammadnejad, F.; Kamari, F.; Gjerstorff, M.F.; Baradaran, B.; Hamblin, M.R. Photodynamic therapy for cancer: Role of natural products. *Photodiagn. Photodyn. Ther.* **2019**, *26*, 395–404. [CrossRef]
94. Josefsen, L.B.; Boyle, R.W. Unique diagnostic and therapeutic roles of porphyrins and phthalocyanines in photodynamic therapy, imaging and theranostics. *Theranostics* **2012**, *2*, 916–966. [CrossRef] [PubMed]
95. Uguz, A.C.; Rocha-Pimienta, J.; Martillanes, S.; Garrido, M.; Espino, J.; Delgado-Adámez, J. Chlorophyll Pigments of Olive Leaves and Green Tea Extracts Differentially Affect Their Antioxidant and Anticancer Properties. *Molecules* **2023**, *28*, 2779. [CrossRef]
96. Ebrahimi, P.; Shokramraji, Z.; Tavakkoli, S.; Mihaylova, D.; Lante, A. Chlorophylls as Natural Bioactive Compounds Existing in Food By-Products: A Critical Review. *Plants* **2023**, *12*, 1533. [CrossRef]
97. Torres-Isidro, O.; González-Montoya, M.; Vargas-Vargas, M.A.; Florian-Rodriguez, U.; García-Berumen, C.I.; Montoya-Pérez, R.; Saavedra-Molina, A.; Calderón-Cortés, E.; Rodríguez-Orozco, A.R.; Cortés-Rojo, C. Anti-Aging Potential of Avocado Oil via Its Antioxidant Effects. *Pharmaceuticals* **2025**, *18*, 246. [CrossRef]
98. Hsu, C.; Chao, P.; Hu, S.; Yang, C. The Antioxidant and Free Radical Scavenging Activities of Chlorophylls and Pheophytins. *Food Nutr. Sci.* **2013**, *4*, 1–8. [CrossRef]
99. Subramoniam, A.; Asha, V.V.; Nair, S.A.; Sasidharan, S.P.; Sureshkumar, P.K.; Rajendran, K.N.; Karunagaran, D.; Ramalingam, K. Chlorophyll revisited: Anti-inflammatory activities of chlorophyll a and inhibition of expression of TNF-α gene by the same. *Inflammation* **2012**, *35*, 959–966. [CrossRef]
100. Marshall, L.J.; Bailey, J.; Cassotta, M.; Herrmann, K.; Pistollato, F. Poor Translatability of Biomedical Research Using Animals—A Narrative Review. *Altern. Lab. Anim.* **2023**, *51*, 102–135. [CrossRef] [PubMed]
101. Hartung, T. The (misleading) role of animal models in drug development. *Front. Drug Discov.* **2024**, *4*, 1355044. [CrossRef]

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Review

Orchestrating Nutrient Homeostasis: RNA-Binding Proteins as Molecular Conductors in Metabolic Disease Pathogenesis

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Abstract

RNA-binding proteins (RBPs) are critical regulators of post-transcriptional processes, playing essential roles in nutrient metabolism and metabolic homeostasis. This literature review explores how RBPs influence the metabolism of glucose, lipid, and amino acid metabolism by controlling processes like mRNA stability and translation regulation. The dysregulation of RBPs, including HuR, PTB, and YTHDF1, is linked to metabolic diseases such as obesity, diabetes, and non-alcoholic fatty liver disease. Advances in techniques like TREX technology and transcriptome analysis have deepened our understanding of RBP functions. Additionally, RBPs show promise as potential biomarkers and targets for new therapies. Future research directions in RBPs could focus on tissue-specific regulation and nutrient–RBP interactions. This could pave the way for more personalized treatments and improved metabolic health.

Keywords: RNA-binding protein; nutrients; obesity; diabetes; LACE-seq

1. Introduction

Nutrients are fundamental to human health, serving as essential components that support bodily functions from the macroscopic level of organ systems down to molecular and genetic processes [1]. Generally, there are six categories of nutrients: carbohydrates, lipids, proteins, vitamins, minerals, and water. Among these, carbohydrates, lipids, and proteins are considered macronutrients, which serve as the energy source of the body; vitamins and minerals are defined as micronutrients, which are needed in smaller amounts but are also vital for life [2]. Beyond energy provision and structural roles [3], nutrients and their metabolites serve as direct modifiers of protein activity [4], potent signaling molecules [5], and regulators of gene expression [6].

RNA-binding proteins (RBPs) are a diverse group of proteins that play a crucial role in the post-transcriptional regulation (within stages after RNA transcription, including RNA processing, stability, and translation efficiency) of eukaryotic transcriptomes [7]. These proteins interact with RNA molecules in various phases, from transcription to translation, and are involved in a wide range of biological processes. The primary function of RBPs is to bind to specific RNA sequences or structures, thereby influencing RNA stability, localization, translation, and degradation [8]. All of the RBPs have one or more RNA-binding domains (RBDs), which are specific motifs that allow them to interact with RNA (Figure 1). The most common RBDs include zinc finger motifs (ZnFs), RNA recognition

motifs (RRMs), cold shock domains (CSDs), K homology (KH) domains, and DEAD/DEAH box helicase domains [9]. The structural and sequence-specific features of RBDs allow RBPs to bind target RNA sequences or structures, such as hairpins, G-quadruplexes, or other secondary structures. This helps RBPs discriminate between different RNA molecules, even in the presence of highly similar sequences, thereby maintaining the fidelity of post-transcriptional regulation.

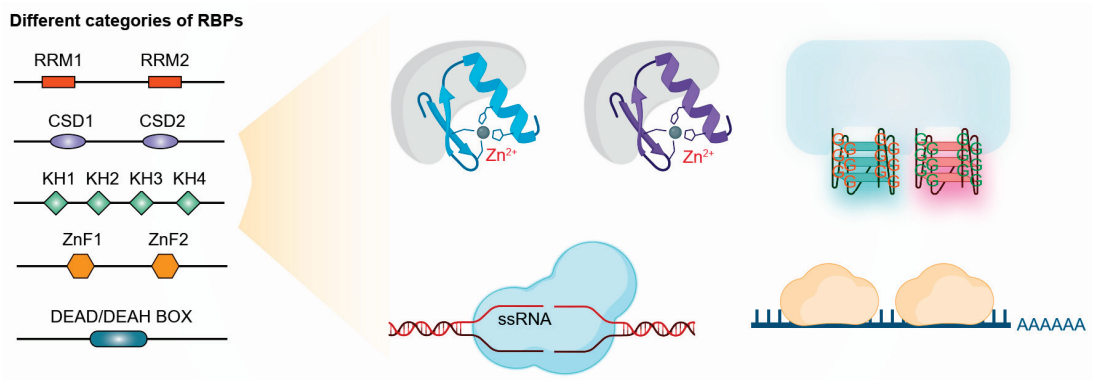


Figure 1. Different RNA-binding proteins categorized by domains and their binding models (RBP: RNA-binding proteins; RRM: RNA recognition motif; CSD: cold shock domains; KH: K homology domain; ZnF: zinc finger motif).

Understanding the roles of RBPs in nutrients is crucial for unraveling the mechanisms of nutrition regulation and for developing therapeutic strategies in various metabolic diseases. Previous reviews have primarily focused on RBPs in gene regulation or disease contexts. However, very few studies establish a relationship between RBPs and dietary nutrients.

In this review, we explore the intersection of RBPs and nutrient metabolism from an interdisciplinary perspective, focusing on their regulatory roles and functional significance in eukaryotic systems. We aim to summarize the current studies on how RBPs regulate macronutrient and micronutrient metabolism by modulating RNA stability, translation, and degradation, thereby shaping metabolic outputs. Then we elucidate the role RBPs play in the pathogenesis of metabolic disorders and medical treatment at present. We also highlight recent advancements in transcriptomics and technologies, such as Linear Amplification of Complementary DNA Ends and Sequencing (LACE-seq) and Capture RIC-seq (CRIC-seq), which have provided unprecedented insights into the dynamic interplay between RBPs and their target networks. Finally, we demonstrate future research directions, like tissue-specific regulation and nutrient–RBP interactions, which are instrumental to personalized therapies' efficiency and overall metabolic health improvement.

2. RBPs in Nutrient Sensing and Macronutrient Metabolism

2.1. Glucose Sensing

RBPs have been reported to transduce the signal of glucose levels in the blood, which promotes downstream reactions such as insulin release and glucagon synthesis.

Poly(C) binding protein 2 (PCBP2) is an RBP that possesses binding specificity for cytosine-rich sequences. The expression of PCBP2 is induced when pancreatic β-cells sense the high glucose level in the blood. β-cells lacking PCBP2 displayed impairments in calcium flux, exocytosis, and insulin secretion [10]. The mRNAs of islet cells were proven to be regulated by the RBP serine/arginine repetitive matrix protein 3 (SRRM3). When the glucose levels are raised, SRRM3 expression also increases. Mice with mutated SRRM3 bear islet cell dysfunction, contributing to hyperinsulinemia hypoglycemia [11].

Apolipoprotein B mRNA editing enzyme catalytic subunit 1 complementation factor (A1CF) is reported to regulate fructose metabolism through alternative splicing in hepatocytes. The target isoforms of A1CF include high-affinity fructose-metabolizing ketohexokinase C and glycerol kinase, both of which are metabolic enzymes participating in hepatic gluconeogenesis (Figure 2A). A1CF-deficient mice demonstrate enhanced glucose tolerance and are resistant to fructose-induced hyperglycemia, liver steatosis, and obesity development [12]. Qiao et al. presented that Sam68 reduces cAMP-response element binding protein-regulated transcription coactivator 2 ubiquitination, a critical transcriptional regulator of gluconeogenesis. Therefore, Sam68 knockouts in hepatocytes have significantly reduced blood glucose levels and the downregulated expression of gluconeogenic genes [13,14].

In hepatocytes, Grb10-interacting GYF protein 2 (GIGYF2) improves Staufen1 stability, which subsequently suppresses PI3K/AKT signaling and induces insulin resistance. When exposed to a high concentration of palmitic acid, the expression of GIGYF2 is upregulated, accompanied by reduced glucose uptake in HepG2 liver cancer cells [15].

Researchers have demonstrated that the formation of protein kinase R (PKR) and TAR RNA-binding protein (TRBP) regulates the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) [16]. TRBP overexpression aggravated glucose homeostasis impairment in obese mice, but the suppression of hepatic TRBP decreased eIF2α phosphorylation and improved systemic insulin resistance and glucose metabolism. Hence, the PKR-TRBP complex serves as a molecular hub linking metabolic stress to translational regulation, contributing to metabolic homeostasis.

2.2. Lipid Signaling and Adipogenesis

In the context of epigenetics, RBPs can influence the expression of genes involved in epigenetic pathways by stabilizing or degrading mRNAs that encode epigenetic enzymes. One well-established mechanism linking RBPs to epigenetic regulation is N6-methyladenosine (m6A), a common form of modification in eukaryotic mRNAs [17]. Fat mass and obesity-associated protein (FTO), a well-known m6A demethylase, promotes autophagosome formation and triglyceride accumulation (Figure 2B). When it is silenced, *Autophagy related 5* (*Atg5*) and *Atg7* transcripts with higher m6A levels were degraded by another RBP, YTH N6-methyladenosine RNA-binding protein F2 (YTHDF2), which alleviates autophagy and adipogenesis [18]. It was found that nicotinamide adenine dinucleotide phosphate (NADP) can directly bind FTO. The NADP/FTO axis increases both FTO activity and the triglyceride fold in 3T3-L1 preadipocytes [19]. Besides, it is reported that human antigen R (HuR) is a major repressor in the process of adipogenesis. HuR binds to and regulates the stability of numerous adipocyte transcripts, including *Insig1*, a key negative regulator of adipogenesis [20]. As a result, the knockout of HuR in the adipose tissue activates the adipogenic gene program, leading to systemic glucose intolerance and insulin resistance. Mynatt et al. demonstrated that the HuR in skeletal muscle modulates a group of genes involved in mitochondrial fatty acid oxidation and oxidative phosphorylation [21]. Another RBP non-POU domain containing octamer binding protein (NONO) was found to accumulate within speckle-like structures in liver cell nuclei. Mice with the removal of NONO have a different mode of energy homeostasis with a high rate of fat oxidation, and they remain lean from infant to adult [22].

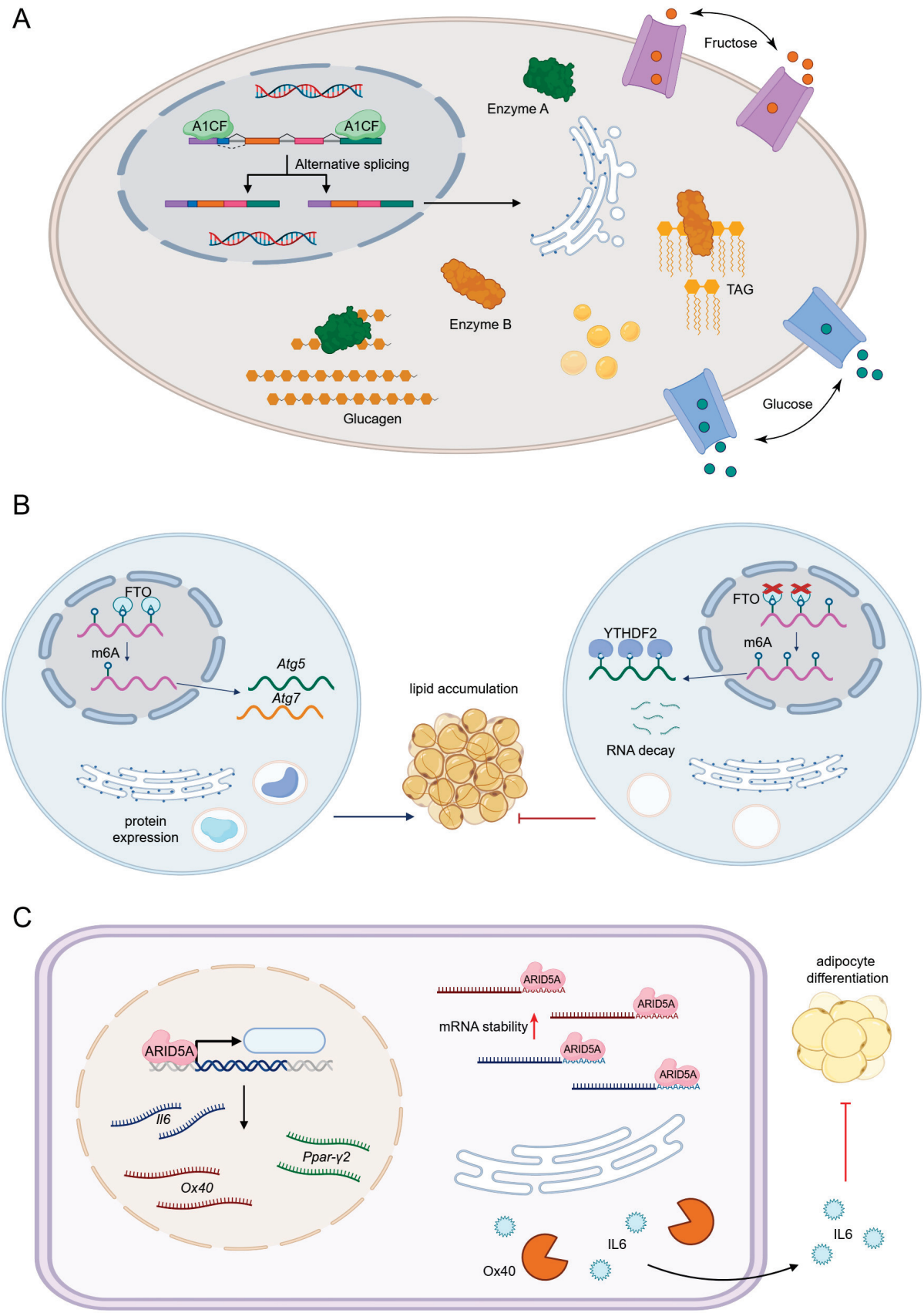


Figure 2. The participation of RBPs in different processes of nutrient metabolism. (**A**) A1CF participates in fructose metabolism through alternative splicing of mRNAs in hepatocytes. (**B**) FTO promotes triglyceride accumulation by m6A demethylation of mRNAs. (**C**) ARID5A regulates adipocyte differentiation

via mRNA 3′ untranslated region stabilization. (TAG: triacylglycerol; A1CF: APOBEC1 complementation factor; FTO: fat mass and obesity-associated protein; m6A: N6-methyladenosine; Atg: autophagy-related protein; YTHDF2: YTH N6-methyladenosine RNA-binding protein F2; ARID5A: AT-rich interaction domain 5A; IL6: interleukin 6; Ox40: TNF receptor superfamily member 4; Ppar-γ2: peroxisome proliferators-activated receptor gamma 2).

It is shown that DEAD-box helicase 1 (DDX1) is phosphorylated in the treatment of palmitate and subsequently releases insulin mRNA, which suppresses insulin translation and elevates glucose level [23]. Sam68, the Src-associated substrate during mitosis of 68 kDa, has demonstrated its vital role in adipogenesis. Through alternative splicing, Sam68 maintains the level of mammalian target of rapamycin (mTOR) transcripts; *Sam68*$^{-/-}$ mice have lower mTORC1/mTORC1 activity and fewer pericytes in white adipose tissue, protecting them against dietary-induced obesity [24].

AT-rich interaction domain 5A (ARID5A) has been identified to stabilize mRNAs by binding to their 3′ untranslated region (UTR) sequences, including Interleukin-6 (IL-6) [25] and Ox40 [26]. Given the connection between IL-6 and ARID5A signaling in adipogenesis, one study highlighted the role of ARID5A in regulating adipogenesis (Figure 2C). IL-6 failed to block adipocyte differentiation in ARID5A-deficient 3T3-L1 cells. Specifically, ARID5A suppressed adipogenesis by inhibiting the transcription of Ppar-γ2, a key regulator of adipocyte differentiation, within the nucleus [27].

2.3. Amino Acid Production and Metabolism

There is a group of RBPs that are indispensable in the transport and absorption of amino acids in cells.

Y-box binding protein 3 (YBX3) can be induced by cold stimulation and β-adrenergic signaling in skeletal muscle cells. It stabilizes thermogenesis-related gene transcripts such as the amino acid transporter solute carrier family 3 member 2 (Slc3a2) and Slc1a5 [28], promoting glutamine and branched-chain amino acid (BCAA) uptake [29]. As a result, it supports the proliferation and differentiation of skeletal muscle cells [30]. Musashi2 (MSI2) has been demonstrated as an oncogenic RBP that regulates branched chain amino acid transaminase 1 (BCAT1), a cytosolic aminotransferase for BCAAs. MSI2 contributes to the aberrant activation of BCAT1 in chronic myeloid leukemia cells, which uplifts the BCAA production [31].

m6A and its associated RBPs have been linked to pathological processes, including hematopoiesis impairment and metabolic disorders. RNA-binding motif protein 15 (RBM15) has been reported as an m6A methyltransferase, which directly binds to target serine and glycine metabolic genes, including PHGDH, PSAT1, PSPH, and SHMT2 [32]. Therefore, RBM15 increases the serine and glycine flux in cells. In a benzene-induced hematopoietic damage mouse model, insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) was found to be a critical RBP in BCAT1 and carnitine palmitoyltransferase 1A (CPT1A) metabolic enzymes, which improve hematopoietic injury via BCAA metabolism and fatty acid oxidation [33]. Moreover, IGF2BP2 has been reported to be involved in acute myeloid leukemia development by regulating a group of genes, including MYC, GPT2, and SLC1A5. By m6A modification, IGF2BP2 activates the gene expression in the glutamine metabolism pathways [34].

Angiogenin (ANG) is an RNase-A-family RBP known for promoting endothelial cell proliferation and angiogenesis [35]. Recent research has revealed a novel, non-vascular role of ANG in neuronal metabolism. Specifically, in glutamatergic neurons of the adult brain, ANG undergoes dephosphorylation-induced translocation from the nucleus to the cytoplasm. Once in the cytoplasm, ANG interacts with tRNAs and catalyzes the precise cleavage of nucleus-encoded $tRNA^{Glu}$, generating a specific transfer-RNA-derived small

RNA known as Glu-5′ tsRNA-CTC. This tsRNA exerts downstream effects by binding to leucyl-tRNA synthetase 2 (LARS2), an essential mitochondrial enzyme responsible for aminoacylating mitochondrial $tRNA^{Leu}$ (mt-$tRNA^{Leu}$) with leucine. Upon complex formation, Glu-5′ tsRNA-CTC and LARS2 translocate to the mitochondria, where the interaction inhibits the aminoacylation activity of LARS2. Consequently, this blockade disrupts the mitochondrial translation machinery by impairing the synthesis of proteins [36].

3. Interactive Influence Between Micronutrients and RBPs

3.1. Vitamins

Retinoic acid (RA), the derivative of vitamin A, is considered a chemopreventive agent for breast cancer. RA treatment recovers the translation level of HOXA5, which controls cell growth. This process needs the participation of HuR, which binds to 3′ UTR of HOXA5 mRNA and increases its stability [37].

Insulin-like growth factor-2 mRNA-binding protein 3 (IGF2BP3) has been implicated in inflammatory responses. In a high glucose (HG)-induced retinal pigment epithelial (RPE) cell model, ascorbic acid (vitamin C) was shown to alleviate HG-induced RPE cell damage by inhibiting the NF-κB signaling pathway through regulation of the MALAT1/IGF2BP3 axis [38]. In one study, dietary vitamin C shows therapeutic potential in Fragile X Syndrome (FX) by reducing DNA methylation at the X-linked Fragile X Messenger Ribonucleoprotein 1 (FMR1) gene locus, thereby restoring its expression in FX-induced pluripotent stem cells (iPSCs) and cerebral organoids [39]. Given that certain RNA-binding proteins possess methyltransferase activity, further research may focus on which RNA-binding protein participates in the FMR1 regulation in the presence of vitamin C.

As for cobalamin (vitamin B12), it has been reported that patients with inborn defects in cobalamin metabolism have altered shuttling and splicing of the mRNA repertoire, caused by the dysfunction of RBPs, including ELAVL1/HuR, HnRNPA1, and RBM10 [40].

Researchers have demonstrated that the levels of the vitamin D receptor (VDR) in the intestinal mucosa decreased significantly in mice with the removal of HuR. In intestinal epithelial cells (IECs), Vdr mRNA is modulated by HuR, which binds to the 3′-untranslated region (UTR) and thus enhances VDR translation [41]. Another study about ovarian cancer has shown the synergistic effect of VDR and RBP. It is reported that the expression of lncRNA TOPORS antisense RNA 1 (TOPORS-AS1) is positively correlated with the overall survival of patients. The vitamin D receptor (VDR) upregulated the expression of TOPORS-AS1 while hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2B1) directly interacted with TOPORS-AS1, both of which elicit the inhibition of β-catenin and suppress the proliferation of ovarian cancer cells [42].

3.2. Iron Homeostasis

IRP1 is an RNA-binding protein that regulates iron homeostasis post-transcriptionally [43]. Researchers have found that it binds to iron-responsive elements (IREs), which are stem-loop structures in the 5′ UTR of ferritin mRNA and the 3′ UTR of transferrin receptor mRNA. IRP1 binds to 5′ UTR IREs to inhibit translation in iron-deficient cells, while it stabilizes the mRNA and prevents degradation when binding to 3′ UTR IREs [44]. In this way, IRP1 maintains appropriate intracellular iron levels.

HAMP encodes hepcidin, another key regulator of systemic iron homeostasis. The 3′ UTR of HAMP mRNA is enriched with canonical cytoplasmic polyadenylation element (CPE) motifs [45], which have been identified as binding sites for the RNA-binding protein CPEB4 [46]. Notably, CPEB4 plays a critical role in modulating cancer cell sensitivity to ferroptosis, a form of regulated cell death driven by iron-dependent lipid peroxidation. Loss of CPEB4 function leads to reduced hepcidin expression, resulting in the upregulation

of ferroportin and decreased intracellular iron levels. These changes collectively suppress ferroptosis, highlighting the significance of the CPEB4-hepcidin axis in iron metabolism and cancer cell death pathways [47].

YTH N6-methyladenosine RNA-binding protein F1 (YTHDF1) and YTHDF2 play an essential role in binding ferritin heavy chain 1 (FTH1) mRNA to improve its stability and translation. The knockdown of FTO, the m6A demethylase, leads to increased m6A modification on FTH1 mRNA. This impairs recognition by the m6A reader proteins YTHDF1 and YTHDF2, resulting in reduced intracellular levels of FTH1. Without sufficient FTH1 to bind and store Fe^{2+}, spermatogenic cells undergo ferroptosis [48].

4. Dysregulation of RBPs and Metabolic Diseases

4.1. Obesity and Insulin Resistance

Obesity, recognized as a globally prevalent metabolic disorder, arises from dysregulated energy homeostasis and lipid metabolism [49]. Emerging evidence has elucidated the mechanistic role of RBPs in modulating obesity-associated pathways through post-transcriptional regulation [50,51]. In general, RBPs can directly govern the expression of genes critical for adipogenesis, thermogenic energy expenditure, and insulin signaling cascades.

RBPs contribute to obesity through regulating genes related to thermogenesis. The RNA-binding protein quaking (QKI) is a vital regulator of the metabolic homeostasis of adipose tissue. Studies have shown that QKI-deficient mice exhibit resistance to high-fat diet (HFD)-induced obesity, primarily due to enhanced energy expenditure. Specifically, QKI depletion promotes thermogenic activity by increasing energy dissipation in brown adipose tissue and stimulating the browning of subcutaneous white adipose tissue. Functionally, QKI suppresses energy consumption in adipose tissue by destabilizing mRNAs and impairing their nuclear export and translation—particularly those encoding key thermogenic regulators UCP1 (uncoupling protein 1) and PGC1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) [52]. Dai et al. found that *Imp2*$^{-/-}$ mice exhibit marked resistance to diet-induced obesity and hepatic steatosis. Adipocytes in the mice model with Imp knockout demonstrate significantly enhanced uncoupled oxygen consumption, indicative of elevated thermogenic activity. IMP2 has been shown to bind to the mRNAs encoding UCP1 and various mitochondrial components, where it acts as a translational repressor. In the absence of IMP2, these target mRNAs display increased translational efficiency, contributing to enhanced mitochondrial function and energy expenditure [53].

Another role of RNA-binding proteins (RBPs) is their active involvement in the regulation of adipogenesis. It is reported that paraspeckle component 1 (PSPC1) promotes obesity by facilitating adipogenesis and enhancing fat storage. As an RNA-binding protein, it regulates key adipogenic RNAs, such as EBF1, and collaborates with DEAD-box helicase 3 X-linked (DDX3X) to export these RNAs during cell differentiation. This process increases adipocyte formation and lipid accumulation. Furthermore, in mice lacking PSPC1 specifically in adipose tissue, there is reduced fat mass and lipid storage, leading to resistance to diet-induced obesity and insulin resistance. This protective effect stems from a compensatory increase in energy expenditure [54]. One clinical trial found that Paral1, a long intergenic noncoding RNA (lincRNA), is a novel biomarker of obesity-related dysregulation of adipose tissue function [55]. Paral1 acts as an obesity-sensitive regulator of adipocyte differentiation and metabolic function. Paral1 exerts its effect through interactions with RBM14, a paraspeckle-associated RNA-binding protein with hnRNP-like features. It promotes adipogenesis by acting as a coactivator of peroxisome proliferator-activated receptor gamma (PPARγ), a key transcription factor governing adipocyte development. One study showcased the pivotal role of YBX1 in driving fat accumulation, a core feature of obesity. YBX1 is a 5-methylcytosine (m5C)-binding protein, and its deficiency inhibits adipocyte

differentiation in murine models while the overexpression of YBX1 in white adipose tissue increases ULK1/ULK2 expression and adipogenesis by promoting autophagy controlled by ULK1 and ULK2. Mechanistically, YBX1 directly targets and stabilizes m5C-modified Ulk1 mRNA, thereby upregulating ULK1 expression. Simultaneously, YBX1 acts as a transcription factor to promote Ulk2 transcription and expression. Collectively, YBX1 controls autophagy and adipogenesis, positioning it as a key regulator for obesity driven by adipose tissue expansion [56].

Recently, researchers developed adipose-specific zinc finger protein 36 knockout (ZFP36 KO) mice and exposed them to a 16-week high-fat diet (HFD). They elucidated that ZFP36 deficiency-driven obesity manifested through white adipose tissue adipocyte hypertrophy, mediated by the reduced expression of lipid metabolism regulators PLIN1, ATGL, and HSL. Mechanistically, ZFP36 directly suppressed RNF128 expression through dual inhibition of mRNA stability and translation, which enhanced Sirt1-mediated metabolic homeostasis. Their findings establish adipose ZFP36 as a critical modulator of diet-induced obesity in the ZFP36/RNF128/Sirt1 signaling axis [57]. Meng et al. demonstrated that hnRNPA2B1 (A2B1) mRNA levels are elevated in the epididymal white adipose tissue (eWAT) and spleen of high-fat diet (HFD)-induced obese mice, as well as in colon tissues during inflammation. Notably, mice with a haploinsufficiency of A2B1 exhibit resistance to HFD-induced obesity, characterized by reduced white adipose tissue expansion. Mechanistically, A2B1 enhanced the RNA stability of pro-inflammatory genes, including TNF-α, IL-6, and IL-1β, for the intensified polarization of pro-inflammatory M1 macrophages in eWAT [58].

As for hormones, PTB (polypyrimidine tract-binding protein) plays a critical role in the post-transcriptional regulation of adiponectin receptor 1 (AdipoR1), which mediates adiponectin's metabolic benefits in muscle and liver. PTB binds to the 3′ UTR of AdipoR1 mRNA and cooperates with microRNA-221 (miR-221) to suppress its translation. In mouse models of obesity, both PTB and miR-221 are upregulated in muscle and liver tissues, contributing to the pathological decline in AdipoR1 protein. Reduced AdipoR1 exacerbates insulin resistance by diminishing adiponectin's ability to enhance glucose uptake and lipid metabolism. Conversely, the depletion of PTB or miR-221 restores AdipoR1 translation, suggesting that targeting PTB could rescue adiponectin signaling and ameliorate metabolic dysfunction in obesity [59]. ZFP36L1 is an RBP that binds to AU-rich elements in the 3′ UTRs of mRNAs for their degradation [60], which was identified as a target gene of the bile-acid-activated nuclear receptor called farnesoid X receptor (FXR). It participates in bile acid metabolism and lipid homeostasis. Experimental studies demonstrate that modulating ZFP36L1 function reciprocally controls Cyp7a1 mRNA stability and bile acid levels in vivo. Mice lacking hepatic ZFP36L1 exhibit resistance to diet-induced obesity and hepatosteatosis. These findings establish ZFP36L1 as a key metabolic regulator linking FXR signaling to obesity and fatty liver disease through bile-acid-dependent pathways [61].

4.2. Non-Alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is characterized by impaired lipid metabolism, fat accumulation, and lipid droplet formation. NALFD has the risk of developing liver fibrosis, cirrhosis, or even hepatocellular carcinoma [62]. RBPs are associated with the pathogenesis of NAFLD for their involvement in triglyceride metabolism.

The RNA-binding protein HuR (ELAVL1), which is popular among researchers, has been proven to take an active part in the development of NALFD [63]. In the cytoplasm, HuR binds to AU-rich elements (AREs) within the 3′ UTR of target mRNAs via its RNA recognition motifs (RRMs), thereby enhancing mRNA stability by preventing degradation

and deadenylation. RRM1 and RRM2 typically recognize A/U-rich or U-rich sequences, while RRM3 specifically binds to U-rich regions [64]. Liu and his colleagues have demonstrated that HuR acts as a dual regulator of intestinal triglyceride (TAG) synthesis by modulating the post-transcriptional stability of Dgat2 mRNA and the splicing efficiency of Mgat2 pre-mRNA. Specifically, HuR binds to the 3′ untranslated region (UTR) of Dgat2 mRNA to enhance its stability, while simultaneously interacting with intron 1 of Mgat2 pre-mRNA to facilitate its processing into mature mRNA. These complementary mechanisms elevate the expression of DGAT2 and MGAT2, key enzymes in intestinal TAG synthesis, thereby promoting dietary fat absorption. Consequently, HuR deficiency mitigates high-fat diet (HFD)-induced non-alcoholic fatty liver disease (NAFLD) and obesity [65]. HuR also plays a protective role against metabolic syndrome-related diseases, primarily by regulating cholesterol transport. ABCB1 is a key mediator of reverse cholesterol transport, facilitating cholesterol efflux and HDL formation. It has been shown that HuR enhances ABCA1 expression by stabilizing its mRNA through binding to the 3′ UTR. Silencing HuR can result in decreased ABCA1 levels and reduced cholesterol efflux to APOA1 in hepatocytes [66].

One study reveals that QKI 5, a critical RNA-binding protein, regulates hepatic triglyceride synthesis in a non-alcoholic fatty liver disease (NAFLD) model. QKI 5 upregulated the expression of PPARα through post-transcriptional regulation, which further inhibited TAG synthesis and NAFLD progression [67]. Insulin-like growth factor 2 mRNA-binding protein 2 (IMP2) is involved in hepatic triglyceride metabolism. Mice with liver-specific IMP2 overexpression are prone to steatosis. Hepatocyte-specific IMP2 knockout exacerbates diet-induced fatty liver by impairing fatty acid oxidation, resulting from the increased degradation of IMP2 target mRNAs, PPARα, and CPT1A. These findings suggest that IMP2 deficiency is attributed to the reduced adiposity [68].

4.3. Diabetes

For mammals, the primary cause of diabetes is the dysfunction or loss of pancreatic β-cells, which are specialized cells located in the islets of Langerhans within the pancreas. These β-cells are responsible for secreting insulin, a hormone essential for maintaining blood glucose homeostasis [69]. However, when these cells become dysfunctional—either through impaired insulin secretion, loss of β-cell mass, or both—this regulation fails, resulting in chronic hyperglycemia, the hallmark of diabetes mellitus [70]. Clinical observations have reported significantly altered expression levels of multiple proteins in patients with diabetes [71,72]. Since the dysregulation of RBPs can induce functional abnormalities in their downstream target genes, these protein-level disturbances correlate with disease progression.

There are two major mechanisms through which RBPs are involved in diabetes. First, RBPs can affect the process of insulin secretion. Stoll et al. demonstrated that there are numerous circular RNAs (circRNAs) that are expressed in pancreatic islets, and many of them regulate β-cell activities. The researchers further found an interaction between the RBP, trans-activator regulatory DNA-binding protein 43 (TDP-43), and intron lariats of ci-Ins2. The abundance of this circularized intron is decreased in the islets of both rodent models of diabetes and individuals with type 2 diabetes. Since ci-Ins2 promotes insulin release, the existence of TDP-43 is crucial for β-cells to secrete insulin [73]. CUG-binding protein 1 (CUGBP1) is a multifunctional RBP that regulates various aspects of RNA metabolism, preferentially binding to GU-rich elements [74]. CUGBP1 is expressed in rodent islets and β-cell lines and is notably upregulated in the islets of diabetic mice [75]. It is demonstrated that pancreas-specific overexpression of CUGBP1 impairs glucose tolerance, as evidenced by reduced plasma insulin levels following glucose challenge and delayed glucose clearance. Conversely, CUGBP1 knockdown improves insulin secretion and glucose handling, indicating a deleterious role for CUGBP1 in glucose homeostasis.

Mechanistically, CUGBP1 overexpression attenuates glucose- and GLP-1-stimulated insulin secretion, which correlates with a marked reduction in intracellular cAMP levels—a key second messenger in insulin exocytosis. CUGBP1 specifically increases the expression of PDE3B, an enzyme known to inhibit insulin secretion by lowering cAMP. This occurs through direct binding of CUGBP1 to an ATTTGTT motif in the 3′ UTR of PDE3B mRNA, thereby stabilizing the transcript and promoting its accumulation. Notably, both CUGBP1 and PDE3B are also overexpressed in diabetic islets, further supporting a pathological link between the CUGBP1–PDE3B axis and β-cell dysfunction. Poly(A)-specific ribonuclease (PARN) is widely recognized for its role in regulating mRNA stability through poly(A) tail shortening [76]. Recent findings have reported its involvement in pancreatic β-cell physiology [77]. From β-cell-specific PARN knockout mice, the absence of PARN leads to a marked reduction in glucose-stimulated insulin secretion (GSIS). Transcriptomic profiling of PARN-deficient β-cells reveals the misregulation of genes essential for insulin secretion. In detail, PARN interacts with the RNA-binding protein polypyrimidine tract binding protein 1 (PTBP1) to regulate the stability of key transcripts, including Slc30a8, which encodes a zinc transporter vital for insulin crystallization, and Chst3, which is implicated in vesicle trafficking and secretion. Disruption of either PARN or PTBP1 compromises the stability of these mRNAs, leading to defects in insulin maturation and exocytosis. Therefore, PARN serves as a crucial regulator of β-cell secretory capacity, and targeting the PARN–PTBP1–mRNA axis may offer novel therapeutic avenues for improving insulin secretion in diabetes.

In addition, RBPs contribute to the maintenance of pancreatic cell homeostasis and the dynamic modulation of the microenvironment. The RNA-binding protein HuD plays a vital role in post-transcriptional gene regulation and has been implicated in the pathogenesis of various diseases, including diabetes [78,79]. Hong et al. found that in both db/db diabetic mice and HuD knockout models, mitochondrial fragmentation was increased, indicating impaired mitochondrial fusion, a main phenotype of β-cell failure in diabetes [80]. HuD binds to the 3′ UTR of mitofusin 2 (Mfn2) mRNA, a key mediator of mitochondrial fusion, and positively regulates its expression. In vitro, HuD depletion in mouse insulinoma βTC6 cells leads to a higher proportion of cells with fragmented mitochondria, accompanied by decreased mitochondrial membrane potential and reduced ATP production, reflecting diminished mitochondrial function. Notably, the ectopic expression of Mfn2 in HuD-depleted cells was sufficient to rescue mitochondrial morphology and function, confirming that HuD exerts its protective role via the HuD–Mfn2 axis. Another group of researchers identified endostatin and Serpin E1—two proteins with well-known anti-angiogenic properties—as HuD-regulated factors by using a cytokine array in mouse insulinoma βTC6 cells [81]. Specifically, HuD knockdown led to elevated levels of Col18a1 (the precursor of endostatin) and Serpin E1 by binding to their 3′ UTRs and repressing their translation. Functional analyses using co-culture systems of βTC6 cells and islet endothelial MS1 cells revealed that HuD downregulation impaired endothelial cell growth and migration, indicating a key role for HuD in mediating β-cell–endothelial cell crosstalk. Moreover, ectopic expressions of HuD in db/db diabetic mice reduced Col18a1 and Serpin E1 expression while enhancing the expression of vascular markers in islet tissue, suggesting a protective role in maintaining islet vascular homeostasis. A genome-wide analysis of circRNA expression in peripheral blood mononuclear cells (PBMCs) from type 1 diabetes mellitus (T1DM) patients identified circular protein phosphatase 1F (circPPM1F) as significantly upregulated compared to healthy controls [82]. In streptozotocin (STZ)-induced diabetic mice, circPPM1F overexpression worsened pancreatic damage through its activation of M1 macrophages. Functionally, circPPM1F promotes lipopolysaccharide (LPS)-induced M1 macrophage activation by enhancing NF-κB signaling, a key inflammatory pathway implicated in β-cell destruction

in T1DM. Mechanistically, circPPM1F acts as a molecular sponge for HuR, competitively binding to it and thereby impairing HuR-mediated translation of PPM1F—a negative regulator of NF-κB signaling. By inhibiting PPM1F translation, circPPM1F removes a critical brake on inflammatory activation, thereby exacerbating macrophage-driven pancreatic injury. The elevated expression of circPPM1F is maintained by the RBPs—EIF4A3 and FUS, highlighting a complex RBP-regulated network in T1DM pathogenesis.

5. Research Methods and Technological Advances

5.1. CRISPR/Cas9 Library Screening: Identifying Metabolism-Related RBPs

Clustered regularly interspaced short palindromic repeats and their associated protein 9 (CRISPR/Cas9)-based functional genomics have emerged as a powerful tool for uncovering metabolism-related RBPs. Pooled knockout screens using sgRNA libraries targeting hundreds to thousands of RBP genes have pinpointed factors like INTS3, whose loss induces apoptosis in colorectal cancer cells [83]. Patrick et al. demonstrated a reporter-based RNA-linked CRISPR (ReLiC), which embeds barcoded RNA reporters at a defined genomic locus to directly assay diverse RNA metabolic events—splicing, translation, and decay—upon gene knockout [84]. Single-cell CRISPR screens such as Perturb-seq (Figure 3A) integrate pooled CRISPR perturbations with single-cell RNA sequencing to map transcriptome-wide effects of RBP loss on metabolic gene programs [85]. Amit et al. adopted focused sub-genomic CRISPR screens and revealed putative RBP vulnerability in MLL-translocated B-ALL [86]. CRISPR interference/activation (CRISPRi/a) platforms modulate RBP expression without double-strand breaks, facilitating the interrogation of essential or dosage-sensitive RBPs in metabolism [87]. Overall, these advanced CRISPR/Cas9 techniques enable more precise validation of the specific functions of RBPs.

5.2. TREX Technology: Mapping RNA–Protein Interaction Networks in Live Cells

TREX, with the full name of targeted RNase H-mediated extraction of crosslinked RBPs, is a revolutionary technique (Figure 3B). It is designed to identify RBPs that interact with specific RNA regions in living cells under physiological conditions [88]. Unlike existing methods, this approach combines RNase H-mediated cleavage with crosslinking to isolate proteins that directly bind to defined RNA regions. TREX uses two-step phase separation: the first one is after UV-C crosslinking, which extracts all the RNA–protein adducts; the second one is after antisense DNA oligonucleotides hybridize to the target RNA, which releases the RBPs for further quantitative mass spectrometry (MS) analysis. Since it enables the precise removal of unrelated RNA–protein adducts and the enrichment of RBPs binding to the RNA sequence of interest, it offers unparalleled sensitivity and specificity compared to other techniques. For example, TREX detects up to 27 direct U1 snRNA interactors, in contrast to the current reported 10 U1 snRNP components [89,90]. By systematically mapping the interactome of 45S rRNA and NORAD long noncoding RNA, TREX has shown its effectiveness in identifying functional interactors of a specific RNA region under endogenous settings. Hence, TREX could serve as a powerful RNA-centric methodology for studying positional RNA–protein interactions across diverse cell types, providing researchers with a robust tool to explore the nutrient conversion and regulation in living systems.

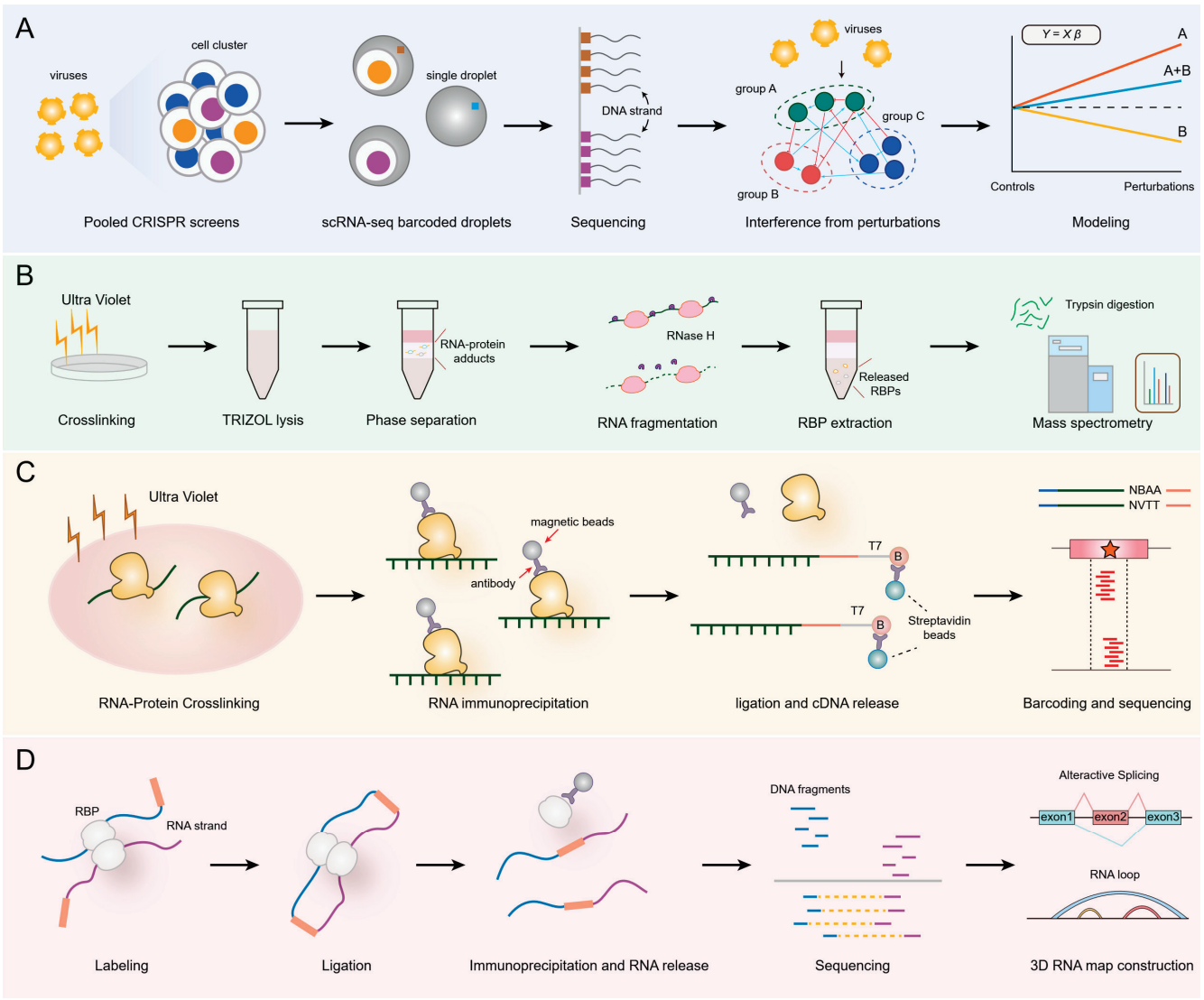


Figure 3. Advanced technologies in discovering the characteristics of RBPs. (**A**) Perturb Sequencing (Perturb-seq). (**B**) Targeted RNase H-mediated extraction (TREX) technology. (**C**) Linear Amplification of Complementary DNA Ends and Sequencing (LACE-seq). (**D**) Captured RNA in situ conformation sequencing (CRIC-seq).

5.3. LACE-Seq

LACE-seq, which is short for Linear Amplification of Complementary DNA Ends and Sequencing, is an advanced technique developed to profile RBP target sites across the transcriptome (Figure 3C), particularly in low-input samples such as oocytes [91] and spermatocytes [92]. This method addresses the limitations of traditional approaches like crosslinking-immunoprecipitation and high-throughput sequencing (CLIP-seq), which often require large amounts of starting material and may not be suitable for studying rare cell populations [93].

This technique has been widely applied to study various RBPs in mouse models. For instance, Su et al. [94] utilized LACE-seq to investigate the binding sites of RBPs such as Argonaute 2 (Ago2), Mili, and Ddx4. Their findings revealed that Ago2 interacts with endogenous small interfering RNAs (endo-siRNAs) to globally repress mRNA translation in oocytes. Furthermore, the Ago2-endo-siRNA complexes were found to fine-tune the transcriptome by slicing long terminal repeat retrotransposon-derived chimeric transcripts. In another study, LACE-seq was employed to analyze the binding patterns of eukaryotic

translation initiation factor 4E family member 1B (eIF4E1B), a translation initiation factor, in mouse oocytes [95]. The results indicated that eIF4E1B preferentially binds to the 5′ UTRs of target mRNAs, particularly those containing GC-rich motifs, thereby selectively activating the translation of maternal mRNAs during the oocyte-to-embryo transition. Lei et al. utilized LACE-seq to demonstrate that SRSF1 plays an essential role in spermatogenesis and male fertility by regulating alternative splicing (AS). Male mice with germ cell-specific deletion of Srsf1 bear complete infertility, which has the abnormal AS of genes involved in the spermatogenesis process, such as Stra8, Dazl, and Dmc [96].

5.4. CRIC-Seq

CRIC-seq is an innovative technique designed to map RNA–RNA interactions mediated by specific RNA-binding proteins (RBPs) within living cells, offering profound insights into post-transcriptional gene regulation [97]. Building upon the foundation of RNA in situ conformation sequencing (RIC-seq), CRIC-seq introduces an immunoprecipitation step to isolate RNA–RNA contacts associated with particular RBPs (Figure 3D), thereby enhancing the specificity and depth of interaction profiling [98]. While earlier techniques like hiCLIP primarily detected RNA–RNA interactions based on sequence complementarity, CRIC-seq's use of formaldehyde crosslinking allows for the capture of protein-mediated interactions, which allows one to detect long-range RNA interactions and provides a more comprehensive view of the RBP-associated RNA interactome [99].

CRIC-seq is a useful tool for elucidating the role of RBPs in alternative splicing. For example, studies utilizing CRIC-seq to investigate PTBP1, SRSF1, and hnRNPA1 in HeLa cells revealed that PTBP1-associated RNA loops within individual introns tend to promote exon inclusion by facilitating asymmetric intron removal. Conversely, loops spanning across exons often repress splicing. These findings underscore the significance of the spatial positioning of RBP-mediated RNA loops in determining splicing outcomes [100].

6. Clinical Translation and Future Directions

6.1. Therapeutic Strategies Targeting RBPs for Metabolic Diseases

Due to the dysregulation of RBPs in metabolic disorders such as obesity, NAFLD, and diabetes, therapeutic strategies aimed at modulating RBP activity are gaining traction.

Zhuang and his colleagues highlight niclosamide (NCS), an HuR inhibitor, as a potential therapeutic agent for Diabetic Nephropathy (DN) [101]. NCS treatment in db/db mice reduced hyperglycemia (lowered blood glucose and HbA1c) without affecting body weight, effectively halting DN progression. HuR activation upregulated Wisp1, a downstream effector of Wnt1 signaling, in diabetic kidneys, while NCS treatment restored Wisp1 to normal levels. HuR inhibition subsequently attenuated renal inflammation by suppressing NF-κBp65, TNF-α, and MCP-1 expression.

Wang et al. reveal that endostatin inhibits adipogenesis and dietary-induced obesity. Endostatin exerts its anti-adipogenic effects by interacting with the RNA-binding protein Sam68 within the nuclei of preadipocytes. This interaction competitively disrupts Sam68's binding to intron 5 of the mammalian target of the rapamycin (mTOR) transcript, resulting in aberrant mTOR mRNA processing. As a consequence, mTOR expression is reduced, leading to diminished activity of the mTOR complex 1 (mTORC1) pathway and impaired adipogenesis [102].

One interesting study showed that BCAA supplementation can inhibit adipogenesis and obesity [103]. It first reduces the levels of nicotinamide adenine dinucleotide phosphate (NADPH) in adipose tissue with decreased expression of FTO. This reduction in FTO activity increases m6A methylation on Ccna2 and Cdk2 mRNAs, which are subsequently recognized by the m6A reader protein YTHDF2. YTHDF2 quickens mRNA decay and

reduces the protein levels of CDK2 and CCNA2. This NADPH-FTO- m6A regulatory axis offers a new option for alleviating obesity.

Another study reported that metformin prevents obesity in a similar way. Metformin can significantly suppress FTO expression, leading to elevated m6A methylation of Ccnd1 and Cdk2 mRNAs. This modification promotes their recognition by the m6A reader protein YTHDF2, resulting in reduced protein levels of CCND1 and CDK2 [104].

A small molecule, ABX300, has been identified as a potential therapeutic agent capable of mitigating diet-induced obesity by modulating LMNA isoform expression through the regulation of serine/arginine-rich splicing factor 1 (SRSF1) in HFD-fed mice [105]. SRSF1 is a key regulator of pre-mRNA splicing that causes abnormal gene expression through post-translational regulation [106]. Treatment with ABX300 was shown to reverse HFD-induced aberrant splicing of LMNA through the inhibition of SRSF1 activity. Beyond its splicing modulatory effects, ABX300 also altered the metabolic rate and energy expenditure in HFD-fed mice by upregulating genes involved in fat utilization and lipid catabolism, without decreasing the lean weight of mice.

Platania et al. identified two indole-containing derivatives, VP12/14 and VP12/110, as modulators of HuR (ELAVL1) expression with therapeutic potential in the context of diabetic retinopath [107]. In human retinal endothelial cells (HRECs) exposed to high glucose (HG) conditions, both compounds significantly reduced the release of vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-α) [107]. VP12/14 and VP12/110 were shown to inhibit VEGF and TNF-α production by disrupting the interaction between HuR and TNF-α mRNA, thereby attenuating post-transcriptional regulation of inflammation.

6.2. Future Directions in Revealing Nutrient–RBP Interaction Mechanisms

The intersection of RBPs and nutrient metabolism represents a burgeoning frontier with transformative potential for understanding diet-related diseases. The current literature on RBPs faces several limitations that constrain the generalizability of findings. One major issue is the tissue-specific variability in RBP expression and function, as the same RBP may exert distinct regulatory roles depending on the cellular context, developmental stage, or metabolic state of a given tissue. Besides, much of the current understanding of RBP-mediated mechanisms is derived from animal models, which, while informative, may not fully recapitulate human-specific regulatory networks. The interspecies difference weakens the feasibility of translating preclinical findings into effective human therapies.

Future research will prioritize elucidating how RBPs dynamically sense and transduce nutrient signals to regulate metabolic pathways. One way is to identify specific RBPs that interact with functional nutrients such as vitamin C, folate, and prebiotics, and their roles in common metabolic diseases like obesity [108]. Also, the dysfunction of RNA-binding proteins is worth studying. They may act as a molecular bridge between transient hyperglycemia and sustained pathogenic gene expression, thereby contributing to the establishment and persistence of metabolic memory in diabetes. A critical focus will be deciphering the "nutrient-RBP-mRNA axis" in pathologies such as malnutrition and metabolic syndromes, particularly in understudied tissues like the gut–liver axis or adipose depots. CRISPR-Cas screens and chemical probes may identify RBPs that act as nutrient sensors, while engineered RBPs or synthetic RNA scaffolds could pioneer nutrient-guided therapeutics.

Advanced techniques such as single-cell omics and spatial transcriptomics, combined with AI-driven integrative multi-omics platforms, will map tissue-specific RBP responses to dietary components like fatty acids, amino acids, and micronutrients. Machine Learning (ML) algorithms can identify subtle patterns in large-scale datasets, predicting how

nutrient fluctuations alter RBP binding affinities or RNA regulon composition (Figure 4). For instance, deep learning models trained on CLIP-seq and metabolomic data may uncover novel "nutrient-RBP-mRNA axes" in pathologies such as obesity or malnutrition. CRISPR-Cas screens coupled with AI-powered drug discovery pipelines could accelerate the identification of nutrient-sensitive RBPs as therapeutic targets, while generative AI models might design synthetic small molecules to modulate RBP activity in a nutrient-dependent manner.

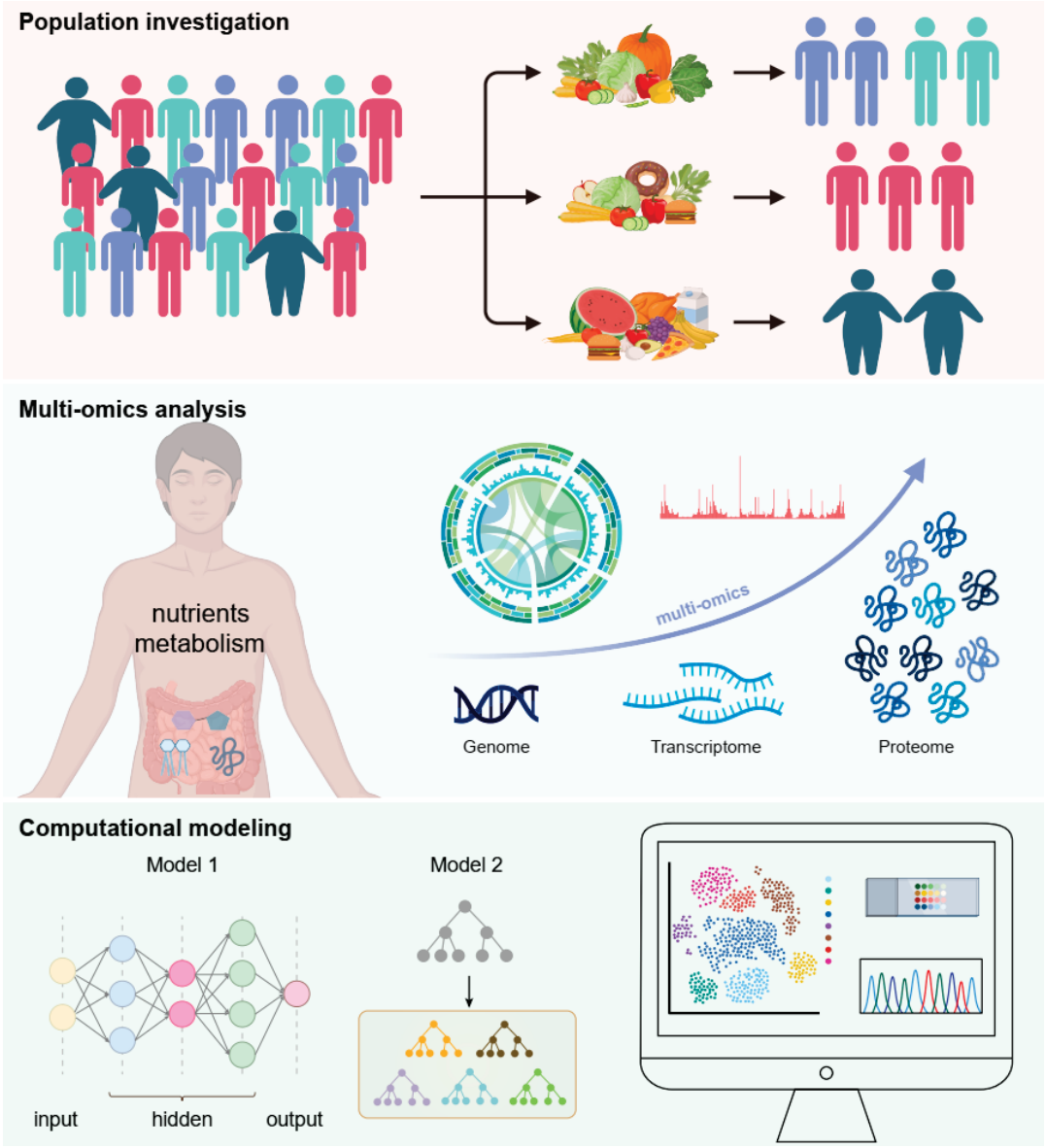


Figure 4. Future directions in RNA-binding protein research in nutrient metabolism.

However, challenges such as inter-individual variability [109], delivery of RNA-targeted therapies, and regulatory hurdles must be carefully addressed before clinical implementation can be achieved. One possible solution would be employing federated ML frameworks to harmonize diverse clinical datasets while preserving privacy. Furthermore, reinforcement learning systems might optimize personalized dietary interventions by predicting how specific macronutrient ratios restore RBP-driven homeostasis in metabolic tissues. By merging RBP biology with precision nutrition, future studies could unlock

diet-responsive RNA regulatory codes, paving the way for individualized therapies that exploit RBPs as dynamic sensors of nutritional status.

7. Conclusions

The emerging field of RBPs as regulators of nutrient metabolism represents a promising frontier in molecular and metabolic research. As our understanding of the intricate relationship between RBPs and metabolic transitions continues to evolve, several key areas warrant further investigation. A primary objective should be the systematic mapping of RBP–nutrient regulatory networks. Although significant progress has been made in identifying individual RBPs involved in metabolic regulation, a large number of interactions remain uncharacterized.

To address this gap, future studies should aim to identify novel RBPs implicated in metabolic control using high-throughput functional screening platforms. Advanced methodologies such as LACE-seq and CRIC-seq have demonstrated the ability to detect transient and context-specific RBP–RNA interactions. Expanding upon these tools and developing novel techniques—particularly those capable of capturing dynamic interactions in three-dimensional RNA structures—will be critical for uncovering mechanistic insights at a high resolution.

A more integrative approach, combining transcriptomic, proteomic, and metabolomic datasets, is essential for constructing a comprehensive framework of RBP-mediated metabolic regulation. Multi-omics integration will allow researchers to understand not only how RBPs affect gene expression and protein abundance, but also how these changes manifest at the level of cellular metabolism and nutrient flux.

With the development of sophisticated computational tools, AI technology is indispensable in identifying key regulators, screening therapeutic targets, and predicting functional outcomes of RBP perturbations. Such integrative and technologically advanced strategies will accelerate the discovery of novel regulatory mechanisms and facilitate the translation of RBP-based findings into therapeutic applications for metabolic diseases.

8. Methods

This review utilized PubMed, a comprehensive biomedical database maintained by the National Center for Biotechnology Information (NCBI), to identify relevant studies. The search covered publications from January 2000 to July 2025 to ensure a thorough analysis of historical and current evidence. Keywords include “RNA binding protein”, “obesity”, “NAFLD”, “diabetes”, “vitamin”, “iron”, “glucose”, “lipid”, “amino acid”, etc.

Given that this review is narrative in design, it may introduce subjectivity or selection bias compared to employing systematic screening protocols.

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References

1. Oliphant, K.; Allen-Vercoe, E. Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. *Microbiome* **2019**, *7*, 91. [CrossRef] [PubMed]

2. Savarino, G.; Corsello, A.; Corsello, G. Macronutrient balance and micronutrient amounts through growth and development. *Ital. J. Pediatr.* **2021**, *47*, 109. [CrossRef] [PubMed]
3. Ispoglou, T.; Witard, O.C.; Duckworth, L.C.; Lees, M.J. The efficacy of essential amino acid supplementation for augmenting dietary protein intake in older adults: Implications for skeletal muscle mass, strength and function. *Proc. Nutr. Soc.* **2021**, *80*, 230–242. [CrossRef] [PubMed]
4. Nakamura, M.T.; Yudell, B.E.; Loor, J.J. Regulation of energy metabolism by long-chain fatty acids. *Prog. Lipid Res.* **2014**, *53*, 124–144. [CrossRef] [PubMed]
5. Bang, Y.J.; Hu, Z.; Li, Y.; Gattu, S.; Ruhn, K.A.; Raj, P.; Herz, J.; Hooper, L.V. Serum amyloid A delivers retinol to intestinal myeloid cells to promote adaptive immunity. *Science* **2021**, *373*, eabf9232. [CrossRef] [PubMed]
6. Chen, Y.; Michalak, M.; Agellon, L.B. Importance of Nutrients and Nutrient Metabolism on Human Health. *Yale J. Biol. Med.* **2018**, *91*, 95–103. [PubMed]
7. Silverman, I.M.; Li, F.; Gregory, B.D. Genomic era analyses of RNA secondary structure and RNA-binding proteins reveal their significance to post-transcriptional regulation in plants. *Plant Sci.* **2013**, *205–206*, 55–62. [CrossRef] [PubMed]
8. Dominguez, D.; Freese, P.; Alexis, M.S.; Su, A.; Hochman, M.; Palden, T.; Bazile, C.; Lambert, N.J.; Van Nostrand, E.L.; Pratt, G.A.; et al. Sequence, Structure, and Context Preferences of Human RNA Binding Proteins. *Mol. Cell* **2018**, *70*, 854–867.e859. [CrossRef] [PubMed]
9. Corley, M.; Burns, M.C.; Yeo, G.W. How RNA-Binding Proteins Interact with RNA: Molecules and Mechanisms. *Mol. Cell* **2020**, *78*, 9–29. [CrossRef] [PubMed]
10. Haemmerle, M.W.; Scota, A.V.; Khosravifar, M.; Varney, M.J.; Sen, S.; Good, A.L.; Yang, X.; Wells, K.L.; Sussel, L.; Rozo, A.V.; et al. RNA-binding protein PCBP2 regulates pancreatic beta cell function and adaptation to glucose. *J. Clin. Investig.* **2024**, *134*, e172436. [CrossRef] [PubMed]
11. Juan-Mateu, J.; Bajew, S.; Miret-Cuesta, M.; Iniguez, L.P.; Lopez-Pascual, A.; Bonnal, S.; Atla, G.; Bonas-Guarch, S.; Ferrer, J.; Valcarcel, J.; et al. Pancreatic microexons regulate islet function and glucose homeostasis. *Nat. Metab.* **2023**, *5*, 219–236. [CrossRef] [PubMed]
12. Nikolaou, K.C.; Vatandaslar, H.; Meyer, C.; Schmid, M.W.; Tuschl, T.; Stoffel, M. The RNA-Binding Protein A1CF Regulates Hepatic Fructose and Glycerol Metabolism via Alternative RNA Splicing. *Cell Rep.* **2019**, *29*, 283–300.e288. [CrossRef] [PubMed]
13. Qiao, A.; Zhou, J.; Xu, S.; Ma, W.; Boriboun, C.; Kim, T.; Yan, B.; Deng, J.; Yang, L.; Zhang, E.; et al. Sam68 promotes hepatic gluconeogenesis via CRTC2. *Nat. Commun.* **2021**, *12*, 3340. [CrossRef] [PubMed]
14. Qiao, A.; Ma, W.; Jiang, Y.; Han, C.; Yan, B.; Zhou, J.; Qin, G. Hepatic Sam68 Regulates Systemic Glucose Homeostasis and Insulin Sensitivity. *Int. J. Mol. Sci.* **2022**, *23*, 11469. [CrossRef] [PubMed]
15. Lv, Z.; Ren, Y.; Li, Y.; Niu, F.; Li, Z.; Li, M.; Li, X.; Li, Q.; Huang, D.; Yu, Y.; et al. RNA-binding protein GIGYF2 orchestrates hepatic insulin resistance through STAU1/PTEN-mediated disruption of the PI3K/AKT signaling cascade. *Mol. Med.* **2024**, *30*, 124. [CrossRef] [PubMed]
16. Nakamura, T.; Kunz, R.C.; Zhang, C.; Kimura, T.; Yuan, C.L.; Baccaro, B.; Namiki, Y.; Gygi, S.P.; Hotamisligil, G.S. A critical role for PKR complexes with TRBP in Immunometabolic regulation and eIF2alpha phosphorylation in obesity. *Cell Rep.* **2015**, *11*, 295–307. [CrossRef] [PubMed]
17. Wang, P.; Xie, D.; Xiao, T.; Cheng, C.; Wang, D.; Sun, J.; Wu, M.; Yang, Y.; Zhang, A.; Liu, Q. H3K18 lactylation promotes the progression of arsenite-related idiopathic pulmonary fibrosis via YTHDF1/m6A/NREP. *J. Hazard. Mater.* **2024**, *461*, 132582. [CrossRef] [PubMed]
18. Wang, X.; Wu, R.; Liu, Y.; Zhao, Y.; Bi, Z.; Yao, Y.; Liu, Q.; Shi, H.; Wang, F.; Wang, Y. m(6)A mRNA methylation controls autophagy and adipogenesis by targeting Atg5 and Atg7. *Autophagy* **2020**, *16*, 1221–1235. [CrossRef] [PubMed]
19. Wang, L.; Song, C.; Wang, N.; Li, S.; Liu, Q.; Sun, Z.; Wang, K.; Yu, S.C.; Yang, Q. NADP modulates RNA m(6)A methylation and adipogenesis via enhancing FTO activity. *Nat. Chem. Biol.* **2020**, *16*, 1394–1402. [CrossRef] [PubMed]
20. Siang, D.T.C.; Lim, Y.C.; Kyaw, A.M.M.; Win, K.N.; Chia, S.Y.; Degirmenci, U.; Hu, X.; Tan, B.C.; Walet, A.C.E.; Sun, L.; et al. The RNA-binding protein HuR is a negative regulator in adipogenesis. *Nat. Commun.* **2020**, *11*, 213. [CrossRef] [PubMed]
21. Mynatt, R.L.; Noland, R.C.; Elks, C.M.; Vandanmagsar, B.; Bayless, D.S.; Stone, A.C.; Ghosh, S.; Ravussin, E.; Warfel, J.D. The RNA binding protein HuR influences skeletal muscle metabolic flexibility in rodents and humans. *Metabolism* **2019**, *97*, 40–49. [CrossRef] [PubMed]
22. Benegiamo, G.; Mure, L.S.; Erikson, G.; Le, H.D.; Moriggi, E.; Brown, S.A.; Panda, S. The RNA-Binding Protein NONO Coordinates Hepatic Adaptation to Feeding. *Cell Metab.* **2018**, *27*, 404–418.e407. [CrossRef] [PubMed]
23. Li, Z.; Zhou, M.; Cai, Z.; Liu, H.; Zhong, W.; Hao, Q.; Cheng, D.; Hu, X.; Hou, J.; Xu, P.; et al. RNA-binding protein DDX1 is responsible for fatty acid-mediated repression of insulin translation. *Nucleic Acids Res.* **2018**, *46*, 12052–12066. [CrossRef] [PubMed]
24. Huot, M.E.; Vogel, G.; Zabarauskas, A.; Ngo, C.T.; Coulombe-Huntington, J.; Majewski, J.; Richard, S. The Sam68 STAR RNA-binding protein regulates mTOR alternative splicing during adipogenesis. *Mol. Cell* **2012**, *46*, 187–199. [CrossRef] [PubMed]

25. Masuda, K.; Ripley, B.; Nishimura, R.; Mino, T.; Takeuchi, O.; Shioi, G.; Kiyonari, H.; Kishimoto, T. Arid5a controls IL-6 mRNA stability, which contributes to elevation of IL-6 level in vivo. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9409–9414. [CrossRef] [PubMed]
26. Hanieh, H.; Masuda, K.; Metwally, H.; Chalise, J.P.; Mohamed, M.; Nyati, K.K.; Standley, D.M.; Li, S.; Higa, M.; Zaman, M.M.; et al. Arid5a stabilizes OX40 mRNA in murine CD4(+) T cells by recognizing a stem-loop structure in its 3'UTR. *Eur. J. Immunol.* **2018**, *48*, 593–604. [CrossRef] [PubMed]
27. Chalise, J.P.; Hashimoto, S.; Parajuli, G.; Kang, S.; Singh, S.K.; Gemechu, Y.; Metwally, H.; Nyati, K.K.; Dubey, P.K.; Zaman, M.M.; et al. Feedback regulation of Arid5a and Ppar-gamma2 maintains adipose tissue homeostasis. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 15128–15133. [CrossRef] [PubMed]
28. Cooke, A.; Schwarzl, T.; Huppertz, I.; Kramer, G.; Mantas, P.; Alleaume, A.M.; Huber, W.; Krijgsveld, J.; Hentze, M.W. The RNA-Binding Protein YBX3 Controls Amino Acid Levels by Regulating SLC mRNA Abundance. *Cell Rep.* **2019**, *27*, 3097–3106.e3095. [CrossRef] [PubMed]
29. Awad, S.; Skipper, W.; Vostrejs, W.; Ozorowski, K.; Min, K.; Pfuhler, L.; Mehta, D.; Cooke, A. The YBX3 RNA-binding protein posttranscriptionally controls SLC1A5 mRNA in proliferating and differentiating skeletal muscle cells. *J. Biol. Chem.* **2024**, *300*, 105602. [CrossRef] [PubMed]
30. Chen, L.Y.; Wang, L.W.; Wen, J.; Cao, J.D.; Zhou, R.; Yang, J.L.; Xiao, Y.; Su, T.; Huang, Y.; Guo, Q.; et al. RNA-binding protein YBX3 promotes PPARgamma-SLC3A2 mediated BCAA metabolism fueling brown adipogenesis and thermogenesis. *Mol. Metab.* **2024**, *90*, 102053. [CrossRef] [PubMed]
31. Hattori, A.; Tsunoda, M.; Konuma, T.; Kobayashi, M.; Nagy, T.; Glushka, J.; Tayyari, F.; McSkimming, D.; Kannan, N.; Tojo, A.; et al. Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. *Nature* **2017**, *545*, 500–504. [CrossRef] [PubMed]
32. Park, S.H.; Ju, J.S.; Woo, H.; Yun, H.J.; Lee, S.B.; Kim, S.H.; Gyorffy, B.; Kim, E.J.; Kim, H.; Han, H.D.; et al. The m(6)A writer RBM15 drives the growth of triple-negative breast cancer cells through the stimulation of serine and glycine metabolism. *Exp. Mol. Med.* **2024**, *56*, 1373–1387. [CrossRef] [PubMed]
33. Wang, J.; Han, L.; Liu, Z.; Zhang, W.; Zhang, L.; Jing, J.; Gao, A. Targeting IGF2BP1 alleviated benzene hematotoxicity by reprogramming BCAA metabolism and fatty acid oxidation. *Chem. Biol. Interact.* **2024**, *398*, 111107. [CrossRef] [PubMed]
34. Weng, H.; Huang, F.; Yu, Z.; Chen, Z.; Prince, E.; Kang, Y.; Zhou, K.; Li, W.; Hu, J.; Fu, C.; et al. The m(6)A reader IGF2BP2 regulates glutamine metabolism and represents a therapeutic target in acute myeloid leukemia. *Cancer Cell* **2022**, *40*, 1566–1582.e10. [CrossRef] [PubMed]
35. Loveland, A.B.; Koh, C.S.; Ganesan, R.; Jacobson, A.; Korostelev, A.A. Structural mechanism of angiogenin activation by the ribosome. *Nature* **2024**, *630*, 769–776. [CrossRef] [PubMed]
36. Li, D.; Gao, X.; Ma, X.; Wang, M.; Cheng, C.; Xue, T.; Gao, F.; Shen, Y.; Zhang, J.; Liu, Q. Aging-induced tRNA(Glu)-derived fragment impairs glutamate biosynthesis by targeting mitochondrial translation-dependent cristae organization. *Cell Metab.* **2024**, *36*, 1059–1075.e1059. [CrossRef] [PubMed]
37. Yang, F.; Miao, L.; Mei, Y.; Wu, M. Retinoic acid-induced HOXA5 expression is co-regulated by HuR and miR-130a. *Cell Signal.* **2013**, *25*, 1476–1485. [CrossRef] [PubMed]
38. Tian, Y.; Cheng, W.; Wang, H.; Zeng, C.; Chen, X. Ascorbic acid protects retinal pigment epithelial cells from high glucose by inhibiting the NF-kappaB signal pathway through MALAT1/IGF2BP3 axis. *Diabet. Med.* **2023**, *40*, e15050. [CrossRef] [PubMed]
39. Gunapala, K.M.; Gadban, A.; Noreen, F.; Schar, P.; Benvenisty, N.; Taylor, V. Ascorbic Acid Ameliorates Molecular and Developmental Defects in Human-Induced Pluripotent Stem Cell and Cerebral Organoid Models of Fragile X Syndrome. *Int. J. Mol. Sci.* **2024**, *25*, 12718. [CrossRef] [PubMed]
40. Ghemrawi, R.; Arnold, C.; Battaglia-Hsu, S.F.; Pourie, G.; Trinh, I.; Bassila, C.; Rashka, C.; Wiedemann, A.; Flayac, J.; Robert, A.; et al. SIRT1 activation rescues the mislocalization of RNA-binding proteins and cognitive defects induced by inherited cobalamin disorders. *Metabolism* **2019**, *101*, 153992. [CrossRef] [PubMed]
41. Zhang, Y.; Cai, J.Z.; Xiao, L.; Chung, H.K.; Ma, X.X.; Chen, L.L.; Rao, J.N.; Wang, J.Y. RNA-binding protein HuR regulates translation of vitamin D receptor modulating rapid epithelial restitution after wounding. *Am. J. Physiol. Cell Physiol.* **2020**, *319*, C208–C217. [CrossRef] [PubMed]
42. Fu, Y.; Katsaros, D.; Biglia, N.; Wang, Z.; Pagano, I.; Tius, M.; Tiirikainen, M.; Rosser, C.; Yang, H.; Yu, H. Vitamin D receptor upregulates lncRNA TOPORS-AS1 which inhibits the Wnt/beta-catenin pathway and associates with favorable prognosis of ovarian cancer. *Sci. Rep.* **2021**, *11*, 7484. [CrossRef] [PubMed]
43. Hentze, M.W.; Muckenthaler, M.U.; Galy, B.; Camaschella, C. Two to tango: Regulation of Mammalian iron metabolism. *Cell* **2010**, *142*, 24–38. [CrossRef] [PubMed]
44. Castello, A.; Hentze, M.W.; Preiss, T. Metabolic Enzymes Enjoying New Partnerships as RNA-Binding Proteins. *Trends Endocrinol. Metab.* **2015**, *26*, 746–757. [CrossRef] [PubMed]
45. Fernandez-Miranda, G.; Mendez, R. The CPEB-family of proteins, translational control in senescence and cancer. *Ageing Res. Rev.* **2012**, *11*, 460–472. [CrossRef] [PubMed]

46. Novoa, I.; Gallego, J.; Ferreira, P.G.; Mendez, R. Mitotic cell-cycle progression is regulated by CPEB1 and CPEB4-dependent translational control. *Nat. Cell Biol.* **2010**, *12*, 447–456. [CrossRef] [PubMed]
47. Delgado, M.E.; Naranjo-Suarez, S.; Ramirez-Pedraza, M.; Cardenas, B.I.; Gallardo-Martinez, C.; Balvey, A.; Belloc, E.; Martin, J.; Boyle, M.; Mendez, R.; et al. $CPEB_4$ modulates liver cancer progression by translationally regulating hepcidin expression and sensitivity to ferroptosis. *JHEP Rep.* **2025**, *7*, 101296. [CrossRef] [PubMed]
48. Wong, C.M.; Xu, L.; Yau, M.Y. Alternative mRNA Splicing in the Pathogenesis of Obesity. *Int. J. Mol. Sci.* **2018**, *19*, 632. [CrossRef] [PubMed]
49. Cooper, A.J.; Gupta, S.R.; Moustafa, A.F.; Chao, A.M. Sex/Gender Differences in Obesity Prevalence, Comorbidities, and Treatment. *Curr. Obes. Rep.* **2021**, *10*, 458–466. [CrossRef] [PubMed]
50. Yan, S.; Zhou, X.; Wu, C.; Gao, Y.; Qian, Y.; Hou, J.; Xie, R.; Han, B.; Chen, Z.; Wei, S.; et al. Adipocyte YTH N(6)-methyladenosine RNA-binding protein 1 protects against obesity by promoting white adipose tissue beiging in male mice. *Nat. Commun.* **2023**, *14*, 1379. [CrossRef] [PubMed]
51. Xu, A.; Zhang, J.; Zuo, L.; Yan, H.; Chen, L.; Zhao, F.; Fan, F.; Xu, J.; Zhang, B.; Zhang, Y.; et al. FTO promotes multiple myeloma progression by posttranscriptional activation of HSF1 in an m(6)A-YTHDF2-dependent manner. *Mol. Ther.* **2022**, *30*, 1104–1118. [CrossRef] [PubMed]
52. Lu, H.; Ye, Z.; Zhai, Y.; Wang, L.; Liu, Y.; Wang, J.; Zhang, W.; Luo, W.; Lu, Z.; Chen, J. QKI regulates adipose tissue metabolism by acting as a brake on thermogenesis and promoting obesity. *EMBO Rep.* **2020**, *21*, e47929. [CrossRef] [PubMed]
53. Dai, N.; Zhao, L.; Wrighting, D.; Kramer, D.; Majithia, A.; Wang, Y.; Cracan, V.; Borges-Rivera, D.; Mootha, V.K.; Nahrendorf, M.; et al. IGF2BP2/IMP2-Deficient mice resist obesity through enhanced translation of Ucp1 mRNA and Other mRNAs encoding mitochondrial proteins. *Cell Metab.* **2015**, *21*, 609–621. [CrossRef] [PubMed]
54. Wang, J.; Rajbhandari, P.; Damianov, A.; Han, A.; Sallam, T.; Waki, H.; Villanueva, C.J.; Lee, S.D.; Nielsen, R.; Mandrup, S.; et al. RNA-binding protein PSPC1 promotes the differentiation-dependent nuclear export of adipocyte RNAs. *J. Clin. Investig.* **2017**, *127*, 987–1004. [CrossRef] [PubMed]
55. Firmin, F.F.; Oger, F.; Gheeraert, C.; Dubois-Chevalier, J.; Vercoutter-Edouart, A.S.; Alzaid, F.; Mazuy, C.; Dehondt, H.; Alexandre, J.; Derudas, B.; et al. The RBM14/CoAA-interacting, long intergenic non-coding RNA Paral1 regulates adipogenesis and coactivates the nuclear receptor PPARgamma. *Sci. Rep.* **2017**, *7*, 14087. [CrossRef] [PubMed]
56. Wu, R.; Feng, S.; Li, F.; Shu, G.; Wang, L.; Gao, P.; Zhu, X.; Zhu, C.; Wang, S.; Jiang, Q. Transcriptional and post-transcriptional control of autophagy and adipogenesis by YBX1. *Cell Death Dis.* **2023**, *14*, 29. [CrossRef] [PubMed]
57. Hu, Y.; Hai, J.; Ti, Y.; Kong, B.; Yao, G.; Zhao, Y.; Zhang, C.; Zheng, X.; Zhang, C.; Ma, X.; et al. Adipose ZFP36 protects against diet-induced obesity and insulin resistance. *Metabolism* **2025**, *164*, 156131. [CrossRef] [PubMed]
58. Meng, M.; Cao, Y.; Zhang, Y.; Liu, S.; Zhong, Y.; Wang, D.; Li, D.; Xu, L.; Ma, X. HnRNPA2B1 Aggravates Inflammation by Promoting M1 Macrophage Polarization. *Nutrients* **2023**, *15*, 1555. [CrossRef] [PubMed]
59. Lustig, Y.; Barhod, E.; Ashwal-Fluss, R.; Gordin, R.; Shomron, N.; Baruch-Umansky, K.; Hemi, R.; Karasik, A.; Kanety, H. RNA-binding protein PTB and microRNA-221 coregulate AdipoR1 translation and adiponectin signaling. *Diabetes* **2014**, *63*, 433–445. [CrossRef] [PubMed]
60. Chen, H.Y.; Durmaz, Y.T.; Li, Y.; Sabet, A.H.; Vajdi, A.; Denize, T.; Walton, E.; Laimon, Y.N.; Doench, J.G.; Mahadevan, N.R.; et al. Regulation of neuroendocrine plasticity by the RNA-binding protein ZFP36L1. *Nat. Commun.* **2022**, *13*, 4998. [CrossRef] [PubMed]
61. Tarling, E.J.; Clifford, B.L.; Cheng, J.; Morand, P.; Cheng, A.; Lester, E.; Sallam, T.; Turner, M.; de Aguiar Vallim, T.Q. RNA-binding protein ZFP36L1 maintains posttranscriptional regulation of bile acid metabolism. *J. Clin. Investig.* **2017**, *127*, 3741–3754. [CrossRef] [PubMed]
62. Drescher, H.K.; Weiskirchen, S.; Weiskirchen, R. Current Status in Testing for Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH). *Cells* **2019**, *8*, 845. [CrossRef] [PubMed]
63. Liu, R.; Wu, K.; Li, Y.; Sun, R.; Li, X. Human antigen R: A potential therapeutic target for liver diseases. *Pharmacol. Res.* **2020**, *155*, 104684. [CrossRef] [PubMed]
64. Fan, X.C.; Steitz, J.A. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J.* **1998**, *17*, 3448–3460. [CrossRef] [PubMed]
65. Liu, C.; Lin, Y.; Wang, Y.; Lin, S.; Zhou, J.; Tang, H.; Yi, X.; Ma, Z.; Xia, T.; Jiang, B.; et al. HuR promotes triglyceride synthesis and intestinal fat absorption. *Cell Rep.* **2024**, *43*, 114238. [CrossRef] [PubMed]
66. Ramirez, C.M.; Lin, C.S.; Abdelmohsen, K.; Goedeke, L.; Yoon, J.H.; Madrigal-Matute, J.; Martin-Ventura, J.L.; Vo, D.T.; Uren, P.J.; Penalva, L.O.; et al. RNA binding protein HuR regulates the expression of ABCA1. *J. Lipid Res.* **2014**, *55*, 1066–1076. [CrossRef] [PubMed]
67. Zhang, W.; Sun, Y.; Liu, W.; Dong, J.; Chen, J. SIRT1 mediates the role of RNA-binding protein QKI 5 in the synthesis of triglycerides in non-alcoholic fatty liver disease mice via the PPARalpha/FoxO1 signaling pathway. *Int. J. Mol. Med.* **2019**, *43*, 1271–1280. [CrossRef] [PubMed]

68. Regue, L.; Minichiello, L.; Avruch, J.; Dai, N. Liver-specific deletion of IGF2 mRNA binding protein-2/IMP2 reduces hepatic fatty acid oxidation and increases hepatic triglyceride accumulation. *J. Biol. Chem.* **2019**, *294*, 11944–11951. [CrossRef] [PubMed]
69. Atkinson, M.A.; Mirmira, R.G. The pathogenic "symphony" in type 1 diabetes: A disorder of the immune system, beta cells, and exocrine pancreas. *Cell Metab.* **2023**, *35*, 1500–1518. [CrossRef] [PubMed]
70. Rorsman, P.; Ashcroft, F.M. Pancreatic beta-Cell Electrical Activity and Insulin Secretion: Of Mice and Men. *Physiol. Rev.* **2018**, *98*, 117–214. [CrossRef] [PubMed]
71. Yuan, S.; Xu, F.; Li, X.; Chen, J.; Zheng, J.; Mantzoros, C.S.; Larsson, S.C. Plasma proteins and onset of type 2 diabetes and diabetic complications: Proteome-wide Mendelian randomization and colocalization analyses. *Cell Rep. Med.* **2023**, *4*, 101174. [CrossRef] [PubMed]
72. Chen, Z.Z.; Gerszten, R.E. Metabolomics and Proteomics in Type 2 Diabetes. *Circ. Res.* **2020**, *126*, 1613–1627. [CrossRef] [PubMed]
73. Stoll, L.; Rodriguez-Trejo, A.; Guay, C.; Brozzi, F.; Bayazit, M.B.; Gattesco, S.; Menoud, V.; Sobel, J.; Marques, A.C.; Veno, M.T.; et al. A circular RNA generated from an intron of the insulin gene controls insulin secretion. *Nat. Commun.* **2020**, *11*, 5611. [CrossRef] [PubMed]
74. Teplova, M.; Song, J.; Gaw, H.Y.; Teplov, A.; Patel, D.J. Structural insights into RNA recognition by the alternate-splicing regulator CUG-binding protein 1. *Structure* **2010**, *18*, 1364–1377. [CrossRef] [PubMed]
75. Zhai, K.; Gu, L.; Yang, Z.; Mao, Y.; Jin, M.; Chang, Y.; Yuan, Q.; Leblais, V.; Wang, H.; Fischmeister, R.; et al. RNA-binding protein CUGBP1 regulates insulin secretion via activation of phosphodiesterase 3B in mice. *Diabetologia* **2016**, *59*, 1959–1967. [CrossRef] [PubMed]
76. Yin, J.; Seo, Y.; Rhim, J.; Jin, X.; Kim, T.H.; Kim, S.S.; Hong, J.H.; Gwak, H.S.; Yoo, H.; Park, J.B.; et al. Cross-talk between PARN and EGFR-STAT3 Signaling Facilitates Self-Renewal and Proliferation of Glioblastoma Stem Cells. *Cancer Res.* **2023**, *83*, 3693–3709. [CrossRef] [PubMed]
77. Xie, X.; Chen, X.; Wang, C.; Sun, L.; Yu, W.; Lv, Z.; Tian, S.; Yao, X.; Wang, F.; Ding, D.; et al. PARN Maintains RNA Stability to Regulate Insulin Maturation and GSIS in Pancreatic beta Cells. *Adv. Sci.* **2024**, *11*, e2407774. [CrossRef] [PubMed]
78. Akamatsu, W.; Fujihara, H.; Mitsuhashi, T.; Yano, M.; Shibata, S.; Hayakawa, Y.; Okano, H.J.; Sakakibara, S.; Takano, H.; Takano, T.; et al. The RNA-binding protein HuD regulates neuronal cell identity and maturation. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 4625–4630. [CrossRef] [PubMed]
79. Ahn, S.; Tak, H.; Kang, H.; Ryu, S.; Jeong, S.M.; Kim, W.; Lee, E.K. The RNA-binding protein, HuD regulates proglucagon biosynthesis in pancreatic alpha cells. *Biochem. Biophys. Res. Commun.* **2020**, *530*, 266–272. [CrossRef] [PubMed]
80. Hong, Y.; Tak, H.; Kim, C.; Kang, H.; Ji, E.; Ahn, S.; Jung, M.; Kim, H.L.; Lee, J.H.; Kim, W.; et al. RNA binding protein HuD contributes to beta-cell dysfunction by impairing mitochondria dynamics. *Cell Death Differ.* **2020**, *27*, 1633–1643. [CrossRef] [PubMed]
81. Jung, M.; Ryu, S.; Kim, C.; Cha, S.; Kang, H.; Ji, E.; Hong, Y.; Lee, Y.; Han, S.; Jeong, S.M.; et al. RNA binding protein HuD mediates the crosstalk between beta cells and islet endothelial cells by the regulation of Endostatin and Serpin E1 expression. *Cell Death Dis.* **2022**, *13*, 1019. [CrossRef] [PubMed]
82. Zhang, C.; Han, X.; Yang, L.; Fu, J.; Sun, C.; Huang, S.; Xiao, W.; Gao, Y.; Liang, Q.; Wang, X.; et al. Circular RNA circPPM1F modulates M1 macrophage activation and pancreatic islet inflammation in type 1 diabetes mellitus. *Theranostics* **2020**, *10*, 10908–10924. [CrossRef] [PubMed]
83. Wang, Z.; Zhang, C.; Guo, J.; Yang, Y.; Li, P.; Wang, Z.; Liu, S.; Zhang, L.; Zeng, X.; Zhai, J.; et al. CRISPR-Cas9 screening identifies INTS3 as an anti-apoptotic RNA-binding protein and therapeutic target for colorectal cancer. *iScience* **2024**, *27*, 109676. [CrossRef] [PubMed]
84. Nugent, P.J.; Park, H.; Wladyka, C.L.; Chen, K.Y.; Bynum, C.; Quarterman, G.; Hsieh, A.C.; Subramaniam, A.R. Decoding RNA Metabolism by RNA-linked CRISPR Screening in Human Cells. *bioRxiv*, 2024; *ahead of print*. [CrossRef] [PubMed]
85. Dixit, A.; Parnas, O.; Li, B.; Chen, J.; Fulco, C.P.; Jerby-Arnon, L.; Marjanovic, N.D.; Dionne, D.; Burks, T.; Raychowdhury, R.; et al. Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens. *Cell* **2016**, *167*, 1853–1866.e1817. [CrossRef] [PubMed]
86. Jaiswal, A.K.; Truong, H.; Tran, T.M.; Lin, T.L.; Casero, D.; Alberti, M.O.; Rao, D.S. Focused CRISPR-Cas9 genetic screening reveals USO1 as a vulnerability in B-cell acute lymphoblastic leukemia. *Sci. Rep.* **2021**, *11*, 13158. [CrossRef] [PubMed]
87. Bock, C.; Datlinger, P.; Chardon, F.; Coelho, M.A.; Dong, M.B.; Lawson, K.A.; Lu, T.; Maroc, L.; Norman, T.M.; Song, B.; et al. High-content CRISPR screening. *Nat. Rev. Methods Primers* **2022**, *2*, 9. [CrossRef] [PubMed]
88. Dodel, M.; Guiducci, G.; Dermit, M.; Krishnamurthy, S.; Alard, E.L.; Capraro, F.; Rekad, Z.; Stojic, L.; Mardakheh, F.K. TREX reveals proteins that bind to specific RNA regions in living cells. *Nat. Methods* **2024**, *21*, 423–434. [CrossRef] [PubMed]
89. Agranat, L.; Raitskin, O.; Sperling, J.; Sperling, R. The editing enzyme ADAR1 and the mRNA surveillance protein hUpf1 interact in the cell nucleus. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5028–5033. [CrossRef] [PubMed]

90. Sharma, S.; Wongpalee, S.P.; Vashisht, A.; Wohlschlegel, J.A.; Black, D.L. Stem-loop 4 of U1 snRNA is essential for splicing and interacts with the U2 snRNP-specific SF3A1 protein during spliceosome assembly. *Genes Dev.* **2014**, *28*, 2518–2531. [CrossRef] [PubMed]
91. Chen, L.; Wang, W.J.; Liu, S.Y.; Su, R.B.; Wu, Y.K.; Wu, X.; Zhang, S.Y.; Qiao, J.; Sha, Q.Q.; Fan, H.Y. NAT10-mediated mRNA N(4)-acetylation is essential for the translational regulation during oocyte meiotic maturation in mice. *Sci. Adv.* **2025**, *11*, eadp5163. [CrossRef] [PubMed]
92. Wang, S.; Cai, Y.; Li, T.; Wang, Y.; Bao, Z.; Wang, R.; Qin, J.; Wang, Z.; Liu, Y.; Liu, Z.; et al. CWF19L2 is Essential for Male Fertility and Spermatogenesis by Regulating Alternative Splicing. *Adv. Sci.* **2024**, *11*, e2403866. [CrossRef] [PubMed]
93. Lv, Y.; Lu, G.; Cai, Y.; Su, R.; Liang, L.; Wang, X.; Mu, W.; He, X.; Huang, T.; Ma, J.; et al. RBM46 is essential for gametogenesis and functions in post-transcriptional roles affecting meiotic cohesin subunits. *Protein Cell* **2023**, *14*, 51–63. [CrossRef] [PubMed]
94. Su, R.; Fan, L.H.; Cao, C.; Wang, L.; Du, Z.; Cai, Z.; Ouyang, Y.C.; Wang, Y.; Zhou, Q.; Wu, L.; et al. Global profiling of RNA-binding protein target sites by LACE-seq. *Nat. Cell Biol.* **2021**, *23*, 664–675. [CrossRef] [PubMed]
95. Guo, J.; Zhao, H.; Zhang, J.; Lv, X.; Zhang, S.; Su, R.; Zheng, W.; Dai, J.; Meng, F.; Gong, F.; et al. Selective Translation of Maternal mRNA by eIF4E1B Controls Oocyte to Embryo Transition. *Adv. Sci.* **2023**, *10*, e2205500. [CrossRef] [PubMed]
96. Lei, W.L.; Li, Y.Y.; Du, Z.; Su, R.; Meng, T.G.; Ning, Y.; Hou, G.; Schatten, H.; Wang, Z.B.; Han, Z.; et al. SRSF1-mediated alternative splicing is required for spermatogenesis. *Int. J. Biol. Sci.* **2023**, *19*, 4883–4897. [CrossRef] [PubMed]
97. Cai, Z.; Cao, C.; Ji, L.; Ye, R.; Wang, D.; Xia, C.; Wang, S.; Du, Z.; Hu, N.; Yu, X.; et al. RIC-seq for global in situ profiling of RNA-RNA spatial interactions. *Nature* **2020**, *582*, 432–437. [CrossRef] [PubMed]
98. Ye, R.; Hu, N.; Xue, Y. CRIC-seq protocol for in situ profiling of proximal RNA-RNA contacts associated with RNA-binding proteins. *STAR Protoc.* **2023**, *4*, 102401. [CrossRef] [PubMed]
99. Iosub, I.A.; Klobucar, T.; Ule, J. Looping in on splicing: CRIC-seq uncovers PTBP1's long-range RNA regulatory rules. *Mol. Cell* **2023**, *83*, 1206–1207. [CrossRef] [PubMed]
100. Ye, R.; Hu, N.; Cao, C.; Su, R.; Xu, S.; Yang, C.; Zhou, X.; Xue, Y. Capture RIC-seq reveals positional rules of PTBP1-associated RNA loops in splicing regulation. *Mol. Cell* **2023**, *83*, 1311–1327.e1317. [CrossRef] [PubMed]
101. Zhuang, L.; Liu, W.; Tsai, X.Q.; Outtrim, C.; Tang, A.; Wang, Z.; Huang, Y. Repurposing Niclosamide to Modulate Renal RNA-Binding Protein HuR for the Treatment of Diabetic Nephropathy in db/db Mice. *Int. J. Mol. Sci.* **2024**, *25*, 9651. [CrossRef] [PubMed]
102. Wang, H.; Chen, Y.; Lu, X.A.; Liu, G.; Fu, Y.; Luo, Y. Endostatin Prevents Dietary-Induced Obesity by Inhibiting Adipogenesis and Angiogenesis. *Diabetes* **2015**, *64*, 2442–2456. [CrossRef] [PubMed]
103. Huang, C.; Luo, Y.; Zeng, B.; Chen, Y.; Liu, Y.; Chen, W.; Liao, X.; Liu, Y.; Wang, Y.; Wang, X. Branched-chain amino acids prevent obesity by inhibiting the cell cycle in an NADPH-FTO-m(6)A coordinated manner. *J. Nutr. Biochem.* **2023**, *122*, 109437. [CrossRef] [PubMed]
104. Liao, X.; Liu, J.; Chen, Y.; Liu, Y.; Chen, W.; Zeng, B.; Liu, Y.; Luo, Y.; Huang, C.; Guo, G.; et al. Metformin combats obesity by targeting FTO in an m(6)A-YTHDF2-dependent manner. *J. Drug Target.* **2022**, *30*, 983–991. [CrossRef] [PubMed]
105. Santo, J.; Lopez-Herrera, C.; Apolit, C.; Bareche, Y.; Lapasset, L.; Chavey, C.; Capozi, S.; Mahuteau-Betzer, F.; Najman, R.; Fornarelli, P.; et al. Pharmacological modulation of LMNA SRSF1-dependent splicing abrogates diet-induced obesity in mice. *Int. J. Obes.* **2017**, *41*, 390–401. [CrossRef] [PubMed]
106. Zhu, G.Q.; Tang, Z.; Chu, T.H.; Wang, B.; Chen, S.P.; Tao, C.Y.; Cai, J.L.; Yang, R.; Qu, W.F.; Wang, Y.; et al. Targeting SRSF1 improves cancer immunotherapy by dually acting on CD8(+)T and tumor cells. *Signal Transduct. Target. Ther.* **2025**, *10*, 25. [CrossRef] [PubMed]
107. Platania, C.B.M.; Pittala, V.; Pascale, A.; Marchesi, N.; Anfuso, C.D.; Lupo, G.; Cristaldi, M.; Olivieri, M.; Lazzara, F.; Di Paola, L.; et al. Novel indole derivatives targeting HuR-mRNA complex to counteract high glucose damage in retinal endothelial cells. *Biochem. Pharmacol.* **2020**, *175*, 113908. [CrossRef] [PubMed]
108. Chen, J.; Yu, Y.; Sun, S.; Yu, W.; Lei, Y.; Lu, C.; Zhai, J.; Bai, F.; Ren, F.; Wang, R. Probiotics and Prebiotics: Meeting Dietary Requirements for Optimal Health and Planetary Sustainability. *J. Nutr.* 2025, *ahead of print*. [CrossRef]
109. Wang, R.; Yu, Y.F.; Yu, W.R.; Sun, S.Y.; Lei, Y.M.; Li, Y.X.; Lu, C.X.; Zhai, J.N.; Bai, F.R.; Ren, F.; et al. Roles of Probiotics, Prebiotics, and Postbiotics in B-Cell-Mediated Immune Regulation. *J. Nutr.* **2025**, *155*, 37–51. [CrossRef] [PubMed]

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