Dairy Product Consumption and Metabolic Health

by

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ABSTRACT

The increasing prevalence of overweight and obesity worldwide has raised the alarm for urgently needed preventative strategies to counter the trend of increased risk associated with these conditions, including type 2 diabetes (T2D) and metabolic-associated fatty liver disease (MASLD). Energy imbalance and suboptimal diets can induce these conditions. The contribution of dairy products remains debatable in this regard. Dairy products are quite heterogeneous regarding nutrient composition, physical state, and structure, and their effect on health will likely vary.

In this dissertation, I aim to unravel the effects of different types of dairy foods on obesity-associated metabolic outcomes. It covers a prospective cohort study, a meta-analysis of observational studies, a preclinical trial, and a scoping review. I hypothesize that the impact of dairy consumption on metabolic health varies significantly based on the type of dairy product, fermentation status, and food matrices, and that dairy products can attenuate the metabolic consequences of a Western-style diet.

To address the first objective, the association of dairy consumption patterns with the incidence of T2D among Alberta's Tomorrow Project (ATP) participants was evaluated. The average follow-up time was 5.2 years. Detailed dietary intake information was assessed using a validated food-frequency questionnaire; health outcomes were collected using a self-report questionnaire. Principal component analysis (PCA) was used to extract the dairy consumption patterns. A mixed intake of low and whole-fat milk decreased the risk of T2D by 36% in males. This effect was more pronounced in high-risk groups such as those with obesity.

The second objective was to document the associations between total dairy intake, intake from specific dairy foods, and MASLD. A meta-analysis of 11 observational studies with 43,649

individuals was performed. Pooled data indicated that high dairy product consumption was significantly inversely associated with MASLD risk. Milk and yogurt consumption were associated with 14% and 12% lower risk of MASLD, respectively. However, no significant association was seen with cheese intake.

Objective 3 was conducted to elucidate the mechanism of action of low/non-fat dairy matrices on metabolism in a mouse model of obesity. Male C57BL/6 obese mice were fed an HFD and subsequently allocated into treatment groups receiving either fat-free milk, plain non-fat yogurt, or reduced fat (19%) cheddar cheese at 10% of daily caloric intake for 8 weeks. Milk-treated mice showed high energy expenditure and lower weight gain. Evidence from immunoblotting of metabolic pathways suggested the activation of brown adipose tissue through the SIRT1-PPARγ-PGC1α pathway, which includes increasing UCP1, one of the major thermogenic regulators. Milk consumption also markedly decreased hepatic steatosis, confirmed by a lower triglyceride content. Immunoblotting evidence supports that the mechanism involved lipogenesis suppression and enhanced liver fat oxidation. Mice in the milk group showed improved glucose homeostasis, possibly by ameliorating the hepatic insulin signaling pathway and suppressing gluconeogenic enzymes. While yogurt supplementation improved hepatic steatosis and insulin sensitivity, these beneficial effects were less potent than milk. Yogurt increased energy expenditure suggesting increased BAT activity. Supplementation with cheese had a more neutral effect, with a modest reduction of body weight gain. Gut microbiota analysis indicated increased beneficial bacteria such as *Streptococcus* in the yogurt and *Anaerotignum* in the milk groups. Serum and liver lipidomics demonstrated that milk and yogurt consumption significantly reduced diacylglycerides and increased levels of beneficial lipid species, indicating enhanced lipid metabolism and reduced hepatic lipotoxicity.

Objective 4 was a scoping review summarizing studies on the impact of dairy products on phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) concentrations in humans and animals. Fifteen publications were included in this review. Generally, total phospholipids were stable after dairy consumption, while specific PC and LPC species increased, especially those with dairy fatty acid biomarkers, including C15:0 and C17:0. In animal models, dairy consumption also resulted in increased fecal excretion of phospholipids and significant changes in certain liver and serum PC and LPC species.

The combination of results from this research, along with a review of the current literature, supports my hypothesis that dairy products can mitigate the adverse effects of a Western diet on metabolic health with shared and unique mechanisms that may depend on the dairy matrix. Specifically, the positive effects of milk and yogurt in elevating energy expenditure, insulin sensitivity, and lowering liver fat underscore the importance of such dairy products in dietary recommendations to prevent T2D and MASLD.

PREFACE

Attached to this thesis is the original work of Emad Yuzbashian.

Through my study at the University of Alberta, I have received a 2020-23 ADI/HRD Graduate Studentship funded by the Alberta Diabetes Institute and International Helmholtz Research School for Diabetes, in addition to the Alberta Graduate Excellence Scholarship 2022 and 2023.

The research projects of which this thesis is a part received research ethics approval from the University of Alberta Research Ethics Board:

Association between dairy consumption pattern and type 2 diabetes; Pro00107023, from Feb 2021 to Nov 2022.

The mouse studies received approval from the University of Alberta Animal Care and Use Committee:

The Effects of Dairy Products on Glucose Homeostasis in Mice Fed High Fat Diet; AUP00003066, from Jan 2019 to Dec 2023.

Chapter 3 of this thesis is a version of a manuscript that has been published as: **Yuzbashian E**, Pakseresht M, Vena J, Chan CB. Association of dairy consumption patterns with the incidence of type 2 diabetes: Findings from Alberta's Tomorrow Project. Nutr Metab Cardiovasc Dis. 2022 Dec;32(12):2760-2771. doi: 10.1016/j.numecd.2022.09.022. Emad Yuzbashian conceptualized, designed, and conducted the analysis and drafted the manuscript; Catherine Chan provided substantial input to the study design, secured funding for data acquisition, assisted in interpreting the data, drafted the manuscript, and critically reviewed the manuscript. Mohammadreza Pakseresht assisted in analyzing the data, interpreting the results, and critically reviewing the manuscript for important intellectual content. Jennifer Vena assisted in developing the study protocol, provided guidance with data from Alberta's Tomorrow Project, and critically reviewed the manuscript for important intellectual content.

Chapter 4 of this thesis is a version of a manuscript that has been published as: **Yuzbashian E**, Fernando DN, Pakseresht M, Eurich DT, Chan CB. Dairy product consumption and risk of non-alcoholic fatty liver disease: A systematic review and meta-analysis of observational studies. Nutr Metab Cardiovasc Dis. 2023 Aug;33(8):1461-1471. doi: 10.1016/j.numecd.2023.04.018. Emad Yuzbashian designed the research, drafted the study protocol, developed search syntaxes, searched

within the databases, screened eligible studies, extracted data from included studies, performed the statistical analyses, and drafted the manuscript. Catherine Chan supervised the study, assisted in designing the research, edited the study protocol, made a consensus between reviewers, drafted the manuscript, and critically reviewed the manuscript. Dineli N Fernando served as a second reviewer for title and abstract screening, full-text selection, data extraction, and quality assessment, and assisted in the drafting of the manuscript. Mohammadreza Pakseresht and Dean T. Eurich reviewed the extracted data, oversaw the statistical analyses, and revised the manuscript.

A version of Chapter 5 of this thesis has been accepted for publication in the *Food and Function* journal as Differential Effects of Milk, Yogurt, and Cheese on Energy Homeostasis and Brown Adipose Tissue Phenotype in High-Fat Diet-Induced Obese Mice. **Yuzbashian E,** Fernando D, Ussar S, Chan CB. Dairy Farmers of Canada fund this study. Emad Yuzbashian conceptualized the study, conducted animal work and lab experiments, performed western blot analysis, analyzed and interpreted data, analyzed and visualized lipidomics data, and drafted the manuscript. Dineli N Fernando performed western blot analysis, including ATGL, HSL, FGF21, and PGC1α (figure 4.3 C, I, J, K). Siegfried Ussar reviewed the data and critically reviewed the manuscript. Catherine Chan initiated and designed the study, secured funding from Dairy Farmers Canada, supervised the entire study, and reviewed and edited the manuscript.

Chapter 7 of this thesis contains excerpts from a manuscript that has been published as **Yuzbashian E**, Moftah S, Chan CB. Graduate Student Literature Review: A scoping review on the impact of consumption of dairy products on phosphatidylcholine and lysophosphatidylcholine in circulation and the liver in human studies and animal models. J Dairy Sci. 2023 Jan;106(1):24-38. doi: 10.3168/jds.2022-21938. Emad Yuzbashian conceptualized and designed the study, prepared the search syntax, conducted the search, selection, extraction, and quality evaluation of studies, and prepared the first draft of the manuscript. Salma Moftah served as a second reviewer for the selection and quality evaluation of studies and collaborated in the drafting of the manuscript. Catherine Chan conceptualized and designed the study, reviewed the extracted data, and critically reviewed and edited the manuscript.

DEDICATION

To the health research scholars who have been devoting their lives to piece together the complex puzzle of the workings of the human body. Our unwavering dedication to unraveling the mysteries of disease and developing cures continues to inspire and pave the way for future discoveries.

ACKNOWLEDGMENTS

This Ph.D. dissertation reports a small part of a long journey of learning experiences and personal development. I could not have made it on my own without the help, guidance, and encouragement of many others. It is essential to mention that I am thankful to all the individuals who helped me through this adventure. Most importantly, I am indebted to my loving wife, Behnaz Mahmoodi. She accompanied me to Edmonton and has been my stronghold in this challenging journey. Behnaz, your belief in me and endless support when I doubted myself have been the building blocks of this success story. I cannot thank you enough for your love and dedication.

First, I want to acknowledge my great supervisor, Dr. Catherine Chan, for her outstanding support. Her encouragement and guidance were the push that kept me going. She has taught me that a perfect supervisor can also be an excellent mentor, someone from whom I can seek advice on everything that comes up and how to strive for success. As her last student, I always felt the need to be the best version of myself to honor her legacy and guidance.

I want to acknowledge the contribution of my committee members, Dr. Rene Jacobs, Dr. Seigfried Ussar, and Dr. Mohammad Reza (Peyman) Pakseresht, who helped me greatly enhance the depth and rigor of this thesis. I appreciate their readiness to facilitate my numerous requests and recommendations and for giving their timely constructive responses, which is crucial in developing my research and career.

I also appreciate Dr. Seigfried Ussar, who accommodated me in his lab at Helmholtz Diabetes Center in Munich, Germany, for a period of six months. Dr. Ussar would make provisions for all the necessary equipment and technology and sometimes give invaluable advice on not spending all my time in the lab. I am also thankful to his lab members, especially Samira Zamani and Andreas Israel, who were very welcoming and helpful and made my stay in Munich productive and comfortable.

I appreciate all Dr. Chan's lab members, Emely Berg, Dr. Stepheny Zani, and Salma Moftah, and the undergraduate students, Dineli Fernando, JJ Diaz, Linden Stuart, Maria Janisz, Aaron Getachew, and Jenn Nguyen. A special thanks go to Dineli Fernando for working with me on a dairy project quantifying thousands of liver images for two consecutive years and, afterward, making a friendship.

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1 BACKGROUND

1.1 OBESITY OVERVIEW

Obesity and its related diseases are considered global health problems. The World Health Organization (WHO) reports that obesity is currently the fifth most common cause of death globally (1). Obesity is described as the abnormal or excessive accumulation of fat that may impair metabolic health. Body mass index (BMI) is a surrogate measure of body fat commonly used to categorize the severity of obesity in adults. The four BMI classifications are depicted in Figure 1.1. The WHO classification of people based on their BMI is advantageous for identifying individuals who may be at higher risk of developing metabolic disorders and death from obesity (2, 3, 4). Despite its acknowledged limitations, such as the lack of accurate index data for all ethnic backgrounds and its inapplicability to specific age groups, BMI is frequently utilized to measure obesity and its prevalence. There is a consensus that obesity or excess body fat mass, whether estimated by skin-fold thicknesses (5), BMI (6), waist circumference (7) or more sophisticated techniques, including dual X-ray absorptiometry (8), magnetic resonance imaging or computed tomography (9) or other measures poses an increased risk of mortality and morbidity (10, 11).

According to the WHO, the primary cause of obesity and overweight is an imbalance between calorie intake and expenditure (1). More precisely, for a genetically predisposed individual, excess food intake increases energy storage and leads to fat accumulation in fat cells (12, 13). The pathological lesions of obesity are the enlargement, with or without an increase in the number of fat cells to accommodate the excess stored fat. When fat cells reach their maximal storage capacity, a redistribution of fat in ectopic regions like the viscera, heart, and muscles is triggered (14).

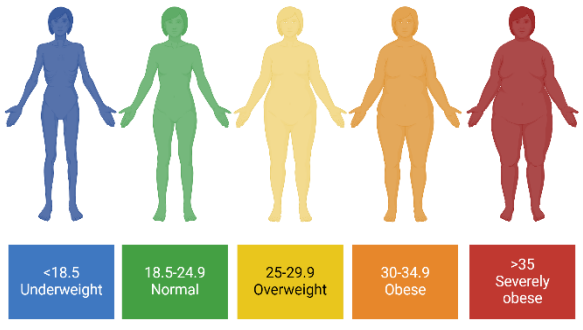


Figure 1. 1 Shows body Mass Index (BMI) categories ranging from underweight to severely obese. (The Image was created by EY using BioRender.com).

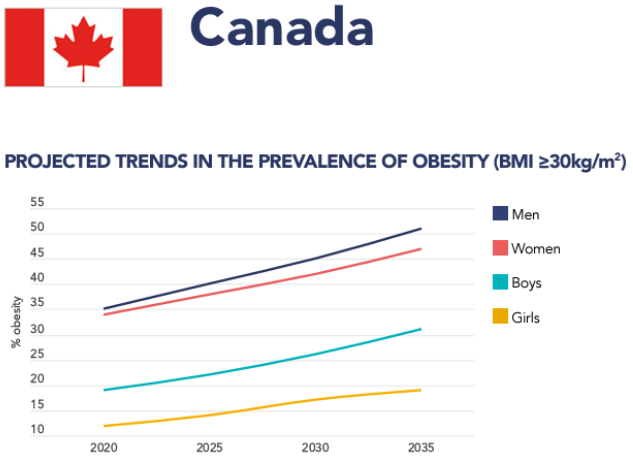
Fat tissues also secrete many regulatory molecules called adipokines. As adipocytes

hypertrophy, the balance of secretion of adipokines will be altered, increasing the circulating concentration of a wide variety of hormones such as leptin, pro-inflammatory cytokines such as interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α), angiotensinogen, adipsin (Complement D), and metabolites such as free fatty acids (FFA) and lactate. Also, an anti-inflammatory adipokine, adiponectin, decreases (12, 15). These products of the adipocyte, in turn, modulate metabolic and inflammatory processes in the host, affecting both the brain and peripheral systems (12). For susceptible individuals, these metabolic changes lead to dysregulation of homeostasis in a number of systems with detrimental consequences on health, which explains why obesity is a serious health concern. These potential consequences are further examined in Section 2.1.3.

1.1.1 Epidemiology of Obesity

The prevalence of obesity has substantially increased, having doubled since 1980. In 2022, the number of people with obesity and overweight exceeded the 2.5-billion mark. According to the latest World Obesity Federation report in 2023, over one-third of the population is classified as obese or overweight worldwide. Although this trend is observed globally, the specific incidence rates differ across regions, nations, and ethnic groups. Without effective public health interventions, approximately 4 billion people, or 51% of the global population, will be affected by overweight or obesity by 2035. Around 25% of the global population, which is over 2 billion individuals, will experience obesity. This problem is no longer limited to adults, and the prevalence is rising more quickly in children. Projections indicate that the rate among boys will increase by 100% to reach 208 million by 2035 relative to 2020, while the rate among girls is anticipated to more than double, increasing by 125% to reach 175 million. Notably, obesity rates are rising most quickly in lower-income countries.Nine out of the top 10 nations worldwide that are expected to have the highest increase in obesity rates for both adults and children belong to poor or lower-middle-income countries (4, 16).

From the recent WHO report in 2023, it appears that both in the United States and in Canada, people have been challenged by the enormous burden of the obesity epidemic and its associated diseases. Despite the fact that the prevalence of obesity in Canada is lower than the United States, the incidence of the disease is relatively high in comparison with other developed countries. Currently, the prevalence of obesity has reached 41.9% of the adult population in the United States, of whom 9.2% have a severe case (BMI>35 kg/m^2). Additionally, 30.7% of the adult population has been classified as overweight based on their BMI. In Canada, the proportion of obese people among the adult population is 31.3%, and the obesity rate has increased 455% between 1985 and 2023. According to the most recent report, approximately 1.9 million Canadians are now classified as being severely obese (Figure 1.2) (16, 17).



IMPACT OF OVERWEIGHT (BMI ≥25kg/m²) 2020–2035

	Healthcare impact of BMI ≥25kg/m², US$ million	Total economic impact of BMI ≥25kg/m², US$ million	Estimated GDP US$ billion	Impact of BMI ≥25kg/m² on GDP
2020	15,240	41,480	1,651	2.5%
2025	17,527	48,763	1,907	2.6%
2030	20,095	57,604	2,117	2.7%
2035	23,003	68,586	2,386	2.9%

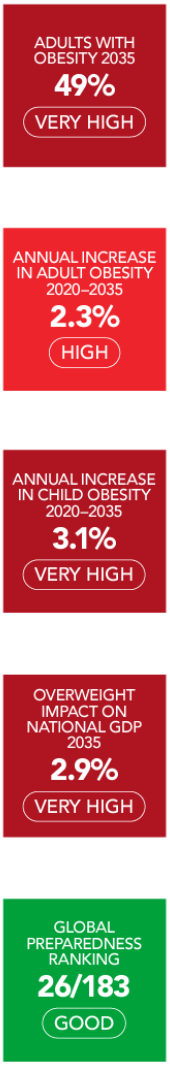


Figure 1. 2 World Obesity Atlas 2023 illustrates the escalating prevalence and economic impact of obesity in Canada

Obesity Atlas 2023 illustrates the escalating prevalence and economic impact of obesity in Canada. The first graph forecasts a significant annual increase in obesity rates across different genders and age groups by 2035. The second graph delineates the projected economic burden of obesity on healthcare costs, productivity losses, and national GDP. The third graph compares the healthcare resources allocated to overweight individuals in 2020 with projections for 2035, highlighting the growing challenge for public health systems. (World Obesity Federation. (2023).

1.1.2 Obesity Risk Factors

Many potential modifiable and non-modifiable risk factors lead to obesity and its complications. The factors, including age (because of changes in body composition and metabolic efficiency), genetics (such as genes controlling metabolic rate and appetite), epigenetics, and social determinants of health (including educational attainment and socioeconomic status), are considered non-modifiable risk factors. These factors are inherently predetermined or less modifiable; understanding how they add to obesity risk enables one to appreciate risk differences and consider them when developing pertinent interventions. In contrast, modifiable risk factors pertain to lifestyle factors regarding diet, physical activity, and behavior patterns, which can be controlled or changed (18). Unhealthy diets consisting mainly of high-calorie, nutrient-poor foods, coupled with sedentary lifestyles, are considered among the primary causes and contributors to obesity consequences (18, 19).

More than 50 medical conditions are related to obesity, including metabolic dysfunctions such as type 2 diabetes, hypertension, metabolic dysfunction-associated steatotic liver disease (MASLD), polycystic ovary syndrome, and cardiovascular diseases. In addition, it is linked to mood disorders like depression and anxiety, dementia, joint problems and osteoarthritis, chronic kidney disease, obstructive sleep apnea, and at least thirteen types of cancer (20).

Obesity has far-reaching consequences that go beyond an individual's health, affecting both the healthcare system and the economy. The World Obesity Atlas 2023 projects the global economic burden of overweight and obesity to escalate to $4.32 trillion yearly by 2035 unless gigantic improvements are made in prevention and treatment (Figure 2). The overall economic burden of disability attributable to obesity in Canada was approximately $11.8 billion Canadian dollars in 2019 (4, 16, 17, 21).

Obesity-induced insulin resistance and fatty liver are two major metabolic abnormalities identified in the initial stages of obesity development and are believed to be key initial mediators of the adverse health effects of obesity (22, 23). Moreover, these abnormalities are not only early steps in metabolic dysfunction but are also considered an exacerbation of other complications related to obesity (24). In the following, I will elaborate on the three important obesity-related metabolic

disorders, including insulin resistance, type 2 diabetes, and metabolic dysfunction-associated steatotic liver disease (MASLD).

1.1.2.1 Insulin resistance

Himsworth first conceptualized the term “insulin resistance” (IR) in 1936, which was a major milestone in the study of glucose homeostasis and comprehension of diabetes (25). He clearly showed that the injection of glucose and insulin together produced a fall in blood glucose in many diabetic patients but also observed an increase in blood glucose in other patients. He deduced that the different responses were caused by a lack of sensitivity to insulin in the latter group (25). IR is defined as a condition in which peripheral tissues, such as skeletal muscle and adipose tissue, lose their responsiveness to insulin to promote glucose uptake from the blood, leading to an abnormal increase in blood glucose. Furthermore, it also includes the dysregulation of lipolysis in the adipose tissue and a decreased ability to restrain glucose production in the liver in the fed state. The impact of IR on tissues is related to their physiological and metabolic roles, resulting in many different clinical manifestations. Collectively, this constellation of symptoms is referred to as IR syndrome or metabolic syndrome (26). Obesity does not always lead to IR; however, a high majority of persons who are insulin resistant are overweight or obese. Thus, obesity is recognized as a major risk factor for developing and worsening IR (26). Skeletal muscle and adipocytes account for 60–70% and 10-20% of glucose uptake, respectively, mediated by glucose transporter 4 (GLUT4) upon stimulation by insulin. In addition, 30% of glucose disposal is attributed to the liver, although its glucose transporter isoform is not reliant on insulin (27, 28). Therefore, understanding insulin physiology is necessary to properly understand the intricacies of IR and its widespread pathophysiological implications.

1.1.2.1.1 Assessment of insulin resistance

The hyperinsulinemic-euglycemic glucose clamp technique is considered the "gold standard" for measuring IR. Insulin is infused intravenously at a steady rate, while glucose is concurrently infused to maintain euglycemia. A higher glucose infusion rate (GIR) indicates increased insulin sensitivity because tissues more efficiently absorb glucose from the bloodstream in response to a given quantity of insulin. Conversely, a lower GIR suggests IR because tissues are less responsive

to a specific amount of insulin, leading to a slower rate of glucose uptake. The infused insulin should also suppress hepatic gluconeogenesis (29).

The practical utility of the glucose clamp technique is restricted due to its complexity. Consequently, several less intrusive surrogate indicator methods may be used to determine IR, such as the quantitative insulin sensitivity test index, homeostatic model assessment (HOMA), fasting insulin test, insulin release test, and glucose tolerance test (30). Post-glucose challenge tests, such as the oral (OGTT) or intravenous glucose tolerance test (IVGTT), are conducted on fasting individuals to assess the body's insulin and glucose responses to a 75-gram glucose load. These tests measure the increase and decrease in blood glucose following glucose administration over the course of time. The area under the curve (AUC) of blood glucose on an OGTT or an IVGTT indicates insulin sensitivity. A lower AUC indicates more effective clearance of glucose and thus higher sensitivity to insulin, whereas a higher AUC indicates reduced clearance of glucose and decreased sensitivity to insulin (31, 32, 33).

The most common method for IR estimation in human studies is the homeostasis model assessment for IR (HOMA-IR) (34, 35, 36). It is based on measurements of circulating glucose and insulin concentrations in the fasting state. Insulin concentrations result from the pancreatic β-cell's response to glucose, and glucose concentrations are determined by hepatic glucose production under the influence of insulin (fasting state). The HOMA-IR model has proven its reliability as a recognized clinical and epidemiological tool in the assessment of IR (34, 37).

Another index developed based on fasting glucose and insulin is the Quantitative Insulin Sensitivity Index (QUICKI) (38, 39, 40). The method is essentially a transformation of the HOMA equations in which the data undergo logarithmic and reciprocal transformations of the glucose-insulin product. QUICKI has a much stronger linear correlation with glucose clamp measurements of insulin sensitivity (gold standard) than other model estimates, particularly among patients with obesity or diabetes. Like HOMA-IR, QUICKI only uses fasting insulin and glucose measurements. The QUICKI is calculated by the following formula by considering fasting plasma glucose (FPG) and insulin (FPI) (41, 42, 43, 44):

$$\text{QUICKI} \frac{1}{\boldsymbol{log}(\text{FPG}[\text{mg/dL}]) + \boldsymbol{log}(\text{FPI}[\mu\text{IU/mL}])}$$

According to formal assessments of the relationship between surrogate measures using indices and clamp measures of IR, both HOMA-IR and QUICKI provide a considerably reliable approximation of IR in both rodents as in humans (45, 46). The continuous use of surrogate indices in animal research is validated by the fact that they accurately represent the variations in hyperinsulinemic–euglycemic clamp data for insulin resistance seen throughout pregnancy in rats (45) and transgenic mice (46).

1.1.2.1.2 Insulin physiology

The normal glucose concentration in the blood is kept within 70 mg/dL to 110 mg/dL and is mainly regulated by the reciprocal secretion of glucagon and insulin. Glucagon, secreted from pancreatic α-cells, increases blood glucose through stimulation of hepatic gluconeogenesis in response to fasting when the blood glucose tends to be low. The pancreatic insulin-producing cells are the β-cells, which synthesize an inactive precursor protein called preproinsulin and process it into proinsulin. Proinsulin is then converted to insulin and C-peptide. It is stored in vesicles inside the cells. Glucose enters pancreatic β-cells through GLUT2, and is then metabolized via glycolysis and the tricarboxylic acid cycle, which raises the ATP/ADP ratio. The high ratio facilitates the opening of ATP-dependent potassium channels, resulting in membrane depolarization that then activates voltage-gated calcium channels.

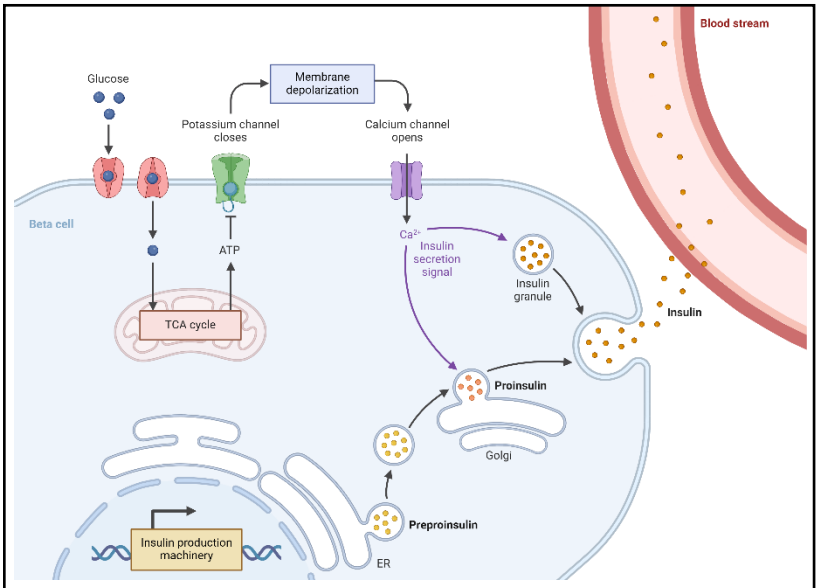


Figure 1. 3 The insulin production and secretion process within a pancreatic β-cell.
Beginning with an increase in plasma glucose followed by glucose uptake, the subsequent membrane depolarization, closure of potassium channels, and opening of calcium channels. Increased ATP/ADP ratio, leading to the rise in intracellular calcium ions that trigger the transport of vesicles containing proinsulin, undergoes conversion into insulin, which is then released into the bloodstream. TCA, tricarboxylic acid cycle (Image was modified by EY using BioRender.com).

This elicits the release of insulin-containing vesicles, thus coupling insulin secretion to the rise in blood glucose (Figure 1.3) (47).

Insulin secretion is controlled by several factors, including hormones, glucose, fatty acids, and amino acids. The gut-derived hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are called "incretins" and are important regulators of insulin secretion. GLP-1 and GIP are rapidly released following food ingestion, potentiating the biosynthesis and secretion of glucose-dependent insulin in pancreatic β-cells. GLP-1 also inhibits glucagon secretion from pancreatic α-cells and has an extra-pancreatic effect that inhibits gastric emptying. GIP stimulates insulin secretion via the GIP receptor (GIPR) on β-cells (48, 49). Insulin molecules are then transported in the bloodstream to the insulin-sensitive tissues, including muscle, liver, and adipose tissues (47). These tissues have specialized, unique functions in metabolic homeostasis, requiring specific insulin signaling routes and actions in each tissue. For instance, insulin increases glucose utilization and storage as glycogen in skeletal muscle by increasing glucose transport and net glycogen synthesis. However, in the liver, insulin activates glycogen synthesis, increases lipogenesis-related genes, and decreases the expression of gluconeogenic enzyme genes. In white adipocyte tissue (WAT), insulin suppresses lipolysis and increases glucose transport and lipogenesis (50).

Although there are pleiotropic effects, the components directly transmitting the insulin signal are strikingly similar in all cells responding to insulin. After a meal, the secreted insulin performs its physiological effects by binding to the insulin receptor (INSR) located on the plasma membrane of target cells. The INSR belongs to the tyrosine kinase receptor subfamily. The INSR structure is a tetrameric glycosylated protein consisting of two α and two β subunits. The binding of insulin with the extracellular α subunit results in the autophosphorylation of tyrosine residues in the intracellular domain of the β subunit. Subsequent to receptor autophosphorylation, receptor tyrosine kinases are activated, leading to the tyrosine phosphorylation of insulin receptor substrates (IRSs). IRSs can bind to the regulatory subunit of phosphoinositide 3-kinase (PI3K), leading to its activation, which causes phosphorylation of membrane phosphatidylinositol 4,5-bisphosphate (PIP2). This complex stimulates the activity of protein kinase Cs (PKCs) and AKT, also called protein kinase B (PKB) (51, 52). This will activate a signaling cascade that eventually increases the translocation of GLUT4 to the cell membrane surface, facilitating glucose absorption from the

bloodstream into skeletal muscle cells and adipocytes (Figure 1.4) (50). In addition to activating insulin-dependent glucose uptake via GLUT4, when AKT is activated, it adds phosphate groups to many different substrates in various functional pathways, triggering many intracellular metabolic effects. This makes AKT an essential checkpoint in the branching of insulin signaling. For instance, AKT mediates the phosphorylation of glycogen synthase kinase 3 (GSK3), which stimulates glycogen synthesis in the liver and skeletal muscle (53).

Cells also possess mechanisms to dampen insulin actions. Protein tyrosine phosphatases (PTPs) are a group of proteins that remove phosphate groups from and deactivate the IRSs. In addition,

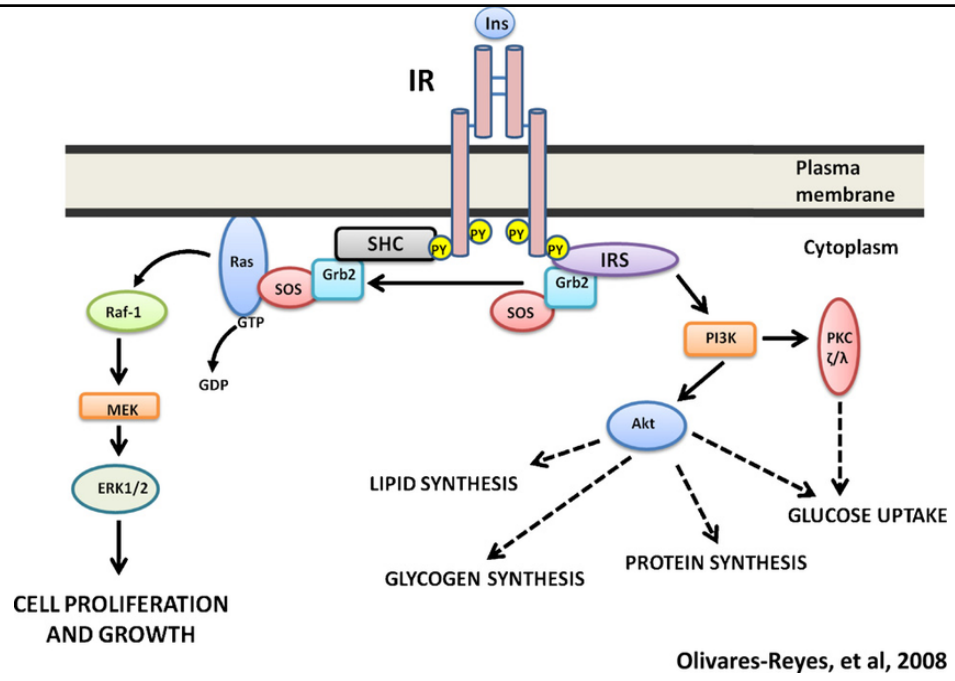


Figure 1. 4 Insulin Receptor (IR) signaling pathways.

Insulin Receptor (IR) signaling pathways, highlighting the complex network of molecular interactions initiated by insulin binding to its receptor. Binding of insulin (Ins) to its receptor elicits autophosphorylation on the insulin receptor (IR)-subunit and the Tyr phosphorylation of insulin receptor substrate (IRS) proteins. Phosphorylated IRS serve as docking proteins for other signaling proteins, such as phosphoinositide 3-kinase (PI3K). PI3K binding to phosphotyrosines on IRS-1 activates PI3K and sets off a cascade of events, ultimately resulting in the phosphorylation and activation of AKT. Activation of these downstream plays roles in lipid synthesis, glycogen synthesis, protein synthesis, and glucose uptake. SHC, Src Homology 2 Domain Containing; Grb2, Growth factor receptor-bound protein 2; SOS, Son of Sevenless; Ras, Rat sarcoma; GTP, Guanosine triphosphate; GDP, Guanosine diphosphate; Raf-1, Rapidly Accelerated Fibrosarcoma 1; MEK, Mitogen-activated protein kinase kinase; ERK1/2, Extracellular signal-regulated kinases 1/2; Akt, Protein Kinase B; PKC ζ/λ, Protein Kinase C zeta/lambda. (Licence: 5803840459299).

phosphatase and tensin homolog (PTEN) plays a crucial role in the insulin signaling pathway. It acts as a negative modulator by hydrolyzing phosphatidylinositol 3,4,5-triphosphate to PIP2, thereby opposing the PI3K pathway. Thus, the balance between phosphorylation and dephosphorylation controls the mechanism of action of insulin in targeted tissues (51).

Insulin signal transduction is a complex process depending on numerous enzymes and modulatory proteins that, by a multistep progression, mediates the signal from the INSR to the final effectors. Defective expression or activity in any of these mediators may impair normal insulin signaling and eventually result in IR in peripheral tissues (54). One of the most important mechanisms contributing to the pathogenesis of IR is hyperphosphorylation of the Ser/Thr sites of IRS proteins. Ser/Thr phosphorylation of IRS decreases its interaction with PI3K, modifying the phosphorylation of AKT and its activation. Also, Ser/Thr phosphorylation of IRS promotes more active degradation of IRS (54).

1.1.2.1.3 Progression of insulin resistance in obesity

Obesity can lead to IR through different pathways and mechanisms solely or in combination by promoting hyperinsulinemia, increased inflammation, mitochondrial failure, and lipotoxicity. Hyperinsulinemia, an abnormally high insulin concentration in the circulation, is associated with obesity. It is commonly accepted that hyperinsulinemia results from resistance to insulin action in glucose metabolism, leading to increased glycemia, which in turn stimulates the pancreatic β-cell to release insulin to avoid more severe hyperglycemia. Chronic hyperinsulinemia, caused by consuming food or administering exogenous insulin directly, has been associated with decreased insulin sensitivity in mouse models (55). Moreover, those diagnosed with hyperinsulinemia show excessive secretion of insulin (insulinoma) and may have a concurrent decrease in insulin sensitivity (56). This excessive secretion of insulin creates a vicious cycle that potently favors energy storage and lipid synthesis over lipid breakdown (57), leading to increased fat accumulation that promotes greater obesity and IR (58, 59, 60).

In parallel with hyperinsulinemia, the persistence of obesity and its risk factors (particularly poor dietary habits) lead to adipose tissue dysfunction. White adipose tissue is categorized into two major subtypes according to its function, location, and role in obesity. Subcutaneous and visceral adipose tissue differ in several aspects, such as adipocyte type, function, lipolytic activity,

vascularity, and innervation. Additionally, these subtypes respond differently to insulin and other hormones (61). Subcutaneous adipose tissue serves as the primary storage site for surplus energy. However, if this storage region gets overloaded or its capacity to produce new fat cells is hindered, lipids will collect in other areas, such as visceral depots and organs like the liver and pancreas. Visceral adipose tissue is highly metabolically active, and as it enlarges, its secretion of non-esterified fatty acids (NEFAs) and glycerol, hormones such as leptin and adiponectin, and proinflammatory cytokines becomes altered. The disrupted metabolic pathways in adipose tissue significantly contribute to the metabolic disorders associated with obesity (62).

The increased release of NEFAs from visceral adipose tissue may be crucial in regulating insulin sensitivity (14). Lipotoxicity occurs when chronically elevated circulating NEFAs accumulate in non-adipose tissues (e.g., liver and muscle), adversely affecting tissue function (63). Elevated NEFA in the blood is seen in individuals with obesity and is linked to IR. Even in healthy individuals, relative IR occurs shortly after a sudden rise in plasma NEFA levels, such as during overnight fasting, in humans, which is appropriate to help switch from glucose to fat oxidation (64). However, chronically elevated intracellular NEFA leads to prolonged competition with glucose for substrate oxidation, which then suppresses the abundance and activation of glycolysis-related enzymes (such as pyruvate dehydrogenase, phosphofructokinase, and hexokinase II). When there is an increase in the delivery of NEFAs or a decrease in the catabolism of fatty acids within cells, there is a buildup of fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA), and ceramides (64). These metabolites then activate a cascade of serine/threonine kinases, which leads to the phosphorylation of IRSs at serine/threonine sites. IRS phosphorylation is diminished in both muscle and adipocytes in individuals with IR, who also have lower expression of IRS in their adipocytes. As a result, the ability to activate PI3K is reduced. In addition to the disruption of PI3K, excessive NEFAs stimulate PKCs, which reduces proximal insulin signaling (65). The immediate consequence of this reduction is a decrease in GLUT4 translocation to the cell surface, which decreases glucose uptake by the skeletal muscle tissue, leading to increased blood glucose concentration (66, 67).

Furthermore, the excessive fat storage, especially in the visceral adipose depots, contributes to a state of chronic low-grade inflammation through the actions of secreted cytokines and chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibitory factor

(MIF), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα), and interleukin-1 beta (IL-1β) (26, 54, 67, 68). Potential mechanisms contributing to developing hypersecretion of these pro-inflammatory molecules include dysregulation of fatty acid homeostasis, increased adipose cell size and death, local hypoxia, mitochondrial dysfunction, endoplasmic reticulum stress, and mechanical stress. These mechanisms are recognized as the link between chronic caloric excess and adipose tissue inflammation or as factors that may perpetuate chronic inflammation (54, 67, 69). Chronic exposure to the inflammatory cytokines, including TNF-α, IL1β, or IL-6, activates numerous kinases, activating Ser/Thr kinases, interfering with insulin signaling and insulin action in adipocytes and hepatocytes (70, 71).

1.1.2.2 Development of type 2 diabetes

Type 2 diabetes (T2D) is defined as long-term hyperglycemia caused by insulin resistance and reduced insulin production relative to demand (Diabetes Canada Clinical Practice Guidelines Expert Committee, 2018) . In the progression of T2D, there is a condition called "prediabetes," in which FPG is higher than normal but is not yet high enough to be diagnosed as T2D. Around 25% of individuals with prediabetes are expected to acquire T2D over a span of 5 years. Although adopting healthier living habits might lower the chances of disease advancement, there is currently no global agreement on the best methods for screening and treating patients with prediabetes (72). Consistent hyperglycemia can result in the progression of diabetes complications such as peripheral neuropathy, retinopathy, nephropathy, lower limb amputation, stroke, and cardiovascular disease (72). Prediabetes and T2D diagnosis criteria are summarized in Table 1.1.

Table 1. 1 Criteria for diagnosis of prediabetes and type 2 diabetes based on the American Diabetes Association criterion

Condition	**FPG (mM; mg/dL)**	**2hPG (mM; mg/dL)**	**HbA1c (%)**
Normal	<5.6; <100	<7.8; <140	<5.7
Prediabetes	5.6-6.9; 100-125	7.8-11.0; 140-199	5.7-6.4
Type 2 Diabetes	≥7.0; ≥126	≥11.1; ≥200	≥6.5

FPG: Fasting Plasma Glucose; 2hPG: 2-hour Plasma Glucose; HbA1c: Hemoglobin A1c

Obesity is a highly predictive factor for the development of T2D. The lifetime risk of developing diabetes in males over the age of 18 rises from 7% to 70% when their BMI increases from 18.5 kg/m^2 to more than 35 kg/m^2. Likewise, the likelihood of developing diabetes during one's lifetime in females rises from 12% to 74% when considering the same BMI criteria (73). Thus, the considerable rise in T2D prevalence worldwide, which affects almost 10.5% of the global population, is attributed to the corresponding increase in the prevalence of obesity. In 2021, the number of diagnosed diabetes cases was 537 million. It is projected to increase to 643 million by 2030 and 783 million by 2045. In 2021, it was estimated that over 6.7 million deaths in the population aged 20 to 79, which accounts for 12.2% of global deaths in this age group, were caused by T2D (74).

As mentioned earlier, the expansion of adipose tissue concomitant with loss of insulin sensitivity in peripheral tissues is initially compensated by increased insulin secretion to regulate glucose balance. However, with time, various factors, such as increased insulin production, lead to a gradual decline in the functioning of β-cells, leading to the death of β-cells and an inability to maintain increased insulin secretion. β-cell depletion coincides with β-cell dysfunction, leading to insufficient insulin synthesis and elevated blood glucose. Hyperglycemia itself contributes to further β-cell dysfunction, and a vicious cycle occurs. IR becomes well established when decreased glucose tolerance occurs, and

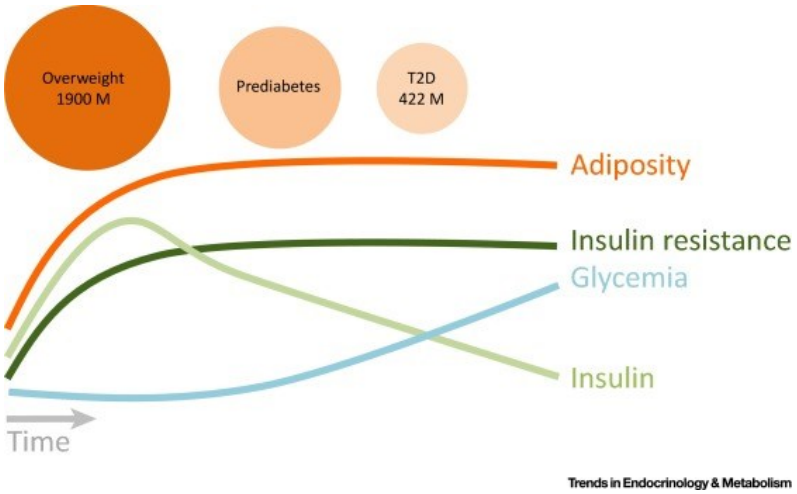


Figure 1. 5 The chronological account of the development and progression of type 2 diabetes.

The chronological account of the development and progression of type 2 diabetes. The primary factors contributing to the development of type 2 diabetes are insulin resistance, reduced insulin secretion, and elevated blood glucose levels. In cases of poor glucose tolerance, beta cell compensation occurs due to the loss of beta cell sensitivity to glucose and the gradual decline of beta cell function. This leads to a delay in the release of insulin when glucose is consumed orally.

further deterioration in β-cell activity results in the onset of T2D (Figure 1.5) (75).

1.1.2.3 Metabolic dysfunction-associated steatotic liver disease (MASLD)

Obesity is also a significant risk factor for metabolic disorders affecting the liver, particularly metabolic dysfunction-associated steatotic liver disease (MASLD) (76). MASLD was previously known as non-alcoholic fatty liver disease (NAFLD); this is a metabolic, chronic hepatic condition characterized by progressive hepatosteatosis, fibrosis, cirrhosis, and hepatocellular carcinoma. The newly proposed nomenclature and definition include the presence of hepatic steatosis detected by imaging or biopsy and the existence of one of the five distinct components of metabolic syndrome, including abdominal obesity, elevated blood pressure, high triglycerides, elevated blood glucose (prediabetes or diabetes), and low HDL cholesterol (77, 78). The incidence of MASLD increases with the expansion of fat mass (79). Population-based estimates of MASLD prevalence average 15-30%, but up to 50-90% for populations with obesity. Hepatic steatosis is detected in 65% of grade I-II obesity (BMI = 30-39.9 kg/m^2) and in 85% of patients with grade III obesity (BMI ≥ 40 kg/m^2) (80).

The liver plays a central role in both glucose and lipid metabolism. The liver regulates glucose in the blood during fasting and after eating. It does this primarily by regulating gluconeogenesis (hepatic glucose production) and storing glucose as glycogen. The liver also functions as a regulator of peripheral insulin by degrading the majority of insulin produced by the pancreas during its first circulation. Furthermore, the liver releases hepatokines and lipids that can function in both an autocrine and paracrine manner, hence regulating insulin sensitivity. The liver plays a vital role in the metabolism of lipids because a large amount of lipogenesis, lipid storage, and lipid consumption takes place there (22, 81, 82).

As mentioned earlier in the pathophysiology of IR (Section 2.1.3.1.3), obesity is usually associated with visceral adipose tissue expansion and dysfunction (Figure 1.6). Insulin-resistant adipose tissue displays enhanced lipolysis, which increases plasma NEFAs and their delivery directly into the liver by the portal vein (83). In response to the high NEFA flux, the liver forms lipid droplets with high TG content (steatosis). This results in a decrease in hepatic insulin clearance and an additional rise in circulating insulin. The increased NEFAs also induce hepatic IR (26). NEFAs in the liver impair insulin suppression of hepatic glucose production by stimulating translocation of the PKCε from the cytosol to the plasma membrane, resulting in reduced hepatic IRS-associated PI3K activity. Elevated adipose tissue release of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and MCP-1 and the reduction in anti-inflammatory adipokines such as adiponectin promotes liver inflammation like in other tissues (84, 85). When inflammation is coupled with steatosis, fibrosis can occur in susceptible individuals, progressing to the onset of severe liver damage like liver cancer (86).

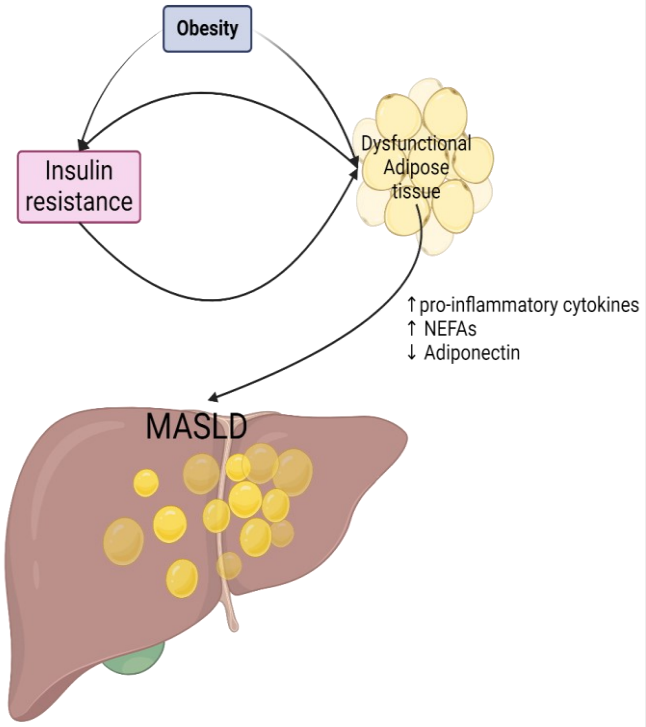


Figure 1. 6 Metabolic dysfunction-associated steatotic liver disease (MASLD) progression

The role of obesity-induced insulin resistance in the progression of accumulation of fat in the liver development and progression (Image was created by EY using BioRender.com).

1.1.2.3.1 Accumulation of fat in the liver

As was elaborated earlier, the liver is a central organ that regulates substrate metabolism and dictates the fate of fuel substrates. Although much is unknown about the mechanisms involved in hepatic fat accumulation, researchers have observed that steatosis occurs when hepatocyte fat accumulation exceeds their oxidation or export. Four major pathways may lead to an imbalance

between inputs and outputs of lipids that finally results in TG accumulating in the liver: (i) an increased influx of NEFA into the liver from the blood (resulting from diet intake or adipose tissue lipolysis); (ii) increased hepatic fatty acid synthesis through the *de novo* lipogenesis (DNL) pathway; (iii) decreased fatty acid oxidation; (iv) decreased very low-density lipoprotein (VLDL)-mediated release of TG into the circulation. The regulation of all these pathways is controlled through a highly networked, intricate interplay among hormones, nuclear receptors, and transcription factors, ensuring precise maintenance of the homeostasis of hepatic lipids. All or some of these pathways may contribute to the pathogenesis of hepatic steatosis (87, 88, 89, 90).

1.1.2.3.1.1 Lipid uptake in the liver

Long-chain NEFA uptake by the liver is mediated by fatty acid transporters; passive diffusion, mainly of short- and medium-chain fatty acids (FFA with <14 carbons), plays a lesser role (91). The main participants in transport are fatty acid transport proteins (FATP) and the cluster of differentiation 36 (CD36), located in the plasma membrane of the hepatocyte (91, 92). A long-term high-fat diet leads to the induction and upregulation of CD36 mRNA and protein along with hepatic steatosis (93, 94). In humans, CD36 mRNA expression is increased in patients with MASLD and is correlated with higher liver fat content (95).

Upon fatty acid uptake from plasma by a fatty acid transporter, specific fatty acid binding proteins (FABP) shuttle hydrophobic fatty acids within the hepatocytes. FABP1 is the most represented isoform in the liver and mediates the transport, storage, and utilization of fatty acids and their derivatives (91). It buffers against the cellular overload of FFA, which could cause lipotoxicity due to its binding sites for FFAs and by facilitating oxidation or incorporation into triglycerides (96). FABP1 also influences PPARα and PPARγ activities by transporting their ligands (i.e., long-chain fatty acids) to the nucleus (97, 98). Elevated FABP1 in early MASLD stages may enhance lipid flux to limit lipotoxicity, but as the disease progresses, diminishing FABP1 abundance can lead to increased lipid accumulation and lipotoxicity, promoting disease progression.

1.1.2.3.1.2 De novo lipogenesis (DNL) in the Liver

Excess carbohydrates obtained through the diet can be utilized to synthesize endogenous fatty acids through a complex DNL pathway in the liver. In this pathway, acetyl-CoA derived from

carbohydrates is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC), then further converted into palmitate by fatty acid synthase (FAS). These new fatty acids can then be desaturated, elongated, and esterified before being stored as TG or exported in VLDL particles (99). ACC and FAS are enzymes that directly enhance FA synthesis. The regulation of FAS in the liver is influenced by hormones such as insulin and glucagon and by nutrients, including carbohydrates and polyunsaturated fatty acids. The enzyme is activated by insulin and its substrate (citrate, isocitrate), whereas its activity is inhibited by glucagon and catecholamines via 3′,5′-cyclic adenosine monophosphate (cAMP)-dependent phosphorylation. The accumulation of fatty acyl-CoA in the cytosol suppresses the ACC activity. The regulation of FAS is also influenced by the quantity of intracellular fatty acids. Elevating fatty acid content leads to decreased FAS activity. The control of lipogenic gene expression by insulin and fatty acids is mostly facilitated by two key transcription factors, including sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate regulatory element-binding protein (ChREBP). Insulin primarily regulates hepatic DNL by influencing the INSR-IRS-AKT pathway, which activates SREBP1c (100).

Increased DNL contributes to hepatic fat accumulation in obesity and associated hyperinsulinemia and IR (101). Individuals who are overweight or obese with concurrent fatty liver exhibit increased DNL compared with those with lower liver fat when matched for adiposity and circulating lipids. Approximately 26% of hepatic TGs in patients with obesity and MASLD are accumulated through DNL, with a proportion being inappropriate relative to the fed/fasting transition. Thus, the lack of DNL suppression is central to liver lipid accumulation in patients with MASLD (102, 103). Even in IR conditions, insulin promotes DNL through SREBP1c while also failing to suppress gluconeogenesis (100, 104, 105, 106). Elevated hepatic SREBP1c in MASLD stimulates the upregulation of downstream pathways, leading to the elevation of ACC and FAS (107, 108). In addition, with the progression of IR and elevated plasma glucose concentrations, glucose can also promote DNL via activating ChREBP, leading to the transcription of genes involved in glycolysis and lipogenesis, thus converting excess glucose into fatty acids through the DNL pathway. This selective IR explains the elevated rates of hepatic DNL under IR conditions. Increased lipogenesis and the subsequent accumulation of harmful lipid species within the liver, such as diacylglycerides, contribute to the development of hepatic IR (109).

1.1.2.3.1.3 Lipid Oxidation in the Liver

The β-oxidation of FFAs serves as an "output" pathway for regulating liver lipid balance. β-oxidation occurs inside the mitochondria, where FFAs are broken down into acetyl-Coenzyme A (acetyl-CoA). FFA-derived acetyl-CoA may either be used for producing ketones or enter the tricarboxylic acid cycle to undergo full oxidation to carbon dioxide and water, resulting in the generation of metabolic energy, ATP, especially when circulating glucose levels are low (110). During fasting, hepatic peroxisome proliferator-activated receptor α (PPARα) is elevated, along with PPARγ-coactivator 1α (PGC-1α), a transcription factor that controls the catabolism of FFAs and the formation of new mitochondria. PGC-1α is stimulated by molecules that indirectly detect food availability, such as 5′ AMP-activated protein kinase (AMPK) (111, 112), and then interact with PPAR-α. SIRT1 also interacts with PPAR-α, hence increasing its transcriptional activity to accelerate FFA β-oxidation. When PPAR-α is activated, it triggers the transcription of many enzymes that help to increase the metabolism of FFAs, such as carnitine palmitoyltransferase 1 (CPT1), which is the rate-limiting enzyme responsible for moving long-chain FFAs into mitochondria (112). In the postprandial state, insulin activates the DNL pathway. In the first stage of DNL, after the conversion of acetyl-CoA into malonyl-CoA by ACC, increased malonyl-CoA concentration down-regulates the action of CPT1 to suppress β-oxidation (113). Furthermore, AKT phosphorylates PGC-1α, suppressing its ability to enhance β-oxidation (114).

In obesity and associated IR states, there are fluctuations in the rates of hepatic β-oxidation and the ability of mitochondria to undergo oxidative processes, which correspond to the development of liver fat accumulation (115). At the initial onset of IR, adipocytes still release NEFAs even when insulin levels are high, leading to higher NEFAs in the blood. This, together with increased DNL, results in excessive accumulation of FFAs in the liver, which then stimulates β-oxidation (115). The increased hepatic DNL and β-oxidation seen in IR seem to contradict the notion that in individuals with normal health, insulin or elevated DNL (linked to higher concentration of malonyl-CoA) should suppress β-oxidation (116). Nevertheless, the collective impact of hepatic β-oxidation and other output paths of TGs, like VLDL-TG secretion, falls short of overcoming the heightened DNL and NEFA flux to the liver (117).

Over time, the increased NEFA influx worsens IR through intensified accumulation of diacylglycerol and ceramides (118). Elevated hepatic IR leads to mitochondrial dysfunction, characterized by producing reactive oxygen species (ROS) and activating inflammatory responses. This can deplete mitochondrial DNA (mtDNA), which leads to fewer mitochondria and reduced mitochondrial function, resulting in inefficient catabolism of fats, increased amounts of harmful lipid compounds, and more oxidative stress, all of which may negatively affect insulin signaling (119). Thus, the state of fatty acid oxidation in MASLD depends on the stage of the disease spectrum, and impaired mitochondrial oxidative capacity is associated with hepatocyte damage caused by enhanced oxidative stress and inflammation (120).

1.1.2.3.1.4 Lipid Export from the Liver

In addition, being oxidized as an energy substrate, lipids can be exported from the liver to reduce hepatic lipid content. TGs are hydrophobic and require association with water-soluble, very low-density lipoprotein (VLDL) particles for transport to adipose tissue for storage or to muscle tissue for combustion (87). VLDL particles are formed in the endoplasmic reticulum, where apolipoprotein B100 (apoB100) receives lipids in a reaction mediated by microsomal triglyceride transfer protein (MTTP). Each VLDL particle contains one molecule of apoB100, which is required for the secretion of VLDL from hepatocytes; however, the TG content of individual VLDL particles is highly variable. In normal physiological conditions, insulin suppresses VLDL-TG secretion by activating the INSR-IRS-PI3K pathway (83) by increasing the degradation of ApoB100 and limiting its synthesis (121). ApoB100 and MTTP are important for hepatic VLDL secretion systemically and for regulating hepatic lipid homeostasis locally. Hepatic steatosis is seen in patients with loss-of-function genetic mutations in either the *Apob* or *Mttp* genes, which results in decreased TG export (121).

While moderate amounts of NEFAs increase apoB100 secretion, prolonged exposure leads to endoplasmic reticulum stress and posttranslational degradation of apoB100, decreasing VLDL-C secretion, thereby contributing to MASLD progression. *Mttp* transcription is positively regulated by PPARα and increased MTTP correlates with changes in apoB100 secretion (87). However, selective hepatic IR in MASLD patients allows increased postprandial insulin to stimulate DNL without inhibiting VLDL production as a compensatory path (122). However, it should be noted

that the VLDL-TG export increases to a certain extent by increasing intrahepatic lipid content, and its secretion plateaus when hepatic fat content exceeds 10% (w/w), surpassing the liver's compensatory capacity (87). Thus, in MASLD, lipid export is biphasic, initially increasing and then plateauing or decreasing, leading to hepatic lipid overload. This overload increases intracellular lipid accumulation, steatosis, lipotoxicity, and liver damage, promoting disease progression and fibrosis, as noted in Section 2.1.3.3.

1.1.3 Energy Balance

Energy balance, defined as the similarity between an individual's energy intake and expenditure, is important in maintaining weight status for all living creatures. Energy balance is zero if the organism takes in the same amount of energy it expends. In such conditions, body mass will be maintained, and there will be enough energy to support tissue functions. When something changes the energy balance from zero, regulatory responses attempt to regain the loss of homeostasis. For example, an increased consumption of food will be followed by an increase in energy expended to store, control, and move the stored energy, thus restoring balance (123). Variable interpersonal differences in response to changes in energy intake may explain the unpredictability of individual responses in weight reduction therapies and other factors that disrupt the energy balance system (124, 125).

The major sources of dietary energy include carbohydrates (4 kCal/g), proteins (approximately 4 kCal/g), fats (9 kCal/g), and alcohol (7 kCal/g) (126). Energy can be expended by performing work or producing heat (thermogenesis). Total energy expenditure refers to converting oxygen and food (or stored energy sources such as fat, glycogen, and protein) into carbon dioxide, water, heat, and work. Heat production occurs because several events involved in energy metabolism, such as those driven by the mitochondrial respiratory chain, plus those that consume ATP (for example, Na^+/K^+ ATPase, Ca^{2+} ATPase, and actinomyosin ATPase) release heat as an unavoidable by-product in the forward reaction. The sum of the organism's environmental work plus the heat produced from the chemical combustion of food is equal to the amount of heat energy produced during the physical combustion of food, measured in calories. During rest, energy expenditure can be measured in two ways. The first is direct calorimetry, in which the organism’s energy expenditure is measured by the heat produced. The second is indirect calorimetry, in which the quantity of

oxygen the organism consumes can be measured and correlated with energy expenditure (127, 128, 129).

Total daily energy expenditure consists of the resting metabolic rate (RMR) (approximately 60-75% of the total) and energy expenditure for physical activity (around 15-30% represents), with the remaining 5-15% available for food thermogenesis (Figure – 1.7). RMR is the energy required to maintain the body's vital functions and keep body temperature constant when a person is in complete rest. Although RMR is relatively constant from day to day, it can be altered across the lifespan (130). Physical activity is the component of total energy expenditure with the most variability because it represents all the activities done, either voluntarily or involuntarily. The last but not least component of total energy expenditure is food thermogenesis, which includes heat created when energy is used in all processes after food consumption, such as digestion, transport, storage, and assimilation of nutrients (131).

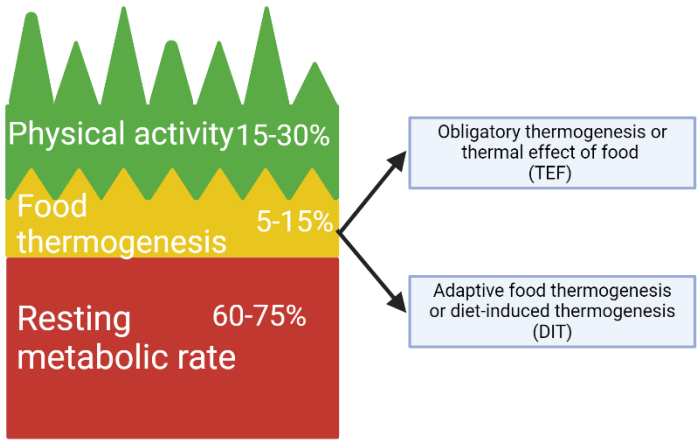


Figure 1. 7 Components of total daily energy expenditure.

Food thermogenesis consists of two components, including obligatory food thermogenesis and adaptive food thermogenesis (Image was created by EY using BioRender.com)

1.1.3.1 Energy regulation

The classical explanation of obesity is that fat accumulation in the body results from the long-term maintenance of a positive energy balance, leading to cumulatively weight gain in the form of fat. However, this simple perspective does not explain the many components of the problem of obesity. For example, this does not explain the fact that not all calories contribute equally to the energy balance equation. Also, although higher caloric intake is strongly correlated with weight gain, the reason for the dysregulation of food intake is not explained (132).

Energy restriction for weight loss produces a negative energy balance, in which the intake is less than the energy expenditure. Regulation of energy balance contains several biological and behavioral factors that regulate and influence both sides of the energy balance equation (133) and those factors associated with the intake of energy may influence other factors associated with the utilization of energy (133, 134). In the context of weight manipulation, reducing food intake alters several components of energy expenditure, including RMR, exercise metabolic rate, and food thermogenesis. Thus, energy restriction leads to weight loss in the short term, but very low energy intake can reduce RMR, cause muscle wasting, fatigue, and nutrient deficiencies, and may even increase the risk of weight regain following diet cessation (135). These consequences underscore the need to consider methods other than caloric restriction to create a negative energy balance.

Increasing energy expenditure to compensate for excess energy intake is an attractive and promising alternative approach to induce a negative energy balance instead of energy-restricted diets. A sustained and substantial increase in activity energy expenditure is followed by a lower RMR associated with improved muscle efficiency (yielding a smaller rise in activity energy expenditure over time) without a substantial net change in total energy expenditure over a long period (136, 137). Although a growing body of evidence considers increasing physical activity a means of boosting energy expenditure, food thermogenesis is equally important, with less research so far.

After a meal, there is an increase in energy expenditure above the RMR, which is ~5-15% for meals in humans and is comprised of so-called obligatory and facultative adaptive effects on metabolic rate. Obligatory food thermogenesis, also known as the thermal effect of food (TEF), refers to the energy required for necessary physiological events of digestion, absorption, and assimilation of nutrients, as well as the synthesis of body fat and protein. These processes are considered essential and non-negotiable in terms of energy expenditure. However, adaptive food thermogenesis refers to an additional energy expenditure from metabolic reactions accompanying dissipated energy consumed beyond the body's immediate requirements, but it is not essential like obligatory thermogenesis (138). It involves mechanisms that are not energy-conserving and thus can serve as a regulatory output to maintain energy balance.

1.1.3.1.1 Diet-induced thermogenesis

Diet-induced thermogenesis (DIT) defines adaptive food thermogenesis as activation and adaptation due to individual meal exposures (138, 139, 140, 141). The distinction between obligatory and adaptive food thermogenesis is not always straightforward. Some obligatory processes may have variable energy costs and could contribute to adaptive responses to nutritional changes. Focusing on increasing food thermogenesis achieved through diet manipulation may effectively establish a long-term negative energy balance sustainable for weight management (142, 143, 144). DIT is predominantly generated by BAT in rodents; however, its role in humans is less investigated and may be influenced by various factors (145). DIT is mediated by the amount and activity of uncoupling protein-1 (UCP1) in BAT. Other tissues, mainly the subcutaneous (primarily inguinal) white adipose tissue, may adopt a brown-like phenotype called “brite” or ”beige” adipose tissue that can participate in DIT (145).

1.1.3.1.1.1 Brown adipose tissue physiology and its relation to obesity

Adipose tissue is a metabolically active endocrine organ categorized into three types: white adipose tissue (WAT), brown adipose tissue (BAT), and beige or “brite” adipocytes. Brown and white adipocytes have opposing actions. White adipocytes can store energy in the form of TG. In contrast, brown adipocytes are thermogenic cells that metabolize substrates to produce heat when activated. Beige adipocytes are brown-like adipocytes derived from specific depots of WAT. They show an intermediate function between white and brown adipocytes and can act thermochemically after activation by appropriate stimulators (146). Adipose tissues are mainly differentiated through the ability to synthesize uncoupling protein-1 (UCP-1) to generate heat. A stimulus such as exposure to cold, medication, or nutrients increases *Ucp1* gene expression in brown and beige adipocytes (147).

The BAT can be activated by the sympathetic nervous system (SNS), which releases norepinephrine, an agonist for β3 adrenergic receptors (β3AR) abundant in BAT. β3AR binding initiates an intracellular signaling cascade leading to protein kinase A (PKA) activation, which stimulates transcription of *Ucp1* and activation of hormone-sensitive lipase (HSL). HSL hydrolyzes TG from lipid droplets to provide FA that are transported to the mitochondria for β-oxidation. The presence of respiratory chain proteins in the mitochondrial inner membrane is

responsible for generating a proton electrochemical gradient between the intermembrane space and the mitochondrial matrix. UCP-1 spans the mitochondria's inner membrane and permits the reentry of protons into the mitochondria matrix instead of producing ATP, dissipating energy as heat. This is called metabolic uncoupling. If BAT activity is constant, the stored TG in the lipid droplets of BAT will eventually be depleted. Subsequently, lipoprotein lipase (LPL) activation in the vessel wall near BAT hydrolyzes fatty acids from TG-rich lipoproteins in the bloodstream, facilitating their uptake into BAT and providing fuel for its activation. The TG-derived FAs are transported into the brown adipocyte via CD36 and FATP, as described for the liver. These FAs are a substrate for β-oxidation. In addition to FAs, BAT can take up glucose from the blood through GLUT4 (insulin-dependent) or GLUT1 (insulin-independent), which can then be utilized in glycolysis. During glycolysis, glucose is broken down to produce pyruvate, which can then enter the mitochondria for further energy production through the citric acid cycle and oxidative phosphorylation. This process provides the necessary energy substrates for BAT's thermogenic function (147, 148).

In addition to BAT, developing beige adipocytes within WAT may contribute to thermogenesis (149). Browning is initiated when WAT is exposed to a stimulator and yields increased mitochondrial content and UCP-1. Activation of BAT and maximal browning of WAT is directly associated with increased energy expenditure and weight loss (150, 151, 152). The gold standard for evaluating BAT activity and volume is ^{18}F-labeled fluoro-2-deoxyglucose (^{18}F-FDG), accompanied by positron emission tomography and computed tomography (PET/CT) imaging, widely used in the research (153).

Stimulating BAT is a practical approach to weight loss. Several human observational studies indicate a reverse association between BAT activity and body fat percentage and BMI (153, 154). Furthermore, BAT volume and activity among individuals with obesity are lower than those with normal BMI (154, 155, 156, 157). Interestingly, it was shown that South Asians, as a representative of unfavorable metabolic phenotype, have reduced BAT quantity, which is associated with lower resting energy expenditure (158). This phenomenon is also seen in animal studies. A/J mice with higher BAT content than C57BL/6J mice are resistant to the accumulation of excess fat and the development of obesity (159, 160, 161, 162, 163). These experimental findings lead to the

hypothesis that the undesirable accumulation of fat in the development of obesity might be because of decreased energy expenditure due to impaired BAT activity.

The most well-known BAT activator is cold exposure. Human studies indicate that exposure to the cold results in increased energy expenditure through the activation of BAT (155, 164, 165, 166). Although body weight remains stable during 6 weeks of cold exposure of 19°C for 2 hours per day among healthy men with low BAT activity, the percentage of body fat significantly decreases compared to the control group (165). Interestingly, among those participants with unmeasurable BAT activity at the beginning of the study, BAT is detectable after 6 weeks of intervention (165, 166). Exposure to a cold environment also induces BAT activation and leads to lower weight gain in HFD mice (167). In addition to activating fat with a cold stimulus, BAT transplantation is also considered an option. Studies in rodents demonstrate that transplantation of BAT prevents obesity and decreases body fat mass in HFD mice accompanied by higher body temperature, increased energy expenditure and oxygen consumption (168, 169, 170, 171, 172). To elucidate how much BAT activity can affect weight status, studies highlight the contribution of BAT to energy expenditure regardless of how BAT is activated. These researchers believe that BAT's contribution to energy expenditure is significant even though its actual impact is still under debate. Among healthy individuals, maximum activation of BAT increases the resting metabolic rate up to 16 % (155).

1.1.3.1.1.2 Activation of BAT thermogenesis through diet

In rodents, a high-fat cafeteria diet delivered *ad libitum* results in lower weight gain than expected based on calorie intake. However, there is an increase in BAT activity and, hence, an increase in energy expenditure. These adaptive changes in response to overfeeding are not seen in UCP1 knockout animals. Therefore, BAT is hypothesized to play a role in mediating the increase in energy expenditure through DIT to regulate energy (141, 145). In 1980, Glick *et al.* first hypothesized the occurrence of BAT stimulation following a meal. They measured an increased respiration rate in BAT within two hours of feeding rats (173). Moreover, they demonstrated that a meal caused an increase in guanosine 5′-diphosphate (GDP) binding to mitochondria that were isolated from BAT (173). This increased GDP binding was a measure of UCP1 activation.

Single nucleotide polymorphisms (SNP) in some genes regulated in BAT support the idea that BAT thermogenesis has a role in DIT and the control of energy balance in humans. The mutation in the *ADRB3* gene encoding β3AR or the *UCP1* gene associated with increased body fat, reduced metabolic rate, and less weight loss when subjected to low-calorie diets (174, 175, 176).

The invention of a new technique to assess the activity of BAT in adult humans was prompted by the limitations of ^{18}F-FDG-PET, which include its inability to differentiate between metabolically active tissues and the necessity for prior activation of BAT, leading to potential inaccuracies in assessing true thermogenic activity (177). ^{15}O [O2]-PET is more direct in measuring the metabolic activity of BAT in humans. It assesses the tissue's oxygen consumption rate, which is closely tied to the primary function of BAT activation to produce heat (178). This method provides more detailed information than ^{18}F-FDG-PET about thermogenesis and mitochondrial substrate oxidation. The magnitude of oxygen consumption and blood flow increases in BAT soon after eating are similar to cold exposure (166). To confirm the function of BAT in DIT, the total energy expenditure of healthy individuals was continually monitored over a 24-hour period using a human whole-body calorimeter (178). Upon categorizing the participants based on their FDG-PET/CT test results into high BAT and low BAT groups, the postprandial energy expenditure was notably greater in the high BAT group, accounting for 9.7% of the total calorie intake, compared to the low BAT group, which accounted for 6.5%. The high BAT group had a lower 24-hour respiratory quotient, suggesting a higher rate of FA oxidation (178). These findings suggest that, at least partially, BAT mediates DIT in humans. This is further supported by the discovery that prolonged exposure to cold increases BAT recruitment, which in turn is accompanied by improved DIT (178).

The major players in regulating DIT are the sympathetic nervous system and the βAR (179), with meal-induced increases in circulating norepinephrine and BAT activation, particularly following high-calorie intake (180, 181). According to human and animal data, the taste and oral detection of food significantly trigger this response (182, 183). Although there is a clear link between DIT and cold-induced thermogenesis via the sympathetic nervous system, some studies illuminate a different possible mechanism of DIT (184). Gut hormones like secretin and cholecystokinin play crucial roles in stimulating BAT activity; secretin has its own specific receptor in the membrane of BAT, and cholecystokinin activates BAT through direct and vagal nerve-mediated pathways (185, 186, 187, 188). Bile acids can activate a unique receptor in BAT, increasing DIT and

suggesting potential anti-obesity effects (189, 190). These synergistic or independent mechanisms collectively show a complex interplay between dietary components, gut hormones, the SNS and BAT in regulating energy expenditure, although more human studies are warranted to elucidate these interactions (145). Regarding dietary ingredients, capsaicin, derived from chili peppers, and its analogs, capsinoids, have thermogenic and anti-obesity effects (191, 192). Studies indicate that capsinoids increase total energy expenditure, induce a negative energy balance, and induced fat oxidation primarily in individuals with metabolically active BAT (193, 194). Interestingly, recent studies highlight that dietary fish oil, primarily eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), increases energy expenditure and prevents fat accumulation in HFD mice liver by increasing UCP1 in BAT (195, 196). Moreover, EPA can increase brown adipogenesis (197). These studies raise the possibility that other dietary components may have specific BAT-activating properties.

1.2 DAIRY PRODUCTS

As mentioned earlier, the accumulation of excess body fat, which begins with excess calorie intake, leads to expanding adipocytes trying to store this surplus of energy, which is a major metabolic stress for the body. The increased prevalence of obesity and its associated metabolic dysfunction, including IR, T2D, and MASLD, is one of the most common health concerns worldwide and is a big burden on healthcare systems. Although environmental and genetic factors increase the risk of these metabolic disorders, modifiable lifestyle factors significantly impact an individual's risk. The marked rise in obesity and its associated metabolic dysfunction can be partly attributed to the transition of diet toward adhering to a Western-style diet characterized by high fat, high sugar, and processed foods with high caloric density, leading to a constant chronic positive energy balance (198). Due to the significant role of nutrition in the development of obesity, an important research focus is on identifying dietary components that could be modified to impact energy and glucose homeostasis.

Most dietary guidelines suggest that eating dairy products, including milk, is an important component of a healthy, balanced diet in meeting nutrient requirements (199). Dairy products have been recognized as a potential dietary factor that could influence weight and metabolic health. The dairy food group is intricate, with multiple elements that can impact its physiological

consequences. These include the lipid content of the dairy products, the process of fermentation, the duration of ripening, the methods employed in production, and the diet of the dairy cows (200).

Since animals were domesticated 8,000 years ago, milk and fermented dairy products have become a widely-consumed component of the human diet. Dairy intake exhibits significant global variation. Developed countries, including those in the European Union and North America, consume more dairy than developing countries; however, the demand for milk and its products in developing nations is increasing due to the rise in incomes, population expansion, urbanization, and dietary changes (201). Data from Canada Statistics shows that per capita milk consumption decreased from 70.76 liters in 2015 to 58.2 liters in 2022 (Figure 1.8) (202). These data reflect a long decay in the amount of dairy consumed, as the Canadian Community Health Survey indicates that, from 2004 to 2015, the proportion of Canadians consuming milk and dairy products significantly decreased from 89.5 to 87.7%, and the number of servings consumed per day dropped from 1.9 to 1.7 (203). The decline in milk consumption is similar to red meat consumption in Canada, and attributed, in part, to the increased number of vegetarians and vegans and also a rise in the availability of plant-based alternatives in Canada's marketplace (203, 204). To address the increasing worldwide obesity crisis, most nutrition guidelines continue to emphasize the importance of limiting calorie and fat consumption. However, this approach fails to take into account the positive health effects of whole foods, which could be quite different that individual nutrients would predict. In recent years, much research has focused on the effects of dairy food as a whole (matrix) on energy and glucose homeostasis (205).

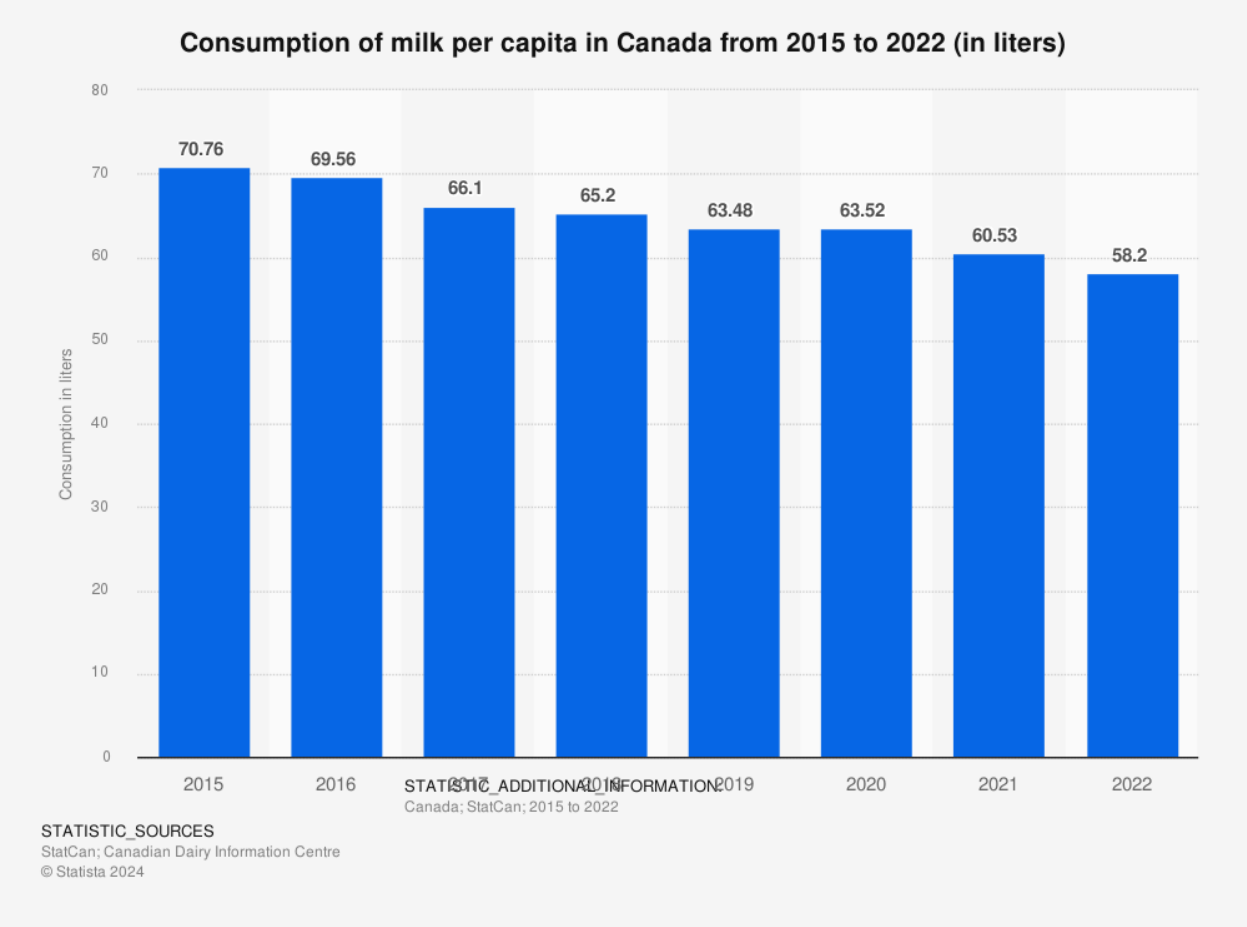


Figure 1. 8 Per capita milk consumption in Canada from 2015 to 2022, measured in liters

A general decline over the observed period, with the highest consumption at approximately 70.76 million liters in 2015 and the lowest at about 58.2 million liters in 2022.

1.2.1 Dairy Composition

Dairy products provide many essential nutrients (206), which vary according to the specific product (Table 1.2). Milk comprises 87% water, while the remaining 13% is made up of carbohydrates (4.8%), fats (3.4%), proteins (3.3%), and several vitamins and minerals. The main carbohydrate present in cow's milk is lactose. Milk fat is a complex mixture of several lipids classes, including TG, phospholipids, and sterols. Milk proteins are classified as high-quality proteins because they contain the 9 essential amino acids and include two major types of classifications: casein (80%) and whey proteins (20%). Among foods made by the processing of milk, in most bodies of literature, dairy foods are considered to include milk, yogurt, and cheese based on the United States Department of Agriculture (USDA) MyPlate definition. Milk-derived

products containing minimal calcium with high-fat content, such as butter and cream, are not included in the dairy food group .

Table 1. 2 Whole-fat dairy product composition(207)

Main Constituent	Whole Milk		Whole Yogurt		Cheddar Cheese	
	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)
Water	85.5-89.5	87.5	81.0-88.0	84.5	35.0-39.0	37
Fat	3.0-4.0	3.5	3.0-4.5	4	30.0-35.0	32.5
Protein	3.0-3.5	3.25	3.5-4.5	4	23.0-27.0	25
Lactose	4.8-5.2	5	4.0-5.0	4.5	0.0-0.5	0.25
Minerals	0.7-0.8	0.75	0.9-1.0	0.95	3.0-4.0	3.5

Regarding vitamins and minerals, milk is the primary source of calcium in the human diet (208) and also provides phosphorus, magnesium, potassium, and trace elements like zinc and selenium. Additionally, milk is an excellent source of important vitamins such as A, D, B12, riboflavin, and niacin. In the diet of Canadians in 2015, dairy products, while contributing only 12.3% of energy intake, played a significant role in providing essential nutrients. They contributed 45.8% of calcium intake, 39.9% of vitamin D intake, and 36.0% of vitamin B12 intake. Dietary intakes of calcium and vitamin D among dairy product consumers are significantly higher than those of non-consumers, by 137.8% and 59.4%, respectively. This highlights the critical role of dairy products in providing essential nutrients that are difficult to replace with other food sources (203).

Dairy products such as milk, cheese, and yogurt may have metabolic bioactivities beyond the classic *"building strong bones"* being a source of calcium (205). A growing body of research indicates that dairy products have complicated effects on the body's metabolism due to complex nutrient profiles and other properties resulting from manufacturing, including homogenization, fermentation, and the addition of probiotics. Regarding the macronutrient constituents of dairy products, milk protein attracts attention because of evidence that it can support weight loss and the reduction of obesity (209). Whey protein from milk reduces body weight and improves obesity metabolic dysfunctions through appetite suppression by increasing the secretion of satiety hormones such as GLP-1 and GIP (210). Whey protein consumption can also maintain fat-free

mass during weight loss and thus can prevent a decline in metabolic rate as a consequence of an energy-restricted diet. Increasing whey protein intake results in higher energy expenditure (211).

Calcium is another nutrient in milk and yogurt that has a key role in the healthy development of bones and teeth, and the clinical evidence supports the claim that it directly improves metabolic health. Calcium in either reduced or whole-fat dairy products decreases the accumulation of body fat and accelerates weight and fat loss during dieting (212). In a meta-analysis of observational studies, higher calcium consumption is associated with an 18% lower risk of T2D (213). Calcium supplementation enhanced glucose-induced insulin secretion in patients with T2D. In addition, higher calcium intake was associated with reduced lipogenesis, increased lipolysis, and lower pro-inflammatory cytokines, indicating the ameliorating effect of higher dietary calcium intake on obesity, inflammation, and, consequently, IR and T2D (214, 215).

Another plausible mechanism by which dairy products can improve metabolic health is through their high amount of phospholipids. Consuming dairy products or supplementing a diet with milk-derived phospholipid could normalize the phospholipid species profile in the liver and plasma, which are disrupted by obesity (216). The potential role of other dairy compounds, including FA biomarkers of dairy fats (217), probiotics (218), and vitamin D (219, 220), on obesity and its related metabolic disorders, have been shown in epidemiological and clinical trial studies.

Along with the abovementioned nutrients, dairy products contain natural bioactive molecules that promote the health benefits of consuming these foods (221, 222). With my co-authors (Yuzbashian, Berg, de Campos Zani, and Chan), I have published a manuscript entitled "Cow's Milk Bioactive Molecules in the Regulation of Glucose Homeostasis in Human and Animal Studies," an offshoot of the research element presented herein (223). In this review, we summarize data from trials investigating the milk's bioactive molecules related to IR and its risk factors, including obesity and MASLD. With regard to the possible beneficial effect of dairy's bioactive molecules in metabolism, in this narrative review, results from clinical trials have been evaluated along with the potential mechanisms of each bioactive molecule obtained using data from *in vitro* and *in vivo* studies. This review improves our understanding of the link between dairy products, their bioactive compounds, and body metabolism, which provides an instrumental role in shaping the arguments and conclusions drawn in the subsequent section of this thesis. We conclude that bioactive compounds are present in carbohydrate, lipid, and protein fractions of milk, which

highlights the importance of considering the effects of whole foods in nutritional and metabolism research (223).

1.2.2 Importance of the Dairy Food Matrix

Traditionally, most studies have investigated the association of single nutrients or compounds in dairy products with health outcomes. However, it should be acknowledged that people do not consume nutrients in isolation in their diet. Nutrients are commonly consumed within a whole food, which is a mixture of nutrients defined by unique composition and structure. Nutrition science has evolved through the past decade, emphasizing the importance of whole foods instead of focusing on their single isolated nutrient in relation to health outcomes, and this is much evident in the framework of dairy products (205).

The interplay of nutrients and bioactive compounds within the unique dairy food's structure is called the dairy matrix (Figure 1.9). Proteins, fats, carbohydrates, vitamins, minerals, as well as certain bioactive molecules form a network within a dairy product's physical structure, interact with each other in the matrix itself, and thereby influence each other in the processes of digestion, absorption, and metabolism (224, 225). For example, dairy fat improves the absorption of fat-soluble vitamins A and D (226), and lactose improves the bioavailability of calcium in such a way that dairy is an excellent source of calcium when compared with supplements and non-dairy foods (227). The processing of dairy products also impacts their physical state and hence the matrix properties of each food type. Fluid milk is changed through manufacturing via processes such as pasteurization, homogenization, and microfiltration, yielding new products like yogurt or cheese, or it can be evaporated and dried to produce powder milk solids.

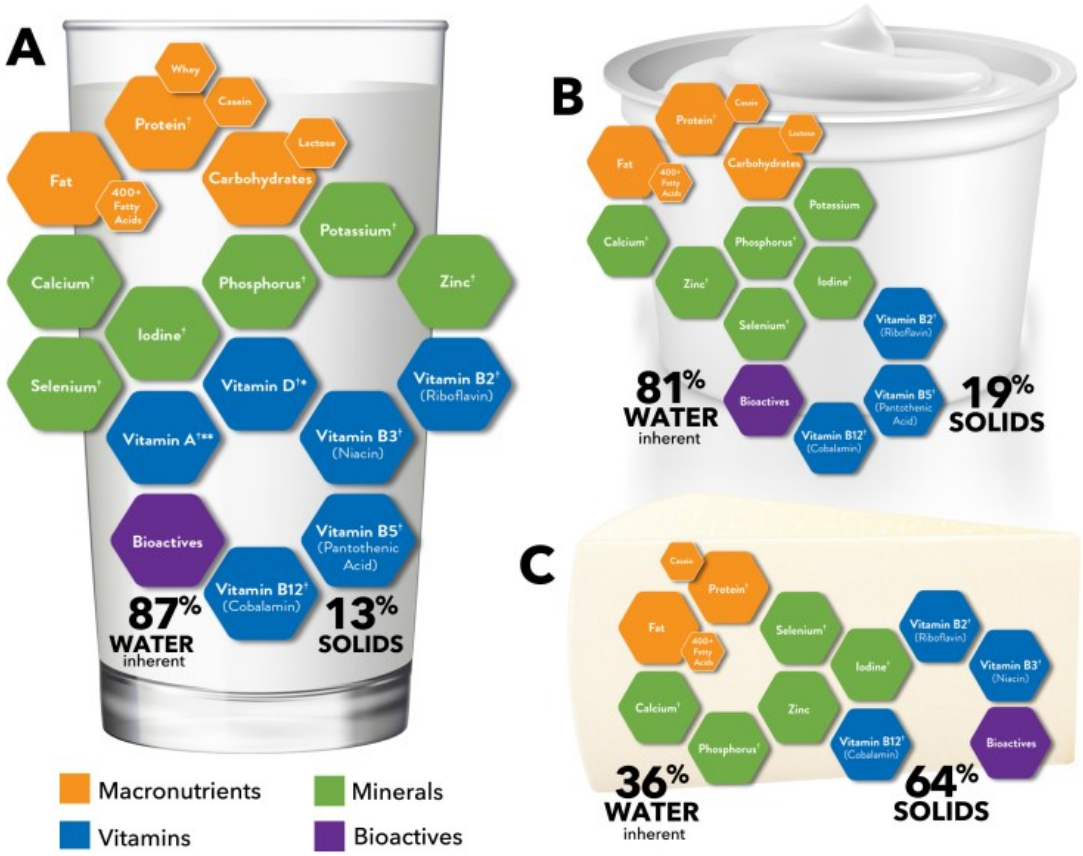


Figure 1. 9 Milk, yogurt, and cheese dairy food matrix.

(A) The image uses the average composition of the whole (3.25%) milk. (B) The image uses the average composition of low-fat (1%) vanilla yogurt. C) The figure uses the typical cheddar (29% fat) cheese composition (Unger et al).

The lipids found in dairy products are of particular interest (Figure 1.10). These molecules, including TG, phospholipids, and sterols, possess distinct chemical characteristics that influence their activity within the dairy matrix and in animal physiology. Milk fat globule membranes (MFGM) of varied diameters are formed by these lipid molecules, which impact the digestion and absorption of milk lipids (228). Metabolic reactions like postprandial lipemia, cholesterol levels, and plasma lipid composition may be influenced by lipid molecules within the dairy matrix (229).

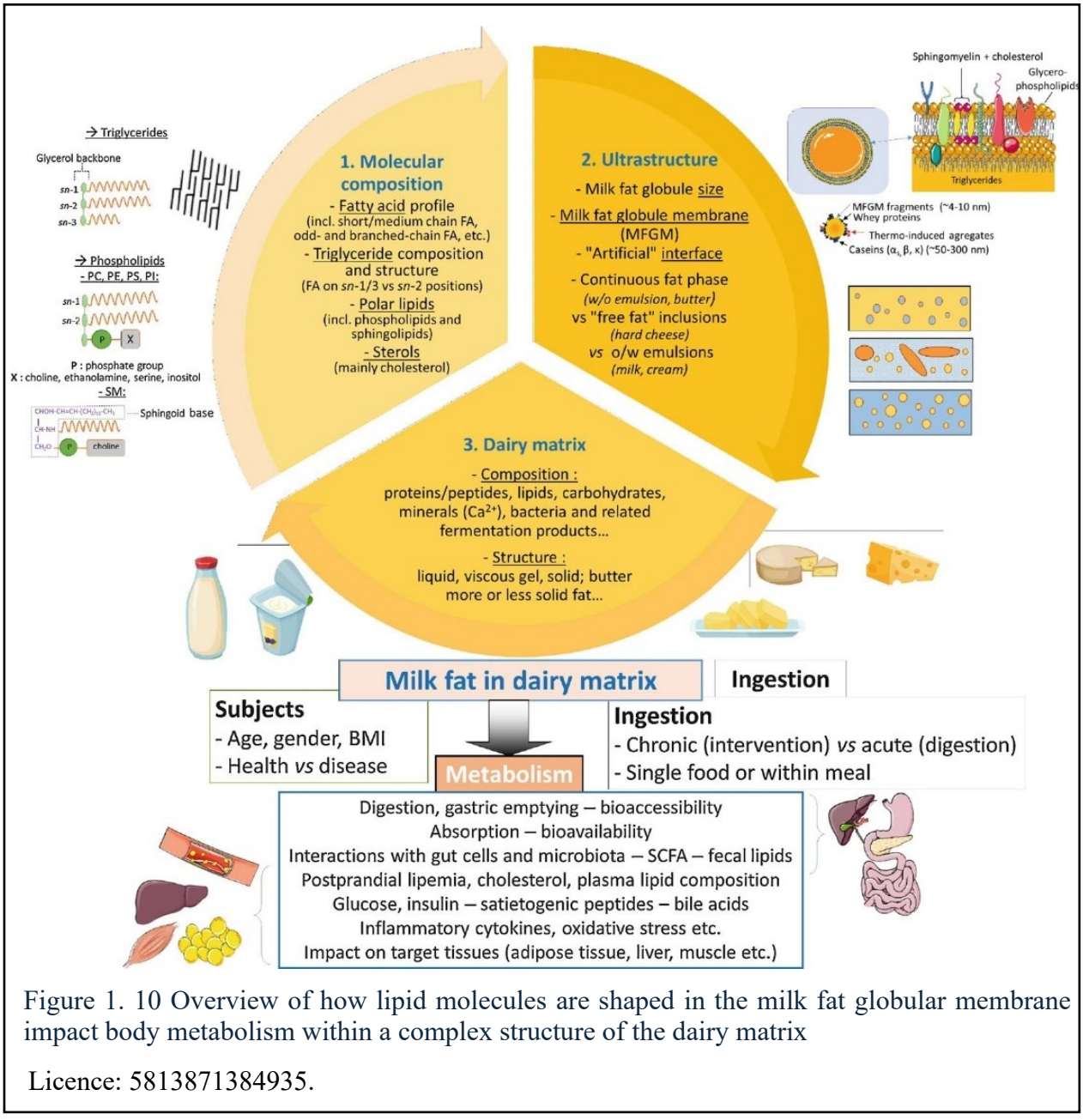


Figure 1. 10 Overview of how lipid molecules are shaped in the milk fat globular membrane impact body metabolism within a complex structure of the dairy matrix

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Considering all of this, the intact dairy matrix should be studied for its nutritional and metabolic effects.

Since milk, yogurt, and cheese are processed differently, they exhibit different physicochemical properties. Milk is a liquid where proteins, fats (lipids), lactose, vitamins, and minerals are evenly distributed. The fats form a stable emulsion, while the other components are dissolved in the water content of the milk. Yogurt is produced by fermentation with a specific starter bacterial culture containing *Streptococcus salivarius* subsp. thermophilus and *Lactobacillus delbrueckii* subsp. Bulgaricus is added to homogenized and pasteurized milk, providing a thicker texture. It also has higher acidity (pH 4.6), resulting from the conversion of lactose into lactic acid, which enhances its probiotic content. Cheese is produced primarily from fermentation or the addition of acid (acid-set) milk. Protein coagulation and aging processes yield a solid structure with concentrated proteins and fats, reduced lactose, and diverse flavor profiles depending on the type and duration of aging. These variations should be taken into consideration when making dietary recommendations and studying the health impacts of dairy products since they highlight the relevance of the matrix in determining these effects (205, 224, 225).

Dairy products' physical state (food texture) affects their digestion, absorption, and subsequent appetite regulation (230). As a liquid, milk is usually digested and absorbed very quickly, hence serving as an efficient vehicle for providing hydration, proteins, and electrolytes. However, the lactose content of milk can cause digestive discomfort for people who are intolerant to it. Yogurt has a semisolid jelly-like texture. This thickened texture and higher acidity of yogurt lead to slower gastric emptying and consequently increasing satiety. In addition, bacterial ferment of the lactose makes yogurt easier to eat for a person who is lactose intolerant. Cheese, as a solid, is nutrient-dense, with low lactose content and high concentrations of available calcium and protein. Combining the cheese's solid matrix and high-fat content contributes to longer absorption times by slowing stomach emptying (230). A crossover design study found that equicaloric meals of grated cheese and yogurt prolonged satiety and delayed gastric emptying compared to when they were provided as a liquid emulsion. Prolonged gastric retention likely resulted from augmented viscosity that then affected intestinal nutrient stimulation of satiety hormone release (231). Further, a recent study by Vien et al. looked at the effects of the dairy matrix on appetite, glycemic and insulin responses, and food intake. In their crossover RCT, test meals included whole milk, skim milk, Greek yogurt, and cheddar cheese provided to adult men and women. It was observed that

appetite suppression was higher with the solid and semisolid products (cheese and yogurt) compared to their liquid forms (skim milk and whole milk) (232).

Dietary recommendations are slowly starting to appreciate the dairy matrix (233). Dairy products and their complex matrices are now being considered in guidelines rather than just specific nutrients like calcium or saturated fats. Research studies should take the dairy matrix concept into account to properly evaluate the health effects of dairy products and to provide informed dietary recommendations.

1.2.2.1 Dairy Consumption and Obesity

1.2.2.1.1 Systematic Reviews and Meta-analyses

Several few systematic reviews and meta-analyses shed light on the current state of evidence regarding dairy consumption and associations with obesity or various adiposity measures. A recent dose-response meta-analysis of pooled prospective cohort studies indicates that the risk of overweight or obesity decreases by 25% for every 200g/d increase in total dairy product consumption. Regarding individual dairy products, the risk of overweight or obesity decreases by 7% and 12% per 200-g/d increase for high-fat dairy and milk, respectively, and by 13% per 50-g/d increment of yogurt. Interestingly, there is no significant association between low-fat dairy products and cheese with overweight or obesity. It should be noted that the neutral association for low-fat dairy products is explained by pooling only 2 studies (234). Another meta-analysis of prospective cohort studies suggests that total dairy consumption is not associated with a risk of overweight and obesity when a high intake of dairy products is compared with low intake (235); however, the researchers did not analyze subgroups of dairy products. A meta-analysis of observational studies focuses on mean differences in body weight rather than the risk of being overweight and obese, indicating no association between total dairy product consumption and body weight change but finding an association between higher intake of yogurt and weight loss (236). Pooled odds ratios of 17 observational studies indicate that the risk of obesity for the highest versus lowest category of total dairy product consumption is decreased by 25%. Furthermore, higher milk consumption is also associated with a 23% lower risk of obesity. In the dose-response analysis, the risk of obesity decreased by 16% for every 200g/d increment of milk consumption (237).

There are further meta-analyses that investigate the association between dairy product consumption and the risk of obesity (238, 239); however, pooling the risk estimates extracted from observational studies has limitations. The contradictory findings between studies in which meta-analyses indicated a lower risk of obesity (234, 237) while others showed a neutral association (235, 236) might be explained by the nature of the studies, given that these meta-analyses include mainly cross-sectional studies, which are more prone to bias. The other limitation is that considerable unexplained heterogenicity was observed in all pooled analyses. Studies including subgroup analysis and meta-regression analyses to explain the possible source of heterogeneity reveal that the heterogeneity might be associated with the characteristics of the participants in addition to study design and variation in follow-up time (234, 236). Moreover, the diverse categories of dairy consumption ("times per week," "grams per day," "serving per day," "high/low intake," "tertiles," etc.), the application of different dietary questionnaires, and adjustment for different confounders may also lead to heterogeneous results.

Numerous randomized controlled trials (RCTs) investigated the effect of dairy consumption on various adiposity indices. A recent meta-analysis of RCTs, which pools the mean difference from 9 original studies with at least 12 weeks of intervention in adults with overweight and obesity, finds that dairy products, regardless of their fat percentage, significantly decrease BMI (MD = −0.46 kg/m^2) and body fat mass (SMD= −0.40); however, there are no differences in changes in body weight and waist circumference (240). The data from 16 RCTs shows that more dairy consumption results in a greater decrease in body fat (MD=0.72 kg) and an increase in lean mass (MD=0.58) with a 2.19 cm further reduction of waist circumference without a significant impact on body weight (241). However, when dairy products are included in calorie-restricted diets for weight loss, studies show a greater reduction in body weight, fat mass, and waist circumference reductions, as well as increased lean mass in the intervention groups compared to controls on habitual diets (241). In parallel with this finding, a meta-analysis of 29 RCTs reports that although dairy consumption does not significantly decrease body weight, in subgroup analysis, there is a reduction in body weight in addition to fat mass when compared specifically with energy-restricted diets (242). Furthermore, a meta-analysis of 37 RCTs finds that dairy consumption reduces fat mass and waist circumference in individuals following a calorie-restricted diet but increases body weight in those restricted in calorie consumption (243). The conclusion of a recent overview of systematic reviews and associated meta-analyses is that increasing total dairy intake in the absence

of caloric restriction has no influence on weight, fat mass, lean mass, or waist circumference. On the other hand, a diet low in calories and high in dairy products causes a greater reduction in body weight and fat mass (244), supporting the idea that the effect of dairy on body composition varies according to dietary context and total calorie intake.

The most important limitation of the meta-analysis of RCTs is that many of the abovementioned studies do not consider the effect of subtypes of dairy products on measures of adiposity. However, as mentioned in the previous section, because the dairy matrix affects the digestion and absorption of the same nutrients, the effect of each dairy product (and interactions between components) may differ in the human body.

1.2.2.1.2 Randomized Controlled Trials and Animal Studies Involving Milk

Several interventional studies in humans investigate the effect of milk consumption on anthropometric measures in the context of energy-restricted or *ad libitum* energy programs. In an RCT, fat-free milk consumption elicits a greater reduction in body weight, body fat mass, waist circumference, and waist-to-hip ratio in adults with type 2 diabetes compared to a control diet in a 12-week experimental session (245). Overweight or obese premenopausal women who receive a total of 4 servings/d of low-fat milk in the context of a weight-maintenance diet for 8 weeks have significantly greater reductions in weight compared to the control group (246). In an energy-restricted diet, overweight and obese adults who consume 400 ml of 3% fat milk do not experience lower weight or BMI compared to a control group (247). A similar finding is observed after 6 weeks of consumption of 2% fat milk within a weight-maintenance diet in postmenopausal women with abdominal obesity compared to a milk-free diet (248). In a 6-month energy-restricted program, adding 3 cups of milk to the diet of women with obesity leads to greater loss of weight and fat mass compared with those provided rice-based beverages (249). Conversely, premenopausal women with overweight or obesity assigned to consume 3–4 servings/d of whole-fat milk in the context of an energy-restricted diet show similar weight loss to controls not consuming dairy. However, the milk consumption group lost more fat mass and visceral and trunk fat and gained lean tissue compared with the non-dairy group [255], indicating that milk intake within an energy-restricted diet for weight loss can maintain or increase body lean mass. Another study compares the effect of 3 cups of low-fat milk with a no-milk group among obese women who followed an energy-restricted diet and found that, after a 6-week intervention, the low-fat

milk group lost significantly more weight than the control group. The low-fat milk group also shows a greater relative fat mass reduction than the control group (250). Kukuljan et al. investigated the effect of 400 mL/d of reduced-fat (1%) milk consumption on the body composition of men with morbid obesity. After 18 months of intervention, this study indicates that there is no significant effect of milk consumption compared to the non-dairy control group (251).

Overall, contradictory findings have been found in clinical trials examining milk intake; whilst some research found benefits in body composition and weight reduction, others found no difference with control diets. The inconsistent results could result from variations in the baseline dairy consumption before the intervention commenced (for example, between studies conducted in the United States, Northern Europe, or Asia). However, it can be inferred that low-fat milk consumption, with or without an energy-restricted diet, may help promote weight loss and changes in body composition, such as reductions in fat mass and increases in lean tissue. There are a number of limitations with the RCTs on this topic. These include small sample sizes, inherent challenges in blinding diet studies, questionable dietary intervention compliance, potentially insufficient duration to significantly impact adiposity measures, and the difficulty of distinguishing the effects of milk alone because of its combination with other interventions.

While clinical trials in humans yield mixed results, animal studies conducted to explore the mechanism by which dairy consumption can change weight and body composition provide additional insights into its potential impacts. However, most animal studies use milk nutrients in an isolated form to elucidate the mechanisms behind milk intake, often ignoring the milk matrix. Experiments incorporating milk are summarized here. Long-term (17-week) milk consumption in young mice fed with a regular chow diet *ad libitum* indicates that while low-fat milk leads to similar caloric intake and body weight gain as the control group, whole milk (3.25% fat) consumption results in higher caloric intake and increased body weight. In addition, compared to control groups receiving water, the low-fat milk group has lower fat mass and higher lean mass, accompanied by reduced DNL in WAT (252). The weight loss effects of the chronic intake of milk are attributed to the increased BAT volume, which is critical for promoting energy expenditure (outlined in section 2.14.1.1) (253). When whole milk is added to the standard diet of rats, the milk group has weight gain and energy intake similar to that of water intake rats, but the fat-to-lean ratio of the milk group is significantly higher (254). In contrast, obesity-prone rats (IIMb/Beta strain) supplemented with skimmed milk showed less weight gain and had lower abdominal fat pads than

rats on the standard diet, pertaining to the increase of fecal fat losses induced by calcium of skimmed milk (255). Interestingly, skimmed milk powder provided in a high-fat, high-sucrose diet (HFHS) reduced weight gain as much as exercise training (256). In conclusion, while clinical trials in humans have shown varied results regarding milk's effects on weight and body composition, animal studies suggest that milk consumption, particularly low-fat and skimmed varieties, may promote favorable changes in body composition.

1.2.2.1.3 Randomized Controlled Trials and Animal Studies Involving Yogurt

The weight management benefits of yogurt have been intensively studied in recent years in part because of a growing appreciation of the probiotic content of yogurt and its potential health benefits. Findings from a meta-analysis summarizing 9 RCTs (up to 2019) indicate that vitamin D-fortified yogurt decreased body weight by 0.92 kg and waist circumference by 2.01 cm compared with controlled treatments (257). In a recent intervention study, a 3-month weight loss regimen using a hypocaloric and fiber-sufficient diet with probiotics, i.e., yogurt, in obese females shows significant reductions in BMI and fat mass (258), which suggests that gut microbiota modulation via increased yogurt consumption may be beneficial for obesity management. Supplementation of 250 g/day of calcium-fortified yogurt and plain yogurt to the diet of healthy individuals with a normal BMI reduces BMI and waist circumference (259). This is concordant with another study finding that intake of both probiotic yogurt and low-fat conventional yogurt over 12 weeks, as part of a weight-loss program, significantly reduces BMI and waist circumference to the same extent, indicating the benefits are independent of fortification (260). Additional RCT examines the effect of fortified or conventional yogurt on adipocyte measures, yielding favorable impacts on these metrics (261, 262, 263). Null findings associated with yogurt intake on anthropometric measures are not common but are not absent. For example, Sandby et al. report no difference in weight loss or body composition in men with abdominal obesity who receive 400 g/day of yogurt for 16 weeks (264). In summary, although many research studies emphasize that yogurt intake will likely support weight loss and body composition improvement, possibly more if it is enriched with vitamins or probiotics, there is also evidence proving a positive impact from consuming plain yogurt, even low-fat, on anthropometric measurements.

The literature on the effect of yogurt on measures of adiposity in animal studies is quite varied and heterogeneous, mainly explained by the variable composition of yogurt (for example, non-fat, low-fat, high-fat, sugar-added, high protein), which can be supplemented with multiple compounds (for example vitamin D, calcium, whey protein). Studies use fermented milk containing different bacteria for making yogurt (265), and several studies supplemented additional probiotic bacteria into the yogurt (265, 266, 267) to potentiate their effects. These differences, in general, make comparisons quite challenging; however, they provide a vast landscape for understanding the relationship of different yogurt formulations to the obesity phenotype. Studies highlight that the negative effects of an HFD on weight gain and fat mass are mitigated by commercially available yogurt in animal models (268, 269, 270, 271). For instance, mice fed with HFD supplemented with a conventional low-fat yogurt gained less weight and had reduced fat mass compared to the control group (272). Another study in mice conducted by Daniel et al. shows that consumption of 2% fat yogurt along with HFD results in significant decreases in body fat percent and total weight gain as compared to HFD (273). Yogurt supplementation significantly contributes to weight management by preventing excessive weight gain, lowering epididymal fat deposition, and reducing the final body weight of rats on an HFD (274). Mechanisms through which yogurt, particularly low-fat yogurt, influences adiposity are mostly attributed to changes in the composition and activity of gut microbiota because of the high content of probiotics in yogurt. In addition, specific probiotic strains in yogurt, such as *Lactobacillus* and *Bifidobacterium*, can also impact energy metabolism and fat storage (275).

In conclusion, although the data from animal studies support that the consumption of low-fat yogurt leads to weight gain attenuation and a decrease in fat mass, there is a need for further research directed toward commercially available yogurt.

1.2.2.1.4 Randomized Controlled Trials and Animal Studies Involving Cheese

Recent RCTs have been conducted to investigate the effect of cheese consumption on body weight and adiposity indices. These are important trials, given the perception of many that cheese causes weight gain because of its high-fat content. Providing 30 g bryndza cheese in an energy-restricted diet for 4 weeks leads to a significantly greater reduction in BMI, body fat, and waist circumference of women with obesity compared with an energy-restricted diet alone. The abundance of lactic

acid bacteria and short-chain fatty acid producers in the gut is also increased (276). Moreover, a combination of 4 weeks of resistance training with 108 g/d cheese supplementation results in a greater reduction in BMI and fat mass compared to resistance training alone, with the possibility of favorable changes in the gut microbiome (277). A combination of 4 weeks of resistance training with 108 g cheese supplementation results in a greater reduction in body weight, BMI, fat mass, and fat percentage compared to resistance training, with the possibility of favorable changes in the gut microbiome (277). In an 8-week RCT in adults with metabolic syndrome, Nilsen et al. report that there is a significant reduction in waist circumference after eating 50 g/day Gamalost cheese (0% fat) compared to the non-cheese group, with effects more pronounced in individuals with higher baseline waist circumference (278). Conversely, participants with abdominal obesity who receive isoenergetic diets containing cheese for 4 weeks show no change in waist circumference, BMI, and body fat compared with those provided a PUFA-rich diet (279). Also, 8 weeks of 80 g/day Gouda-type cheese (27% fat) has no significant effect on waist circumference in adults with metabolic syndrome (278). These studies show that cheese might contribute to weight management and changes in body composition when taken within an energy-restricted diet or with exercise, or at least not worsen these outcomes. Cheese with higher protein and lower fat increases the feeling of satiety, leading to slightly lower energy intake compared with high protein/high-fat cheese and low protein/high-fat cheese, and thus the composition or type of cheese may lead to different outcomes for body weight (280).

Animal experiments also indicate that cheese has beneficial or neutral effects on body composition and weight. When rats are fed with HFD supplemented by Gouda-type cheese, they accumulate less abdominal fat compared with those fed an HFD (281). A study comparing the effects of diets with equal calcium and fat content from cheese, butter, and palm oil finds that the cheese-fed group (80 g lipids per kg diet) has higher fecal fat excretion and does not gain weight compared to the control group (40 g lipids per kg diet), despite consuming more fat (282). On the other hand, an investigation into the effects of low-fat versus high-fat cheese added to an HFD reports no effect on body weight compared with HFD alone (283). Overall, these results suggest that cheese has unique properties related to fat metabolism and energy intake that may be different from those of milk or yogurt.

To summarize, human RCTs and evidence from animal experiments demonstrate that cheese inconsistently lowers body weight and improves body composition. Animal studies show that these

influences of cheese differ between varieties of cheese and the larger dietary context within which it was consumed. More research is needed to establish the nature of the impact that cheese has on obesity and weight, including mechanistic studies. This would allow more accurate dietary recommendations for optimizing health benefits from cheese consumption while minimizing risks.

All in all, there are still gaps in our understanding of the effect of dairy intake consumption on obesity and body composition. Although systematic reviews and meta-analyses generally report an association of higher dairy intake with a lower risk of obesity, the extent to which these effects can be attributed to low-fat milk, low-fat yogurt, and reduced-fat cheese is unclear. Heterogeneity extends also to the study designs, populations, and dairy food items under study. Current evidence shows that low-fat milk and low-fat yogurt tend to favor reducing fat mass and increasing lean mass, particularly when combined with energy-restricted diets. The influence of low-fat cheese is, however, less defined.

1.2.2.2 Dairy Consumption and Insulin Resistance

Obesity-induced IR is the main factor contributing to the long-established connection between obesity and T2D. The presence of IR is also associated with a broad range of additional pathophysiologic consequences, such as metabolic syndrome. Recommendations emphasize that it is more cost-effective and reduces the treatment burden to begin intervention during the period of IR rather than after a diagnosis of T2D (284). Recent research has investigated the distinct health effects of milk and dairy products, including the risk of metabolic syndrome, IR, and T2D, as elaborated in this section.

1.2.2.2.1 Systematic Reviews and Meta-analyses

A recent (2021) meta-analysis of 22 observational studies shows that the highest versus the lowest total dairy consumption is associated with a 20% lower risk of metabolic syndrome. Regarding the type of dairy products producing a health benefit, both the highest compared with the lowest milk and yogurt consumption decrease the risk of metabolic syndrome by 19%. There is no significant association between cheese and metabolic syndrome (285). In a dose-response meta-analysis of observational studies, pooled risk estimates for a one-serving/d increase of total dairy food (9 studies), milk (6 studies), and yogurt (3 studies) reduce by 9%, 13%, and 18%, respectively, the risk of the metabolic syndrome. It is noteworthy that among subtypes of dairy products, only for

yogurt is a one-serving/d increment associated with a lower risk of hyperglycemia (by 16%) (286). The important limitation of this meta-analysis, besides pooling the data of both prospective cohort and cross-sectional studies, is that the authors do not separate the dairy products based on their fat content in their subgroup analysis. Another meta-analysis of cohort studies indicates that consumption of total and low-fat dairy products, milk, and yogurt is inversely associated with the risk of incidence of metabolic syndrome (287).

There are several meta-analyses of observational studies with a dose-response analysis indicating a favorable association of total dairy product consumption with risk of incident or prevalent T2D (288, 289, 290). To summarize these meta-analyses published so far (up to Jun 2024), 8 meta-analyses of cohort studies consistently show an inverse association between total dairy product consumption and risk of T2D (288, 289, 290, 291, 292, 293, 294, 295), except for a study conducted by Schwingshackl et al. (296) which demonstrates a neutral association. In 6 of 7 studies (but not reported in 2 studies), when stratified based on fat content, there is an inverse association between low-fat dairy products and T2D risk (289, 290, 293, 294, 295, 296). Of the various dairy products, 4 of 7 studies that investigate subgroups of dairy products find a significant favorable association for total milk consumption [310, 313-315], and all of those analyzing milk data based on fat content find a significant inverse association for low-fat milk consumption (293, 294). All studies indicated a significant association between yogurt consumption and lower T2D risk (293, 294, 295, 297, 298, 299, 300).

In the most recent dose-response meta-analysis of 20 prospective cohort studies investigating the association between dairy consumption and T2D, Feng et al. report that per 200-g/d increase in total dairy consumption, the risk of T2D decreases by 3%. However, when considering the type of dairy products in their analysis, they observe that only yogurt is associated with a lower risk of T2D (234). A recent unique meta-analysis synthesizes the data of cohort studies that substituted dairy products with plant-based foods, other animal-based foods, and specifically other dairy products and assesses the risk of T2D in the general adult population. Although the number of included studies was quite low (2-3 studies for each analysis), the findings are interesting: substituting low-fat dairy (200 g/d) with red meat (142 g/d), processed red meat (57 g/d), or unprocessed red meat (142 g/d) is associated with a 20%, 40%, and 19% higher risk of T2D incidence, respectively. Regarding the types of dairy products, substituting milk (200 g/d) with

processed meat (50 g/d) is associated with a 10% lower risk of T2D incidence, while for yogurt, when 200 g/d is substituted with red meat, processed meat, or eggs (all 143 g/d) a 21%, 43%, and 44% increased risk of T2D are detected. Furthermore, replacing cheese (30 g/d) with processed meat or eggs (both 50 g/d) is associated with a 14% higher risk of T2D incidence (301). This meta-analysis has the advantage of considering the potential differential influence of dairy depending on the substituted foods [317], not single-exposure models as in the previous meta-analysis.

We previously conducted a prospective cohort study within the framework of the Tehran Lipid and Glucose Study, which is an ongoing prospective study among the population of the capital of Iran, Tehran. In this analysis, we examined the association of changes in total consumption of dairy and subtypes of dairy foods through time among participants with prediabetes with subsequent risk of incident T2D (302). We observed that reducing dairy intake by ≥0.50 servings/d was linked to a 56% increased risk of diabetes at the following 3-year follow-up, compared with prediabetes patients who maintained a steady consumption (±0.50 servings/d). After grouping dairy products based on their fat content, when compared to maintaining steady consumption, increasing low-fat dairy intake by 0.50 servings per day was linked to a 44% reduced risk of T2D. No association was found between the risk of T2D and either increasing or reducing intake of high-fat dairy products. Increased consumption of low-fat milk and low-fat yogurt through the 6 years of follow-up, but not cheese, was associated with a lower risk of subsequent T2D. Interestingly, in the substitution analysis, we consistently observed that replacing 0.5 daily servings of high-fat dairy and its subtypes with the same amount of low-fat dairy was associated with lower T2D risk in the subsequent 3-year follow-up period (302). As mentioned above, the protective effect of fermented dairy products attracts considerable attention because it is hypothesized that the presence of probiotic bacteria provides an added health value to the dairy matrix. However, our findings did not show a superiority of fermented dairy products like yogurt and cheese over non-fermented milk. Nevertheless, a meta-analysis specifically investigating the association of fermented dairy products on T2D and pooled data from 15 observational studies, including a total of 485,992 participants and 20,207 incidences of diabetes, finds that a higher intake of fermented dairy foods (yogurt and cheese) is associated with an 8% lower risk of T2D, in which higher yogurt consumption was significantly associated with 18% decreased T2D risk (303).

Experiments conducted on humans and animals consistently link higher dairy consumption to enhanced lipid fecal excretion and oxidation by tissues, which theoretically should result in better glucose homeostasis; however, some evidence challenges this idea by presenting evidence that milk and dairy products are strong insulin secretagogues (304), which may cause acute hyperinsulinemia and exacerbate insulin resistance (305, 306). In 2019, a meta-analysis pooled data from 14 RCTs demonstrating that consuming low-fat dairy products elicits lower HOMA-IR (standard mean difference (SMD)=-1.21) compared with control groups, which in turn may decrease the chance of developing T2D and IR (307). Interestingly, when only RCTs recruiting participants with BMI >25 kg/m^2 are included, the dairy invention has a slightly greater reduction in HOMA-IR (SMD=-1.39) compared to including participants from all BMI classes (307). Another systematic review of RCTs evaluates the impact of consuming more dairy products on insulin sensitivity and glucose metabolism and concludes such interventions enhance insulin sensitivity, with longer interventions having stronger effects (308). In contrast, one previous meta-analysis of RCTs finds no effect of dairy product interventions on HOMA-IR (309). In a subgroup analysis, they report that individuals with BMI>30 and longer intervention (>23-week) consuming higher dairy had lower insulin concentrations compared with a control group (MD=-5.46 and -4.40, respectively). Moreover, they demonstrate that dairy product consumption decreases HbA1c (MD: −0.09%) in studies with follow-ups of more than 10 weeks. Given that HbA1c is often used as the primary criterion for T2D diagnosis and long-term hyperglycemia management, its inclusion as an outcome provides a more comprehensive picture of the possible beneficial effect of dairy products on long-term glucose management in T2D (309). However, as it relies on the turnover of red blood cells, it changes little in the short term, and studies longer than 12 weeks are required. In addition, no superiority of low-fat compared to whole-fat dairy consumption is seen in a meta-analysis of RCT on IR based on HOMA-IR (310). According to a meta-analysis, yogurt lowers plasma glucose compared to a diet without yogurt (311).

1.2.2.2.2 Randomized Controlled Trials and Animal Studies

Regarding the subtypes of dairy products, there are RCTs investigating specific dairy products on T2D parameters (309). For example, a recent well-designed parallel-arm RCT conducted on males with abdominal obesity examines the effect of milk, yogurt, heat-treated yogurt, and acidified milk as part of a habitual diet on metabolic risk markers after 16 weeks of intervention. Although results

do not demonstrate that consuming yogurt products had any health benefits above non-fermented milk products, by considering the pre-and post-intervention results, it is indicated that 400 g/d of whole milk leads to lower insulin, c-peptide, and HOMA-IR (264). However, in another trial, 220 g/d of conventional yogurts are more effective than 220 g/d whole-fat milk in reducing IR, as evidenced by a greater decrease in fasting insulin, 2h-insulin AUC, as well as HOMA-IR. Notably, in the after-before analysis, a higher intake of milk is also effective in reducing FBG, insulin, and HOMA-IR (312). The effect of milk consumption on glucose homeostasis among individuals with obesity and T2D has been investigated and showed that 3 servings per day of skim milk for 12 weeks when added with energy-restricted diets, increase insulin sensitivity (marked by an increase in HOMA2-%B) and lower FBG and HbA1c compared with those with minimum dairy intake (245). In healthy males and females, 250 g of plain yogurt per day for 120 days leads to a better glucose tolerance test than at baseline (259). Another RCT compares the beneficial effect of regular yogurt with vitamin D-probiotic-fortified yogurt during a 10-week calorie restriction period among males and females with obesity, yielding a significantly greater reduction in the HOMA-IR and fasting serum insulin when compared with the baseline values (313). In contrast, including conventional yogurt in the weight-maintenance diet of adults with overweight does not have a significant effect on glucose-related parameters after 12 weeks of intervention (314). Regarding cheese consumption, neither 120 g of full-fat or reduced-fat Irish cheddar cheese significantly affects the metabolic parameters of overweight adults after a 6-week intervention (225). This null finding is supported by another RCT intervening with 240 g/day cheese for 6 weeks [332] or 120 g/day cheese for 2 weeks (315). Due to variations in trial design, length, and geographic location (background diet), the available data from RCTs with dairy intake as the primary intervention and diabetes-related outcomes are inconsistent.

Animal studies have increasingly provided evidence of mechanisms underlying the effects of dairy consumption on IR and metabolic markers . Mice fed with HFD supplemented with low-fat yogurt (1% fat) show lower weight gain, less fat mass, reduced FPG, OGTT-AUC, and HOMA-IR index, and increased serum FGF21compared with HFD-fed mice. Compared with HFD-fed mice, yogurt decreases FAS and PEPCK but increases the abundance of CPT-1α, PI3K, and p-AKT in the liver of HFD-fed mice, improvements attributed to the adiponectin pathway (266). Similarly, Hasegawa et al. finds that yogurt (2% fat) supplementation in the diet of obese mice (6-week HFD before or HFD concurrent with the intervention) both mitigated and prevented IR. The mechanism involves

reducing systemic inflammation through the modulation of gut microbiota, which reduces metabolic endotoxemia and improves intestinal barrier function (271). Six weeks of supplementation with skim milk or yogurt in rats ameliorates the adverse effects of a high fructose diet on IR accompanied by reduced plasma TC and TG compared to the control group (316). Male rats fed skim milk powder have lower weight gain than controls, though glucose tolerance remains unchanged between the groups (317), and milk administration to *db/db* mice significantly increases muscle mass and decreases visceral fat mass, which results in enhanced glucose tolerance and insulin sensitivity. This study highlights the role of the gut microbiota, particularly an increase in the genus *Akkermansia*, in mediating these beneficial effects (318). In order to understand the effect of fat content in cheese on insulin resistance, our group shows in diet-induced IR rats that both low-fat and regular-fat cheese improve insulin sensitivity, confirmed by increased hepatic G6Pase and PEPCK. In addition, it is speculated that the benefits occur through normalizing specific lysophosphatidylcholine and PC species that are reduced by HFD feeding (283).

In conclusion, the findings of observational studies indicate conflicting results on the effects of dairy intake on metabolic health, but a substantial body of research indicates that low-fat dairy products, particularly low-fat milk and yogurt, may protect against T2D and IR. RCTs on dairy products and T2D parameters show mixed results. While some studies indicate that milk and yogurt improve insulin sensitivity and glucose homeostasis, others find no significant benefits. Furthermore, since yogurt includes probiotics, it may provide additional health advantages, although many trials do not support these conclusions. Moreover, the research on cheese intake is still conflicting, and the varied characteristics of market cheeses investigated in studies, influenced by their manufacturing processes, may contribute to these inconsistencies. In order to fully understand the function of low-fat dairy products, including non-fat milk, low-fat yogurt, and reduced-fat cheese, in managing obesity-related IR and T2D, research on animals consuming these foods is necessary. This study allows a more accurate investigation of the molecular processes by which reduced-fat dairy products might enhance glucose homeostasis and decrease IR, eventually providing a new avenue for future research to prevent and treat metabolic diseases.

1.2.2.3 Dairy Consumption and Liver Function

As elaborated in section 2.1.3.3, over 25% of adults worldwide may be at risk for MASLD, and this prevalence will only increase as the obesity pandemic continues to spread. Observational

studies have examined the relationships between dairy intake and MASLD. Some of them show that higher dairy consumption is associated with a reduced risk of MASLD (319, 320, 321). While others find no significant association (322, 323). The most common limitations are the sample size in such studies and the different methods applied for diagnosing NAFLD, including ultrasound, liver biopsy, and blood tests. These limitations can compromise the reliability and affect the comparability of findings between different studies. Furthermore, no meta-analysis is conducted to pool the data from these studies, which would otherwise enhance the power of the test and a more robust analysis with a larger sample size.

A new well-executed RCT by Sandby et al. investigates the effect of 400 g/day of either milk, yogurt, heat-treated yogurt, or acidified milk as part of participants' habitual diet on liver fat as a primary outcome among males with abdominal obesity during 16 weeks of intervention. They report that all intervention foods exhibit similar but minor beneficial improvements in various metabolic risk indicators but had no effect on liver fat, concluding that there is no superiority for fermented versus non-fermented dairy products on liver health (264). In contrast, an RCT among women with obesity, metabolic syndrome, and MASLD obtained contrary results. It shows that 220 g/d yogurt as compared to 220 g/d milk for 24 weeks, significantly decreases oxidative stress, alanine aminotransferase (ALT), and hepatic fat fraction, which is partially mediated by the changed composition of the gut microbiota (312). Another study highlights the benefit of low-fat dairy food in combination as Dugan et al. observe a significant decrease in hepatic steatosis index, ALT, aspartate aminotransferase (AST), and mRNA expression of *IL6* and *IL1B* in peripheral blood mononuclear cells after low-dairy consumers (n = 37) with metabolic syndrome consume 3 daily servings of low-fat dairy (296 mL 1% milk, 170 g non-fat yogurt, and 56.7 g 2% cheese) in a 6-week RCT (324).

Animal studies are being conducted to better understand how dairy product consumption can affect liver metabolism, with a focus on reducing the accumulation of fat within the hepatocytes. Golden hamsters fed whole-fat milk (2 mL), high-dose whole-fat yogurt (2 mL), or low-dose whole-fat yogurt (1 mL) on an HFD background reveals that compared with the HFD controls and the milk group, yogurt-fed animals gain less weight, and have lower adipose and liver weight, and reduced plasma TG, total cholesterol, AST, and ALT. The higher dose yields a more pronounced benefit. Also, the TG and total cholesterol content of the liver is lower in yogurt than in the HFD and milk

groups, accompanied by lower liver gene expression of *Srebp1*, *Acaca*, and *Fas*, which regulate DNL. Interestingly, the BAT weight is also increased by yogurt (325). In BALB/c mice on an HFD, 0% fat yogurt for 8 weeks demonstrates decreased weight gain, fat mass, average epididymal adipocyte size, and lipid droplets within hepatocytes. Yogurt intake suppresses the liver gene expression of *Fas* and *Acaca*, activates p-AMPK, reduces fat synthesis, and controls the disrupted lipid metabolites promoted by the HFD diet (270). In another study, adding skim milk powder to a standard diet of rats (AIN-93M) yields less weight gain and hepatic fat accumulation, in addition to suppression of liver DNL as indicated by lower FAS and ACC abundance in the liver compared with the control rats (317). The direct effect of cheese consumption on hepatic fat accumulation is less investigated; however, there is some indirect evidence indicating that cheese consumption improves liver metabolism because it reduces IR (283), lowers hepatic gene expression of *Acaca* and *Fasn* (326), and decreases plasma TG and total cholesterol (326, 327, 328). Dr. Chan's lab is conducting a set of studies that will help explain the possible underlying mechanisms of the effects of higher versus lower/non-fat dairy on whole-body glucose and lipid metabolism, focusing on the liver as the control center of metabolism. Previously, Moftah et al. fed mice with a HFD supplemented with approximately 2% of energy from high-fat milk, high-fat yogurt, and regular-fat cheddar cheese. None of these dairy products improved insulin sensitivity after 8 wk of intervention as measured by the insulin tolerance test. Milk reduced the number and size of lipid droplets within the liver tissue compared with HFD controls, and interestingly, these mice had lower serum TG compared to those fed high-fat yogurt. This pilot study does not delve further into the metabolism of lipids inside the liver to unravel the mechanism of action of milk to mitigate hepatic steatosis. It does prove the feasibility of the study design, in particular, that mice can be given different types of dairy products using specialized cages so that all mice get a fixed amount of dairy (329). Further studies to compare the effects of low or non-fat dairy products were proposed within this set of studies and will be described in this thesis.

All in all, although total dairy products are suggested to protect against MASLD in human observational studies, at the time my PhD commenced, there had been no systematic reviews with meta-analysis conducted, and it was unclear how consistent the data were. One objective of my research, as described below, is to fill this gap in the literature. Interventional studies support the potential benefits of low-fat dairy products on liver health. RCTs show fairly consistently that higher intakes of low-fat milk and yogurt significantly improve metabolic risk indicators, liver

enzyme levels, and hepatic fat content. However, the evidence lacks a strong mechanistic explanation. In animal models, intervention with low-fat yogurt and milk reduces hepatic fat deposition, improves lipid metabolism, and suppresses DNL. These results thus suggest that low-fat dairy intake might be a preventing factor in reducing the detrimental effect of obesity-induced MASLD. Therefore, further animal study is warranted to further discover mechanisms through which low-fat milk, low-fat yogurt, and reduced-fat cheese affect liver metabolism.

2 RESEARCH OBJECTIVES

2.1 OVERALL OBJECTIVES AND SPECIFIC AIMS

The detailed overview of recent literature in Chapter 1 provides comprehensive insights into the effects of dairy consumption on obesity and its related metabolic abnormalities. In humans, systematic reviews with meta-analyses along with RCTs report that high dairy consumption, especially low-fat milk and yogurt, is associated with reduced risk of obesity, better body composition, lower risk of T2D, improved insulin sensitivity, and suggestion of enhanced liver function. These studies provide support for the potential benefit of low-fat dairy. However, despite these promising findings, the argument for the optimum kinds and quantity of dairy intake is still ongoing. There is an expression of caution regarding high dairy consumption. It concludes with the potentially negative consequences of the consumption of dairy foods while saying at the same time that dairy consumption depends on overall diet quality, indicating that the health benefits of dairy consumption are totally low, and their health-beneficial effect can indeed be obtained from other foods. Dairy products might improve health when the diet quality is low (330). It is also believed that yogurt is probably the best dairy pick, but still recommended for 1 or even fewer servings daily (330, 331). On the contrary side, others argue that much more emphasis should be placed on the advantages of dairy products, considering them as a functional food. This view is supported by several meta-analyses, suggesting that at least 2 servings/day of low-fat dairy products yield metabolic health benefits without the harmful impact of high intake of saturated fat.

Such consumption is aimed at mitigating the risk of obesity and improving insulin sensitivity (332, 333).

Our group has been addressing the overarching hypothesis that the effects of dairy on metabolic health are different according to the type of dairy product, fat content and bioactive components in the dairy product, fermentation status, food matrices, overall dietary pattern associated with dairy consumption, and health status of an individual. As the debate mainly centers on the fat content of dairy products, all observational studies have been limited to categorizing dairy products based on their fat content. However, "low" and "high" fat terms lack a universal standard definition and can lead to dairy products being differentially categorized by different researchers (205). Given the wide range of available dairy products in the market and the fact that they can be consumed in various forms, a new perspective on dairy consumption could help to unravel some of the conflicting evidence. Based on our literature review, the health benefits of low-fat dairy products on metabolic health have been more consistently reported, whereas high-fat dairy products often show neutral effects. Dairy processing, such as fermentation in yogurt and cheese, has been proposed to have further potential health effects by influencing gut microbiota composition and nutrient bioavailability beyond its native form; however, it needs to confirm this superiority of yogurt and cheese in improving metabolic health, if there is any. Furthermore, the beneficial effects of dairy products are more pronounced when they are accompanied by a Western-style diet, but the mechanisms for metabolic improvement are unclear. In light of these nuanced findings, more detailed investigations need to be conducted to precisely delineate how various food matrices of dairy products, ranging from milk to yogurt and cheese, impact obesity-linked metabolic outcomes. **Thus, the primary objective addressed by this thesis was to advance understanding of the impact of consuming different types of dairy products on obesity-related metabolic outcomes.**

Objective 1: To assess the association between dairy consumption patterns and risk of type 2 diabetes

Rationale: Traditionally, nutrition research has focused on dairy as a food group or single specific foods. However, individual dairy foods are not consumed alone. People may choose a different type of dairy to eat daily based on taste, accessibility, and underlying problems (e.g., lactase deficiency). It is probable that higher intakes of combinations of dairy foods concurrently influence

the risk of T2D. Thus, newer nutritional epidemiologic approaches, which rank participants based on the frequency and quantity of dietary habits they habitually consume in their overall diet, may be useful in studying the association between dairy consumption and T2D and may help explain inconsistencies in the published studies. Principal component analysis (PCA) is commonly used as a powerful data-reduction technique. By maximizing the information from dairy food intake and accounting for previously unrecognized relationships between people's behavior in choosing dairy foods, PCA may provide novel insights into the effects of dairy consumption on human health and disease. Indeed, total dairy intake might be lower or higher than current dietary guidelines, but these amounts, in combination with people's preference to choose certain dairy food group items, might be more powerful in determining association with diseases like T2D than simpler epidemiological approaches. Extracting the dairy consumption pattern using PCA, is a suitable approach to overcome this limitation. To the best of our knowledge, no study has been published examining the association between dairy food patterns and the incidence of T2D. Information obtained from this exploratory research will provide an opportunity to understand population behavior regarding dairy consumption patterns.

Specific aims:

1.1 - To extract dairy consumption patterns of men and women using data dimensionality reduction techniques to derive consumption traits in the population through principal component analysis (PCA);

1.2 - To assess the association of extracted dairy consumption patterns with the risk of T2D.

Objective 2: To document the association between total dairy intake, intake from specific dairy foods, and MASLD

Rationale: IR, obesity, and metabolic syndrome are all linked to MASLD, making it the most frequent form of chronic liver disease globally. While results of recent observational studies suggest that dairy consumption may influence liver health, quantitative data specifically connecting dairy intake to MASLD is limited. This knowledge gap necessitates comprehensively evaluating existing research to understand these relationships better. I, therefore, decided to conduct a systematic review and meta-analysis of observational trials to fill this gap, in other words, to explore the relationship between MASLD and the consumption of total dairy and individual dairy products, including milk, yogurt, and cheese. Summarizing the evidence from

observational studies on dairy product consumption and MASLD will help elucidate the relationships and establish a potential predictive role for dairy foods in MASLD.

Specific aims:

2.1 - To perform a systematic review and meta-analysis of observational studies focusing on the link between MASLD and total and individual dairy product (milk, yogurt, cheese) consumption.

2.2 - To identify potential causes of heterogeneity among studies using subgroup, meta-regression, and sensitivity analyses.

Objective 3: To understand how low/non-fat dairy foods with different matrices impact metabolism

Rationale: Previous RCTs and animal studies report that the type of dairy products used in interventions affects metabolic health outcomes, highlighting the importance of studying dairy matrixes. As noted, our laboratory previously conducted a comparison of the effects of feeding whole-fat milk (3.25% milk fat), high-fat yogurt (4% milk fat), and regular-fat cheddar cheese (28% milk fat) on insulin sensitivity and MASLD-related outcomes (329). However, the literature suggests that low-fat dairy foods generally elicit greater effects on obesity-related metabolic disorders, whereas high-fat dairy has been associated with neutral or slightly protective effects. In addition, it has been reported that certain dairy products added to a Western diet are metabolically beneficial. Preclinical studies indicate that adding dairy foods to a Western-style diet ameliorates the negative consequences of this diet on metabolic health through various pathways. Despite these results, a direct comparison of low-fat milk, low-fat yogurt, and reduced-fat cheese in terms of response to metabolic health with a high-fat diet mimicking a Western-type diet has not been studied. Thus, I aimed to understand the mechanisms underlying the ameliorating effect of low-fat milk, low-fat yogurt, and reduced-fat cheese with distinct matrices when supplemented in the context of a HFD.

Specific aims:

3.1 - To assess the effect of low-fat milk, low-fat yogurt, and reduced-fat cheese on the obesity and metabolic phenotypes in HFD-induced obese mice;

3.2 - To determine the effect of low-fat milk, low-fat yogurt, and reduced-fat cheese on energy metabolism in HFD-induced obese mice;

3.3 - To identify mechanisms by which low-fat milk, low-fat yogurt, and reduced-fat cheese improve liver lipid and glucose metabolism in HFD-induced obese mice

Objective 4: To map the impact of dairy product consumption on circulating and hepatic phosphatidylcholine and lysophosphatidylcholine levels

Rationale: Phospholipids (PL), particularly phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), play an important role in lipid metabolism, energy storage, membrane structure, and signal transduction pathways indispensable for metabolic health maintenance. Cumulative evidence indicates that alterations in the distribution and species of these phospholipids are associated with impairment of metabolic pathway functions and contribute to the development of metabolic disorders, including insulin resistance, T2D, and MASLD. Dairy products are rich in phospholipids, specifically PC and LPC, and have shown potential in ameliorating metabolic health. Dr. Chan also, in her previous study in insulin-resistant rats, suggested that the beneficial effect of regular and low-fat cheese on metabolic health might be attributed to altered proportions of species of PC and LPC in the blood (283). Yet, it remains unknown how the provision of dairy products may differently affect these phospholipids' circulating and hepatic levels and whether the effects are similar in human and rodent models. A review of the current literature would provide insights into the underlying mechanisms regarding how dairy consumption influences metabolic health and help to direct future research. This scoping review aims to summarize existing literature from both human studies and animal models regarding the effect of dairy consumption on PL, PC, and LPC levels.

Specific aim:

4.1 - To conduct a scoping review to synthesize existing research on the impact of dairy product consumption on the amount and species of phosphatidylcholine and lysophosphatidylcholine in circulation and the liver in human studies and animal models.

3 ASSOCIATION OF DAIRY CONSUMPTION PATTERNS WITH THE INCIDENCE OF TYPE 2 DIABETES: FINDINGS FROM ALBERTA'S TOMORROW PROJECT

Association of dairy consumption patterns with the incidence of type 2 diabetes: findings from Alberta's Tomorrow Project

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3.1 ABSTRACT

Background: We aimed to extract dairy consumption patterns of men and women from a population-based cohort and then assess the association of each consumption pattern with incident T2D risk.

Methods: This prospective study was conducted within the framework of Alberta's Tomorrow Project (ATP), in which 8615 men and 15016 women provided information on dietary intake by completing a food-frequency questionnaire at baseline, and then were followed up over time to determine incidence of T2D via questionnaires. Principal Component Analysis (PCA) was used to extract dairy consumption patterns (DCPs). The association between each extracted pattern and T2D incidence was estimated using multivariable logistic regression models.

Results: The incidence of T2D among men and women was 3.8 and 3.2%, respectively, and mean duration of follow-up was 5.2 years. Three major DCPs were identified. After controlling for potential confounders, the OR for risk of T2D in men in the highest compared with those in the

lowest quartile of the DCP3 (whole milk, regular cheese, and non-fat milk as a beverage and in cereal) was 0.64 (95%CI:0.47to0.88, P-trend=0.001), whereas it was not significant for women. DCP1 and DCP2 were not associated with incident T2D in men or women.

Conclusion: Adherence to a DCP characterized by higher consumption of whole milk, regular cheese and non-fat milk was associated with decreased risk of incident T2D only in men. Our results support current evidence that a combination of different dairy products regardless of their fat content might be favorable for health maintenance, at least in men.

Keywords: Dietary pattern, Principal component analysis, Type 2 diabetes, Alberta's Tomorrow Project

3.2 INTRODUCTION

Dairy products have long been regarded as an essential part of a healthy diet because they can provide considerable quantities of high-quality proteins, lipids, vitamins, and minerals (334, 335, 336, 337). Saturated fatty acids (SFA) are also abundant in dairy foods. Because SFAs are considered detrimental to human health and predispose the consumer to increased risk of cardiometabolic diseases (338, 339) dietary guidelines generally recommend the consumption of low-fat dairy to minimize SFA intake. However, recent literature has identified apparent contradictions regarding the association between dairy foods with various fat content and cardiometabolic health (340, 341). Contrary to increased intake of high-fat dairy being harmful, a recent meta-analysis of observational studies reported no consistent association between whole-fat dairy consumption and the risk of type 2 diabetes (T2D) (342). As a result, the epidemiological evidence does not support the concept that high-fat dairy consumption raises the risk of cardiometabolic diseases. By contrast, low-fat dairy products are consistently associated with a reduced risk of T2D (302, 342). However, there is no standard way to assign dairy foods into low-fat or high-fat groups. For example, studies have combined non-fat milk (0% fat) and low-fat milk (1.5% fat) with reduced-fat cheese (containing 19% to 23% fat) and considered these to comprise a low-fat dairy group (302, 343). Thus, these *a priori* groupings based on arbitrary fat content are a source of heterogeneity in observational studies and may not represent the combinations of dairy foods consumed by people.

Previous systematic reviews and meta-analyses support an inverse association of greater total dairy product intake with the risk of T2D (291, 295, 344). However, total dairy consumption cannot fully explain the association of dairy products with T2D because certain intrinsic, physicochemical features, including the structure, type, and fat content of dairy foods (i.e., the food matrix), may differentiate their effects on T2D risk. In this regard, current evidence presents a mixed conclusion on the association of various dairy foods with T2D risk (293, 345). Although a favorable association of low-fat milk and yogurt with T2D has been described, data on the relationship between other commonly consumed dairy foods such as cheese, ice cream, and high-fat dairy are controversial. In addition to the inherent diversity of dairy food matrices, there is a wide spectrum of dairy products marketed to consumers' tastes, nutritional and other priorities (e.g., with or without sugar, lactose-free or with artificial sweeteners or probiotics), acceptability of animal-based foods, or ethnocultural considerations that could influence what combinations and volume of dairy products people consume, and thus the association between dairy intake and the risk of T2D (346).

So far, all studies have focused on dairy as a food group or single specific dairy food. However, individual dairy foods are not usually consumed in isolation, as people consume a variety of dairy foods. It is probable that higher intakes of certain combinations of dairy foods concurrently influence the risk of T2D. Thus, nutritional epidemiologic approaches that score individuals based on the frequency and quantity of dietary items they habitually consume in their overall diet, in addition to precise statistical techniques such as Principal Component Analysis (PCA), may be useful to study the association between dairy consumption and T2D. PCA, a powerful data-reduction technique, can maximize the information from dairy food intake and account for previously unrecognized relationships between the combinations of dairy foods consumed (herein referred to as dairy consumption patterns, DCPs). Thus, PCA may provide deeper insights into the association of dairy consumption with T2D and may be more powerful in determining the association of dairy with diseases like T2D than quantity alone.

Information obtained from this exploratory research will enhance our understanding of dairy consumption patterns and may elucidate why the association of individual dairy products with the risk of T2D is inconsistent. Therefore, we aimed to extract the DCPs of men and women in Alberta's Tomorrow Project (ATP), a longitudinal population cohort study, and assess the association of DCPs with the risk of incident T2D.

3.3 METHOD

Study population

This study was conducted using data from Alberta's Tomorrow Project (ATP), a prospective population-based cohort study of 55,000 adults established in 2000 and designed to investigate the etiology of cancer and other chronic diseases. Detailed methodologies for the study design and recruitment have been reported elsewhere (347, 348). In brief and pertaining to this study, between 2000-2008, male and female Albertans from the general population in both urban and rural communities, aged 35–69 years and free of cancer, were recruited using a random digit dialing method. Participants were asked about their medical history, family health, smoking status, anthropometric measurements, sociodemographic variables, dietary intake, and habitual physical activity. Follow-up occurred in 4-year cycles, and participants provided additional questionnaire data.

In the current study, we selected men (n=10,144) and women (n=16,823) who completed the baseline Health and Lifestyle Questionnaire (HLQ), Canadian Diet History Questionnaire-I (CDHQ-I), and at least one follow-up questionnaire. We excluded participants with a self-reported history of T2D at baseline (n=1,266). Participants were also excluded if they were pregnant (n=56), had a personal history of cancer or stroke (n=142), and over-or under-reported energy intakes (<800 or >2,400 kcal/d for men, <600 or >3,500 kcal/d for women; n=1,045). After exclusion, the final sample size was n=24,467 participants (men=8,615, women=15,016).

Written informed consent was obtained from all ATP participants. The current study was approved by the Health Research Ethics Board at the University of Alberta (Pro00107023), Canada, as a secondary analysis of the data.

Dietary assessments

The Canadian Diet History Questionnaire (CDHQ-I) is a 257-item food frequency questionnaire (FFQ) of meals, drinks, and nutritional supplements and was used to assess the habitual dietary intake of participants (335, 349). The CDHQ-I is a modified version of the Diet History Questionnaire from the U.S. National Cancer Institute, modified to account for differences in Canadian food fortification and nutrient composition. Participants reported their usual frequency of consumption, serving size, and preparation of foods in the past year. Each food was transformed

into grams per day based on the size of the portion and how often it was eaten. The CDHQ-I nutrient database was used to estimate the average daily intakes of energy and nutrients of food items. There were 22 dairy-related items in the FFQ, including milk (0%, 2% or full-fat), yogurt (any fat content), cheese (regular, reduced- and non-fat), cream cheese, ice cream (low-fat and regular), butter, frozen yogurt, and milkshakes. The Healthy Eating Index Canada (HEI) 2015 was used to evaluate the diet quality of participants, which was based on the American Healthy Eating Index (HEI) 2015 (350) scoring criteria, then converted to the number of servings or daily intake age- and sex-specific guidelines from Canada's Food Guide 2007 (351).

Assessment of outcome

At each time point of follow-up, all participants completed a questionnaire detailing the self-reported presence of diagnosed chronic health conditions, including diagnosed T2D.

Assessment of covariates

For the current study, covariates including potential dietary and other risk factors associated with the development of T2D were identified by literature review. Data on education attainment and smoking were gathered using the HLQ at the time of enrollment. The validated Past-Year Total Physical Activity Questionnaire (PYTPAQ) was used to obtain information about the frequency, duration, and intensity of recreational, household, transport, and occupational, physical activities during the past year, and was completed by participants at the time of enrollment alongside the CDHQ-I. Metabolic equivalent (MET) values (MET-min/wk) were calculated. Self-reported body weight and height were collected, and BMI was calculated as weight in kg divided by height in meters squared (kg/m^2). Dietary-related information, including alcohol consumption, total energy intake, and food group consumption, was derived from the CDHQ-I.

Statistical Analyses

All dietary components were adjusted for total energy intake using the residual method (352). PCA was used as a dimension-reduction technique to derive major DCPs (353). Kaiser-Meyer-Olkin (KMO) index and Bartlett's test of sphericity were used to examine the sample adequacy and factor analysis suitability. The KMO ranges between 0 and 1, and a minimum value for good factor analysis is 0.6, and Bartlett's test should be significant ($p<0.05$). For this analysis, the input data was a correlation matrix of 22 dairy products. For both male and female DCPs, the KMOs were

>0.62 (sampling sufficiency of components) and Bartlett's test was significant at $p < 0.001$ (indicating the dataset's suitability). We used eigenvalues, scree plots, interpretability, and absolute factor loadings to decide the number of factors to retain. An orthogonal rotation with a varimax option was applied to derive optimally distinctive extracted patterns and simplify data interpretation. The loading factor for each dairy item represents the coefficient between the item and the given pattern. Dairy products with positive coefficients were positively correlated with an extracted pattern, while the opposite was true for negative coefficients All participants were given a score based on how closely they adhered to each extracted DCP. Quartiles of factor scores were determined and considered in further analysis.

The normality of the distribution of variables was assessed by the Kolmogorov-Smirnov test and visually confirmed by histogram. Descriptive data are presented as the mean values ± SD (normally distributed variables), medians and 25th and 75th percentile (non-normally distributed variables) for continuous outcomes, and percentage (%) for categorical variables. Student's t test and Chi-square test were used to compare continuous and categorical outcomes, respectively. Since alcohol intake was skewed, a log transformation was used. Within each DCP, we reported the distribution in terms of baseline participant characteristics separately for men and women. Linear regression and chi-square tests were used for continuous and categorical variables to investigate the trend of variables according to the quartile of DCPs.

We used multivariable binary logistic regression to estimate the odds ratio (OR) and associated 95% confidence intervals (CIs) of the associations of each DCP with incident T2D. The lowest quartile served as a reference category. We also examined significant linear trends for each DCP and outcome using the median diet scores at each quartile. Two regression models were developed. The first model was adjusted for age, BMI, total energy intake, physical activity, education level, menopausal status and menopausal hormone use (women participants only), hypertension, smoking status, and alcohol consumption. In addition to the variables in the first model, intake of food groups categorized as fruit, vegetables, whole grains, refined grains, processed and red meat, and added sugar were adjusted for in the second model. We also adjusted for further covariates including hypercholesterolemia, hepatitis, stress index, and dietary factors including intakes of sugar-sweetened beverages, legumes, and nuts. Since they did not change the ORs and 95% CI, they were excluded from the final model.

In subgroup analyses, we used multivariable logistic regression to assess potential effect modification by BMI (normal weight, 18.5–24.9 kg/m^2; overweight, 25–29.9 kg/m^2; and obese, >30 kg/m^2), age (>65 years and <65 years), and physical activity METs (above and below the median for each sex). In each subgroup analysis, the covariates were mutually adjusted for each model. Two-sided p>0.05 was considered statistically significant, and statistical analyses were performed using the Statistical Package for Social Sciences (version 15.0; SPSS, Chicago IL).

3.4 RESULTS

Baseline clinical, biochemical, and dietary intake characteristics of men and women are reported in Table 3.1. Participants (36% men, 64% women) had a mean age of 50.0 ± 9.0 years at baseline. Over the 5.2-year follow-up, the incidence of diabetes was 3.8% and 3.2% among men and women, respectively. Men had a lower prevalence of family history of diabetes, were more physically active, and more likely to be hypertensive than women. Regarding diet, the general macronutrient distribution were similar, but men had higher total energy intake while the % energy of carbohydrates was statistically higher in women. Men reported consuming more alcohol, whole and refined grains, and less intake of vegetables, fruits, and fiber. Women consumed slightly more dairy and had a better overall dietary quality, based on the HEI 2005 score, than men.

Table 3. 1 Baseline characteristics of men and women [*]

	Men (n=8615)	Women (n=15016)	P-value[‡]
Age, years	50.7±9.0	50.6±9.2	0.659
Duration of follow-up (years)	5.7 (3.9, 7.8)	5.2 (3.6, 7.4)	<0.001
Family history of diabetes (%)	24.1	27.7	0.002
Incident diabetes[§], %	3.8	3.2	0.016
Body mass index, kg/m^2	27.9±4.2	27.0±5.6	<0.001
Physical activity, MET-h/week	172.3±73.8	156.6±64.2	<0.001
Did not complete high school, %	9.7	8.4	0.001
Current smoker, %	17.1	16.8	0.536
Prevalent hypertension, %	22.5	19.8	<0.001
Dietary intake			
Total energy intake, kcal/day	2130±718	1612±546	<0.001
Fat, % of energy	33.1±6.5	32.4±6.8	<0.001
Saturated fatty acid, % of energy	11.0±2.7	10.5±2.8	<0.001
Protein, % of energy	15.7±2.8	16.1±2.9	<0.001
Carbohydrate, % of energy	48.9±8.3	51.4±8.3	<0.001
Alcohol intake, drinks/day	0.46 (0.13, 1.23)	0.16 (0.05, 0.49)	<0.001
Vegetables[†], servings/day	1.8±1.0	2.4±1.2	<0.001
Fruit[†], servings/day	1.6±1.4	2.1±1.3	0.003
Whole grains[†], servings/day	1.2±0.9	0.8±0.6	<0.001
Refined grains[†], servings/day	4.2±2.0	3.0±1.4	<0.001
Meat[†], servings/day	2.5±1.7	1.5±1.0	<0.001
Added sugar[†], g/day	11.9±4.5	11.7±4.4	0.052
Fiber, gram/1000 kcal/day	9.6±3.3	11.4±3.8	<0.001
Total dairy[†], servings/day	1.5±1.2	1.7±1.1	<0.001
Total dairy, % of energy	12.8±9.4	14.8±10.4	<0.001
Healthy Eating Index 2005[†]	48.6±8.6	56.4±9.2	<0.001

[*] Data are baseline values (except for incident diabetes) and presented as mean±SD or median (25th, 75th percentiles) for continuous variables and percentages (%) for categorical variables.
[†]Adjusted for total energy intake
[‡]P-value was determined using an independent t-test for continuous variables and a Chi-square test for categorical variables.
[§]Defined as a self-report of a previous diabetes diagnosis by a physician as reported in the follow-up surveys

Three distinct major DCPs were identified using PCA for both men and women, explaining 22.6% of the variance in dairy intakes in both sexes. The distribution of the 3-factor scores (loadings of each dairy item) is shown in Figure 3.1, where each bar represents the magnitude of the loading of each dairy item to each DCP. The first factor, DCP1, loaded heavily with non-fat milk as a beverage or in cereal, reduced-fat and non-fat cheese, reduced-fat ice cream, and yogurt, with inverse loadings for 2%-fat milk including in cereal, regular cheese, butter, and whole milk. The second factor had high positive loadings with 2% fat milk, including in cereal and coffee, and non-fat milk as a beverage or in cereal, and high negative loadings for 1% fat milk, including in cereal, and so this pattern was denoted DCP2. The third identified factor, DCP3, was characterized by consumption of whole milk, including in cereal and coffee, regular cheese, and non-fat milk as a beverage or in cereal, with less intake of 2% fat milk, including cereals and coffee. The first to third DCPs explained 8%, 7%, and 6%, respectively, of the variance in measured dairy items.

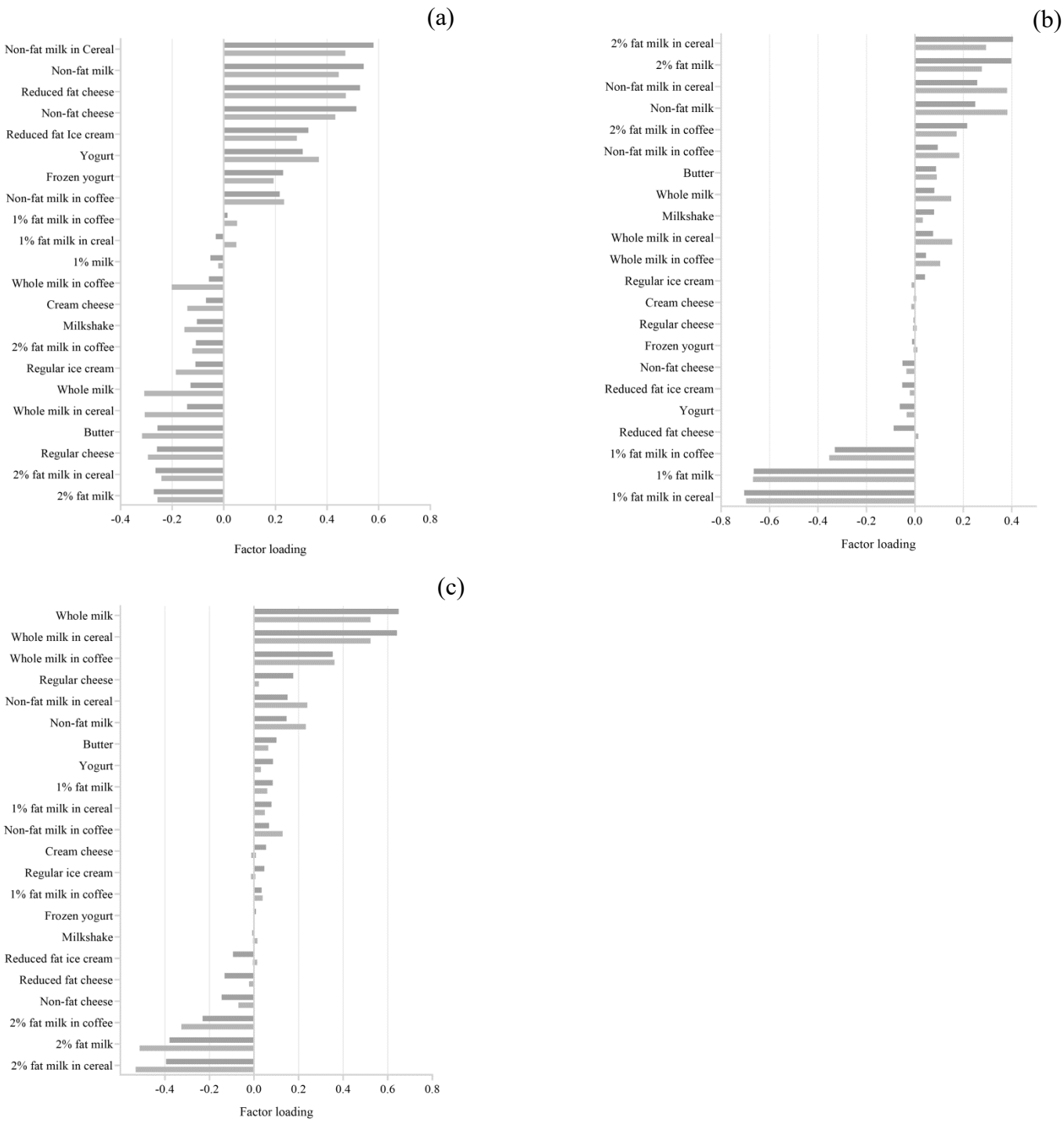

Figure 3. 1 Dairy consumption patterns.

Factor loadings for the three dairy consumption patterns derived using principal component analysis (PCA). Factor 1 (a) is considered a dairy consumption pattern 1, Factor 2 (b) dairy consumption pattern 2, and factor 3 (c) dairy consumption pattern 3

The baseline demographic and clinical characteristics of men by quartile categories of the DCP scores are shown in Table 3.2. Men with higher scores in DCP1 were less physically active and less educated. Fewer were current smokers, but had higher BMIs, and drank more alcohol. They also had a lower % of energy from fat and SFA and a higher % of energy from protein and carbohydrates. Higher adherence to DCP1 was accompanied by a lower intake of added sugar, processed and red meat, and total dairy, and a higher intake of vegetables, fruit, whole and refined grains, and fiber. Men with a higher DCP2 score smoked and consumed alcohol more and were more active than those in the lowest quartile. Men in the highest quartile of DCP2 had a slightly higher % energy from fat and SFA and a lower % energy from protein. They also had lower intakes of vegetables, fruit, whole grains, fiber, and HEI score and a higher intake of added sugar and total dairy. Men who had higher adherence to DCP3 reported less physical activity, higher intakes of fat, SFA, vegetables, fiber, and lower carbohydrate consumption. Across the DCP3 score quartiles, although participants consumed fewer servings of total dairy product per day, a higher proportion of energy intake was from total dairy products.

Table 3. 2 Baseline sociodemographic and clinical characteristics by quartiles of dairy consumption patterns (DCP) among men *,†

	DCP1		P-trend§	DCP2		P-trend§	DCP3		P-trend§
	Q1	Q4		Q1	Q4		Q1	Q4	
Men									
Number	2238	2238		2238	2239		2238	2239	
Age, year	50.0±8.9	50.8±9.3	0.008	50.0±9.1	50.3±9.3	0.028	50.5±9.2	50.3±9.1	0.551
Family history of diabetes (%)	23.1	24.6	0.835	24.4	23.8	0.945	24.5	23.9	0.755
Incident diabetes, %	3.6	3.8	0.603	4.1	3.9	0.980	4.7	2.9	<0.001
Body mass index, kg/m^2	27.7±4.2	28.2±4.2	<0.001	27.9±4.1	27.8±4.1	0.341	27.8±4.2	27.8±4.2	0.635
Physical activity, MET-h/week	182.9±75.5	167.6±73.1	<0.001	168.6±72.2	175.8±75.7	<0.001	180.1±76.4	171.0±73.2	<0.001
Did not complete high school, %	12.7	8.0	<0.001	6.7	10.1	<0.001	11.2	9.5	0.043
Current smoker, %	23.5	9.7	<0.001	12.0	16.7	0.001	18.2	16.1	0.119
Prevalent hypertension, %	19.5	25.2	0.071	21.6	22.1	0.008	22.4	21.9	0.887
Alcohol intake, drink/day	0.41 (0.08, 1.2)	0.48 (0.07, 1.18)	<0.001	0.42 (0.08, 1.17)	0.47 (0.07, 1.18)	0.041	0.47 (0.09, 1.17)	0.41 (0.08, 1.01)	0.316
Dietary intake									
Total energy intake, kcal/day	2400±720	2178±711	<0.001	2157±687	2285±722	<0.001	2357±734	2174±718	<0.001
Fat, % of energy	36.2±5.9	30.3±6.0	<0.001	31.8±5.8	32.4±6.2	<0.001	32.8±5.9	33.7±7.3	<0.001
Saturated fatty acid, % of energy	13.2±2.5	9.7±2.3	<0.001	10.7±2.3	11.3±2.8	<0.001	11.2±2.4	11.6±3.2	<0.001
Protein, % of energy	15.4±2.6	16.7±2.7	<0.001	16.3±2.6	15.8±2.6	<0.001	15.9±2.7	15.9±2.8	0.289
Carbohydrate, % of energy	46.6±7.8	51.5±7.9	<0.001	49.9±7.5	50.5±7.5	0.591	49.7±7.7	48.5±8.8	<0.001
Vegetables‡, servings/day	1.9±1.0	2.4±1.0	<0.001	2.2±1.0	2.0±1.0	<0.001	2.1±1.0	2.2±1.0	0.001
Fruit ‡, servings/day	1.4±1.4	2.3±1.4	<0.001	2.0±1.4	1.9±1.4	<0.001	1.9±1.4	1.9±1.4	0.722
Whole grains‡, servings/day	1.1±0.8	1.4±0.8	<0.001	1.3±0.8	1.2±0.8	<0.001	1.2±0.8	1.2±0.8	0.101
Refined grains‡, servings/day	3.9±1.4	4.3±1.4	<0.001	4.2±1.4	4.1±1.4	0.171	4.2±1.4	4.1±1.4	0.078
Processed and red meat‡, servings/day	2.5±1.4	2.3±1.4	<0.001	2.4±1.4	2.3±1.4	0.792	2.4±1.4	2.4±1.4	0.211

Added sugar[‡], g/day	15.2±4.5	13.6±4.5	<0.001	13.5±4.5	15.2±4.5	<0.001	14.7±4.5	14.3±4.5	0.166
Fiber, gram/1000 kcal/day	8.2±2.6	10.9±3.4	<0.001	9.9±3.1	9.5±3.2	<0.001	9.3±3.0	9.6±3.4	<0.001
Total dairy[‡], servings/day	2.2±1.2	2.1±1.2	0.049	2.3±1.1	2.5±1.1	<0.001	2.2±1.1	2.0±1.2	<0.001
Total dairy, % of energy	17.3±10.4	14.5±9.3	<0.001	15.6±9.2	15.9±9.1	0.056	15.1±9.4	16.4±10.4	0.001
Healthy Eating Index 2005	48.2±7.7	50.6±8.6	<0.001	53.6±7.7	50.6±8.6	<0.001	52.6±7.9	50.6±8.6	<0.001

[*]The results of only Q1 and Q4 of dairy consumption patterns are shown for simplicity.

[†]Data are presented as mean±SD or median (25th, 75th percentiles) for continuous variables and percentages (%) for categorical variables.

[‡]The values were adjusted for total energy intake.

[§]Linear regression was used for continuous variables and the Chi-square test for categorical variables.

The baseline demographic and clinical characteristics of women by quartile of DCP score are shown in Table 3.3. Women in the highest quartile of DCP1 were older and more were post-menopausal, less physically active, more educated, and less were smokers compared to those in the lowest quartile. The % of energy from fat and SFA was lower among those in the highest quartile of DCP1 than in those in the first quartile, but % energy from carbohydrates and protein and the energy-adjusted intake of vegetables, fruit, whole grain, fiber, total dairy, and overall diet quality was higher. Women with higher adherence to DCP2 were younger, more educated, more physically active, had lower BMI, and reported drinking less alcohol but more were current smokers. Higher adherence to DCP2 was accompanied by a lower HEI score and intake of whole and refined grains, and higher added sugar and total dairy intake. Women in the highest quartile of DCP3 had lower physical activity, and fewer were current smokers and post-menopausal, were more educated and drank more alcohol than those in the lowest quartile. The % energy from fat and SFA was lower, and that from carbohydrates and protein was higher in those women with the highest DCP3 score along with intake of more vegetables, fruit, whole grains and fiber and less refined grains and added sugar.

Table 3. 3 Baseline sociodemographic and clinical characteristics by quartile of dairy consumption pattern (DCP) among women *,†

	DCP1		P-trend§	DCP2		P-trend§	DCP3		P-trend§
	Q1	Q4		Q1	Q4		Q1	Q4	
Women									
Number	3878	3879		3878	3878		3879	3878	
Age, year	50.1±9.1	50.9±9.1	<0.001	50.1±9.2	50.8±9.4	<0.001	50.6±9.2	50.6±9.3	0.485
Family history of diabetes (%)	23.8	25.2	0.835	25.1	24.7	0.717	25.0	24.8	0.580
Incident diabetes, %	3.5	3.3	0.983	3.0	3.4	0.317	3.3	3.5	0.855
Body mass index, kg/m^2	26.8±5.6	26.9±5.4	0.557	27.2±5.6	26.7±5.5	<0.001	26.8±5.6	26.9±5.6	0.349
Physical activity, MET-h/week	161.9±68.3	155.4±61.5	<0.001	153.2±60.4	158.2±65.4	<0.001	160.3±66.4	157.1±64.8	0.004
Did not complete high school, %	12.2	4.9	<0.001	6.9	8.7	<0.001	11.5	6.4	<0.001
Current smoker, %	24.7	10.0	<0.001	13.0	16.2	<0.001	22.2	13.1	<0.001
Post-menopausal, %	43.3	48.0	<0.001	44.4	46.1	0.071	46.7	44.3	<0.001
Hormone replacement therapy use	14.7	14.9	0.320	14.1	16.3	0.396	15.1	15.6	<0.001
Hypertension , %	17.7	20.1	0.120	20.3	18.0	0.924	19.1	18.3	<0.001
Alcohol intake, drink/day	0.12 (0.05, 0.5)	0.18 (0.05, 0.51)	0.618	0.17 (0.05, 0.47)	0.14 (0.05, 0.48)	<0.001	0.12 (0.05, 0.42)	0.18 (0.05, 0.48)	<0.001
Dietary intake									
Total energy intake, kcal/day	1743±574	1694±522	0.161	1665±517	1754±557	<0.001	1684±563	1743±560	0.021
Fat, % of energy	36.4±6.3	28.8±6.1	<0.001	31.4±5.8	31.2±6.7	0.023	33.4±6.3	31.3±7.2	<0.001
Saturated fatty acid, % of energy	12.9±2.6	8.9±2.1	<0.001	10.4±2.2	10.5±3.0	<0.001	11.2±2.6	10.4±3.2	<0.001
Protein, % of energy	15.3±2.9	17.5±2.7	<0.001	16.7±2.6	16.6±2.7	<0.001	15.8±2.8	16.8±2.8	<0.001
Carbohydrate, % of energy	48.2±8.0	54.3±7.8	<0.001	52.2±7.2	52.8±7.7	0.738	50.9±8.0	52.3±8.2	<0.001
Vegetables‡, servings/day	1.9±1.0	2.3±1.0	<0.001	2.9±1.0	2.3±1.1	0.148	2.0±0. 1.0	2.1±1.1	0.008

Fruit‡, servings/day	1.5±1.2	2.2±1.2	<0.001	1.9±1.2	1.9±1.2	0.074	1.8±1.2	1.9±1.2	<0.001
Whole grains‡, servings/day	0.75±0.55	0.99±0.56	<0.001	0.93±0.55	0.92±0.55	<0.001	0.81±0.55	0.92±0.56	<0.001
Refined grains‡, servings/day	2.9±1.0	2.9±1.0	<0.001	3.0±1.0	2.9±1.0	<0.001	3.0±1.0	2.9±1.0	<0.001
Processed and red meat‡, servings/day	1.5±0.9	1.3±0.9	<0.001	1.4±0.9	1.4±0.9	0.779	1.5±0.9	1.4±0.9	<0.001
Added sugar‡, gram/day	10.7±4.1	9.8±4.1	<0.001	9.6±4.1	10.3±4.1	<0.001	10.7±4.1	9.9±4.1	<0.001
Fiber, g/1000 kcal/day	9.6±3.2	12.9±3.7	<0.001	11.5±3.5	11.4±3.7	0.019	10.7±3.5	11.5±3.8	<0.001
Total dairy‡, servings/day	1.65±1.0	1.99±1.0	<0.001	2.1±0.8	2.2±0.8	<0.001	1.6±0.9	2.1±1.0	<0.001
Total dairy, % energy	17.4±11.8	18.0±9.9	<0.001	18.1±9.9	18.3±9.6	0.105	15.8±10.5	18.7±11.1	<0.001
Healthy Eating Index	50.8±8.4	55.4±9.2	<0.001	58.5±8.1	55.4±9.2	<0.001	54.2±8.8	55.4±9.2	<0.001

*The results of only Q1 and Q4 of dairy consumption patterns are shown for simplicity.
†Data are presented as mean±SD or median (25th, 75th percentiles) for continuous variables and percentages (%) for categorical variables.
‡Adjusted for total energy intake.
§Linear regression was used for continuous variables and the Chi-square test for categorical variables.

In men, the unadjusted model revealed that DCP3 was inversely associated with incident T2D (OR of 0.61 [95% CI, 0.44 to 0.83] for the 4th vs. 1st quartile). After adjusting for demographic, lifestyle, and clinical factors (model 1), the odds ratio remained similar (OR of 0.60 [95% CI, 0.43 to 0.83] for 4th vs. 1st quartile) for risk of incident T2D. This association remained significant even after further adjusting for dietary intakes (model 2). In our analysis, DCP1 and DCP2 were not associated with the risk of incident T2D. By contrast, there were no significant associations between the extracted DCPs and T2D in women (Table 3.4).

Table 3. 4 Crude and multivariable-adjusted odds ratio for two logistic regression models of associations of dairy consumption patterns (DCP) and risk of Type 2 diabetes among men and women*

	Men				Women			
	Total/cases	Crude	Mode11†	Model 2‡	Total/cases	Crude	Mode1 1†	Model 2‡
DCP1								
Q1	2238/80	Ref.	Ref.	Ref.	3878/134	Ref.	Ref.	Ref.
Q2	2239/84	1.05 (0.77-1.44)	1.08 (0.78-1.49)	1.03 (0.74-1.42)	3879/109	0.80 (0.62-1.03)	0.88 (0.68-1.15)	0.90 (0.69-1.17)
Q3	2238/90	1.13 (0.83-1.54)	1.16 (0.84-1.59)	1.08 (0.78-1.50)	3877/126	0.93 (0.72-1.19)	1.02 (0.79-1.31)	1.04 (0.80-1.35)
Q4	2239/85	1.06 (0.78-1.45)	1.12 (0.82-1.54)	1.07 (0.77-1.48)	3879/128	0.94 (0.74-1.21)	1.01 (0.78-1.30)	1.02 (0.78-1.32)
P for trend§		0.617	0.423	0.648		0.925	0.700	0.656
DCP2								
Q1	2238/91	Ref.	Ref.	Ref.	3879/115	Ref.	Ref.	Ref.
Q2	2239/75	0.81 (0.59-1.11)	0.74 (0.54-1.01)	0.70 (0.51-0.97)	3877/127	1.11 (0.86-1.44)	1.12 (0.87-1.46)	1.15 (0.88-1.49)
Q3	2239/85	0.93 (0.69-1.26)	0.85 (0.62-1.15)	0.82 (0.60-1.11)	3879/122	1.06 (0.82-1.38)	1.04 (0.79-1.35)	1.05 (0.81-1.37)
Q4	2238/88	0.96 (0.71-1.30)	0.89 (0.66-1.21)	0.93 (0.68-1.26)	3878/133	1.17 (0.91-1.51)	1.10 (0.85-1.42)	1.09 (0.85-1.41)
P for trend§		0.978	0.693	0.851		0.285	0.609	0.649
DCP3								
Q1	2238/106	Ref.	Ref.	Ref.	3878/129	Ref.	Ref.	Ref.
Q2	2239/99	0.93 (0.70-1.23)	0.90 (0.67-1.21)	0.89 (0.66-1.19)	3879/131	1.03 (0.80-1.32)	1.14 (0.88-1.46)	1.16 (0.90-1.50)
Q3	2238/68	0.63 (0.46-0.86)	0.58 (0.42-0.81)	0.57 (0.42-0.80)	3878/101	0.78 (0.59-1.02)	0.86 (0.65-1.12)	0.87 (0.67-1.14)
Q4	2239/66	0.61 (0.44-0.83)	0.60 (0.43-0.83)	0.60 (0.43-0.83)	3878/136	1.07 (0.84-1.37)	1.12 (0.87-1.43)	1.12 (0.87-1.44)
P for trend§		<0.001	0.001	0.001		0.937	0.839	0.798

*Values are odds ratio (95% confidence interval) relative to the reference category

†Model 1: Adjusted for age, body mass index, total energy intake, physical activity, education level, menopausal status and menopausal hormone use (women participants only), hypertension, smoking status, and alcohol consumption

‡Model 2: Additionally adjusted for food groups including intakes of fruit, vegetables, whole grains, refined grain, processed and red meat, and added sugar

§P for trend was calculated across quartiles using multivariable logistic regression models when each quartile was assigned the median value of each DCP score

The relationship between the extracted DCPs and T2D was similar among subgroups defined by age and physical activity volume for both men and women. However, we detected a statistically significant effect modification by body size for DCP3 among men (Table 2.5). The risk of T2D for obese and overweight individuals in the highest quartile of DCP3 was reduced by 47% and 41%, respectively, compared with the lowest quartile (OR: 0.53, 95% CI: 0.33 to 0.82 and OR: 0.59, 95% CI: 0.36 to 0.96, respectively), whereas the association was null in normal-weight men.

Table 3. 5 Multivariable-adjusted odds ratio models of incidence of Type 2 diabetes according to quartiles of dairy consumption patterns scores within potential confounder subgroups*,†

	Men				P-trend‡	Women				P-trend‡
	Q1	Q2	Q3	Q4		Q1	Q2	Q3	Q4	
BMI (kg/m^2)										
DCP1										
18.5–24.9	Ref.	1.37 (0.66-2.11)	1.17 (0.42-1.95)	1.23 (0.51-2.00)	0.392	Ref.	1.09 (0.61-1.94)	1.29 (0.74-2.26)	1.09 (0.61-1.94)	0.756
25–29.9	Ref.	1.44 (0.87-2.37)	1.20 (0.71-2.03)	1.27 (0.75-2.15)	0.630	Ref.	0.63 (0.39-1.03)	0.68 (0.43-1.10)	0.93 (0.59-1.46)	0.915
≥30	Ref.	0.71 (0.45-1.12)	0.85 (0.58-1.32)	0.76 (0.49-1.18)	0.393	Ref.	0.85 (0.58-1.25)	0.95 (0.66-1.38)	0.90 (0.61-1.32)	0.711
DCP2										
18.5–24.9	Ref.	1.29 (0.57-2.55)	0.75 (0.26-2.44)	0.63 (0.23-2.01)	0.618	Ref.	1.26 (0.70-2.25)	1.10 (0.61-2.00)	1.20 (0.68-2.09)	0.541
25–29.9	Ref.	0.77 (0.47-1.25)	0.95 (0.60-1.53)	0.90 (0.56-1.45)	0.690	Ref.	1.08 (0.67-1.75)	1.07 (0.66-1.72)	1.15 (0.71-1.85)	0.579
≥30	Ref.	0.61 (0.38-0.96)	0.79 (0.51-1.21)	1.04 (0.68-1.57)	0.851	Ref.	1.08 (0.74-1.57)	1.03 (0.70-1.52)	1.17 (0.81-1.69)	0.443
DCP3										
18.5–24.9	Ref.	1.42 (0.59-4.02)	1.27 (0.54-4.27)	1.17 (0.42-4.18)	0.234	Ref.	1.14 (0.63-2.05)	1.02 (0.57-1.82)	1.44 (0.84-2.46)	0.224
25–29.9	Ref.	0.87 (0.57-1.35)	0.55 (0.33-0.90)	0.59 (0.36-0.96)	0.014	Ref.	1.08 (0.68-1.72)	0.81 (0.49-1.32)	1.05 (0.65-1.67)	0.986
≥30	Ref.	0.79 (0.58-1.18)	0.53 (0.34-0.82)	0.53 (0.33-0.82)	0.002	Ref.	0.96 (0.66-1.38)	0.75 (0.50-1.11)	0.97 (0.67-1.39)	0.675
Age (years)										
DCP1										
<65	Ref.	1.05 (0.75-1.47)	1.06 (0.75-1.49)	1.08 (0.76-1.53)	0.682	Ref.	0.77 (0.58-1.04)	0.89 (0.68-1.78)	0.88 (0.67-1.16)	0.587
≥65	Ref.	1.21 (0.42-3.53)	1.81 (0.66-4.89)	1.34 (0.39-3.30)	0.992	Ref.	0.96 (0.45-2.05)	0.87 (0.40-1.91)	1.49 (0.71-3.13)	0.233
DCP2										

<65	Ref.	0.74 (0.53-1.04)	0.86 (0.63-1.19)	0.93 (0.67-1.28)	0.521	Ref.	1.07 (0.81-1.42)	1.05 (0.79-1.39)	1.13 (0.86-1.49)	0.401
≥65	Ref.	0.64 (0.23-1.78)	0.91 (0.35-2.39)	0.95 (0.38-2.36)	0.896	Ref.	1.68 (0.76-3.71)	1.33 (0.61-2.91)	1.66 (0.79-3.48)	0.189
DCP3										
<65	Ref.	0.94 (0.69-1.28)	0.65 (0.46-0.91)	0.65 (0.47-0.91)	0.005	Ref.	0.95(0.72-1.25)	0.80(0.60-1.07)	1.00(0.76-1.31)	0.767
≥65	Ref.	0.74 (0.33-1.68)	0.34 (0.12-0.99)	0.32 (0.11-0.95)	0.031	Ref.	1.85 (0.87-3.91)	0.82 (0.34-1.97)	1.87 (0.88-3.94)	0.143
Physical activity (MET-h/week)										
DCP1										
<median	Ref.	1.34 (0.84-2.13)	1.38 (0.87-2.19)	1.17 (0.72-1.89)	0.910	Ref.	0.82 (0.57-1.17)	0.79 (0.55-1.13)	0.85 (0.57-1.23)	0.498
≥median	Ref.	0.86 (0.54-1.37)	0.96 (0.60-1.53)	1.06 (0.67-1.68)	0.658	Ref.	0.79 (0.53-1.18)	1.07 (0.74-1.54)	1.06 (0.73-1.54)	0.538
DCP2										
<median	Ref.	0.65 (0.43-1.00)	0.99 (0.67-1.47)	0.75 (0.49-1.15)	0.243	Ref.	1.25 (0.87-1.81)	1.23 (0.85-1.77)	1.35 (0.94-1.94)	0.099
≥median	Ref.	0.89 (0.55-1.44)	0.72 (0.44-1.18)	1.16 (0.75-1.81)	0.733	Ref.	0.96 (0.65-1.41)	0.89 (0.60-1.32)	0.99 (0.68-1.44)	0.899
DCP3										
<median	Ref.	0.91 (0.61-1.36)	0.63 (0.40-0.98)	0.48 (0.27-0.72)	<0.001	Ref.	1.02 (0.71-1.46)	0.82 (0.56-1.20)	1.16 (0.81-1.65)	0.566
≥median	Ref.	0.93 (0.62-1.39)	0.62 (0.39-0.99)	0.38 (0.23-0.64)	<0.001	Ref.	1.07 (073-1.54)	0.82 (0.56-1.22)	1.03 (0.72-1.48)	0.971
Menopausal status										
DCP1										
Premenopausal							0.81(0.55-1.18)	0.97(0.67-1.40)	0.87 (0.97-1.41)	0.869
Postmenopausal							0.90 (0.62-1.32)	0.93 (0.64-1.36)	0.93 (0.64-1.41)	0.947
DCP2										

Premenopausal	1.21 (0.84-1.74)	1.00 (0.69-1.47)	0.96 (0.69-1.31)	0.965
Postmenopausal	1.10 (0.75-1.63)	1.23 (0.84-1.79)	1.34 (0.92-1.94)	0.104
DCP3				
Premenopausal	1.11(0.77-1.58)	1.18 (0.82-1.70)	0.89(0.71-1.29)	0.480
Postmenopausal	0.74 (0.11-1.53)	0.81(0.55-1.21)	1.25 (0.87-1.77)	0.360

*Values are odds ratio (95% confidence interval) relative to the reference category

†The model was adjusted for age, body mass index, total energy intake, physical activity, education level, menopausal status and menopausal hormone use (women participants only), hypertension, smoking status, alcohol consumption, and food groups, including intakes of fruit, vegetables, whole grains, refined grain, processed and red meat, and added sugar, except for the stratified variable

‡P for trend was calculated across quartiles using multivariable logistic regression models when each quartile was assigned the median value of each DCP score

3.5 DISCUSSION

This study provides evidence that, for men, adherence to a pattern characterized by high consumption of whole milk, regular cheese, and non-fat milk reduces the probability of developing T2D up to 40%, even taking into account age, BMI, total energy intake, physical activity, and other potential confounders. This association was strongest among men with greater body size and age (age≥65 years), suggesting that adherence to a DCP with a mix of whole milk, regular cheese, and non-fat milk may have greater benefits for high-risk subgroups. Conversely, none of the identified DCPs were associated with increased T2D risk in women. Interestingly, while DCP1 was focused almost exclusively on low-fat dairy, DCP2 and DCP3 both had products ranging in fat content. Thus, the results imply that concentrating public health messaging only on individual dairy products and their fat content may not be an appropriate approach.

To our knowledge, this is the first study that prospectively assesses a data-driven posterior approach, PCA, to identify DCPs and their association with incident T2D. Because of the complexity of diets consumed by free-living individuals, the application of PCA to derive DCPs helps to identify underlying interactions and correlations between dairy items that may improve our understanding of eating behaviour (354) accompanying dairy consumption and therefore add more robust evidence from which to provide dietary advice. Thus, comparing our results to the current literature is limited since other studies grouped dairy based on their fat content *a priori* or analyzed individual dairy products. Because whole fat milk and regular cheese were the major components of DCP3, the favorable association of DCP3 with the risk of T2D could underlie the inverse relationship between dairy fat and T2D risk reported in previous studies (205, 355, 356, 357). A meta-analysis that synthesized the results of 16 prospective cohort studies found that higher concentrations of C15:0, C17:0, and Ct16:1n7 fatty acids in the blood, as biomarkers of dairy fat, were associated with lower incidence of T2D (217). Unlike our study, their subgroup analyses indicated that the favorable relationship between biomarkers of dairy fat and T2D was more robust in females than in males (217). The biomarker approach used in previous studies as an indicator of dairy fat focused on circulating and tissue levels of C15:0, C17:0, and Ct16:1n7 fatty acids is without recall bias and overestimation related to the application of FFQs. At the same time, investigating associations with biomarkers is prone to limitations, including the lack of

consideration of the totality of the participants' habitual diets. In addition, this approach does not differentiate among the various food sources of dairy fat.

The large prospective urban and rural epidemiology (PURE) study, including 147,812 individuals aged 35-70 years from 21 countries, indicated that participants in the highest category of intake of whole-fat dairy products (whole milk, whole fat yogurt, whole fat cheese, whole fat yogurt drinks, and mixed dishes prepared with whole fat dairy products) compared with those with zero dairy intake had a lower risk of diabetes, metabolic syndrome and hypertension (358). These associations were not seen with intake of low-fat dairy products (low fat (1–2%) milk, skimmed milk, low-fat yogurt, low-fat cheese, and low-fat yogurt drink). Similar inverse associations of high-fat dairy (whole fat yogurt, full-fat fruit yogurt, full-fat curd, high fat cheese, full-fat luxury cheese) intake with a lower risk of pre-diabetes were also reported in the longitudinal Hoorn Studies (355). However, the data linking high-fat dairy food intake to diabetes risk is inconsistent, and some researchers find a neutral association between high-fat dairy and the risk of T2D. When high-fat versus reduced-fat dairy was evaluated in a systemic review and meta-analysis of 26 cohort studies, there was no significant association between risk of T2D and high-fat dairy consumption (290). Data from observational studies show that dairy foods, no matter how much fat they have, lower T2D risk at best and are neutral at worst. This inconsistency highlights the complexities inherent in studying the effects of dairy foods on health outcomes, implying that using techniques to assess DCPs may address these issues. It is, however, difficult to directly compare these findings with current study because the grouping of dairy products based on their fat content is a source of heterogeneity among the studies. The extracted DCPs in the present study are based on whole dairy foods, so not only have the complex interactions among dairy products and combinations been considered, but also the characteristics of the food matrices has been incorporated.

The reason for the lack of a relationship between dairy consumption and the risk of T2D in females in the present study is unclear, but the mean daily consumption of high-fat dairy was approximately 2.5-fold higher in men than in women (e.g. 0.34 servings/d in men versus 0.13 servings/d in women for whole milk, data not shown), which might explain the lack of effect. Furthermore, there were sex differences in the factor loadings for components in the DCP3, i.e., the factor loadings for whole milk and cheese were higher in men than in women, which might influence the association of this pattern with the incidence of T2D in men vs. women. Additionally, women had a slightly

overall healthier diet, as indicated by the HEI 2015 score (359), so the putative protective effects of high-fat dairy may be less detectable.

Previous observational studies indicate that people who are more prone to T2D may gain more preventative benefits from dairy products. A modifying effect of glycemic status at baseline on the association of intake of dairy products with the risk of incident T2D has been proposed (302, 360). Although data regarding the glycemic status of included participants was not available, subgroup analyses were performed to examine how the baseline characteristics of participants associated with insulin resistance (i.e., BMI, age) may have influenced the association between DCPs and incident T2D. We found that the inverse association between DCP3 and T2D was more pronounced in people with greater body size. The BMI was equal across the quartiles of DCP3, and it was accompanied by lower total energy intake, meaning that individuals may efficiently modify their consumption of non-dairy foods to compensate for the caloric content of high-fat dairy foods. Some physiological evidence is consistent with the benefits of high-fat dairy on weight control. Compared with 0% fat milk, consumption of high-fat milk before a meal subsequently reduced appetite and energy intake in the subsequent meal 4 hours later, increased postprandial concentrations of the satiety hormones pancreatic polypeptide and cholecystokinin, and delayed gastric emptying (361, 362). Lower post-meal plasma ghrelin after consumption of whole milk and regular cheese but not skim milk compared to water was also reported (232). Consumption of high-fat dairy provides higher amounts of medium-chain triglycerides and conjugated linoleic acid, whose ability to suppress appetite and energy intake is suggested in several studies (363, 364, 365). Metabolic responses to a dairy meal, including decreased appetite, subsequent food intake, and post-meal appetite hormones, are reported to be different in magnitude between sexes (232), which may explain why a higher DCP3 score was not associated with T2D in females.

Our study has some limitations to acknowledge. First, self-reported questionnaires were used to identify T2D cases; however, the diabetes diagnosis was confirmed after careful consideration of subsequent questions, including symptoms, diagnostic tests, and treatment. Notably, high sensitivity and specificity of self-reported diabetes have been reported when it is verified using administrative health databases (366). Second, the assessment of dietary intake using self-reported FFQ has known measurement errors, including recall and desirability bias. In addition, participants' dietary intake was assessed only once at baseline; therefore, extracted DCPs do not reflect any modifications in dietary intake during the subsequent follow-up period. Although we

used PCA to group dairy without *a priori* biases, the extracted pattern is likely due to how dairy foods were characterized in the CDHQ-I, which was based on fat content and did not take into account other nutritional properties such as sugar content. More specifically, the fat and sugar content of yogurt consumed by participants was not collected. Also, our participants were limited to middle-aged males and females; thus, future studies of younger populations are warranted. Finally, even though we took into account a wide range of established and possible risk factors for T2D, the possibility of residual or unmeasured confounding cannot be ruled out.

In this population-based prospective study of Canadian men and women, a DCP with consumption of products with a range of fat content (whole milk, regular cheese, and non-fat milk) was associated with a reduced risk of incident T2D among men, with stronger protective associations suggested in individuals with greater body size. Focusing on the effects of combinations of dairy products may be more appropriate for the understanding their contribution to the prevention of T2D.

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4 DAIRY PRODUCT CONSUMPTION AND RISK OF NON-ALCOHOLIC FATTY LIVER DISEASE: A SYSTEMATIC REVIEW AND META-ANALYSIS OF OBSERVATIONAL STUDIES

Dairy product consumption and risk of non-alcoholic fatty liver disease: a systematic review and meta-analysis of observational studies

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4.1 ABSTRACT

Background and Aims: It is unclear whether regular consumption of dairy products is associated with the risk of developing non-alcoholic fatty liver disease (NAFLD). Thus, we conducted a systematic review followed by a meta-analysis of studies reporting on the association of dairy consumption with NAFLD risk.

Methods and Results: We comprehensively searched PubMed, Web of Science, and Scopus for observational studies that evaluated the association between dairy intake and NAFLD likelihood that were published before September 1, 2022. The reported odds ratios (ORs) of fully adjusted models and their 95% confidence intervals (CIs) were pooled using a random-effects model for the meta-analysis. Out of 1,206 articles retrieved, 11 observational studies, including 43,649 participants and 11,020 cases, were included. Pooled OR indicated a significant association between dairy intake and NAFLD (OR=0.90; 95% CI: 0.83, 0.98; I^2 = 67.8%, n=11). Pooled ORs

revealed that milk (OR: 0.86; 95% CI: 0.78, 0.95; I^2 = 65.7%, n=6), yogurt (OR: 0.88; 95% CI: 0.82; I^2 = 0.0%, n=4), and high-fat dairy (OR: 0.38; 95% CI: 0.19, 0.75; I^2 = 0.0%, n=5) consumption was inversely associated with NAFLD while cheese was not linked to NAFLD risk.

Conclusion: We observed that consumption of dairy products is linked to a reduced risk of developing NAFLD. Overall, the data in the source articles is of low to moderate quality; therefore, further observational studies are required to support the current findings (PROSPERO Reg. number: CRD42022319028).

Key words: milk, yogurt, cheese, dairy products, meta-analysis, non-alcoholic fatty liver disease

4.2 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease worldwide with an estimated prevalence of 25% and is characterized by intrahepatic fat deposits (>5% of liver weight) in the absence of secondary causes of steatosis (367). NAFLD encompasses a range of disease states that gradually progress from simple steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (367, 368). It associates closely with metabolic syndrome, a cluster of metabolic abnormalities including obesity, insulin resistance, hypertension, and hyperlipidemia (368, 369). An unhealthy lifestyle underpins the onset of metabolic dysfunction leading to NAFLD. Lifestyle modifications, including adoption of healthy eating patterns, remain the primary and most effective strategy for preventing NAFLD (370, 371, 372, 373). Excess consumption of red and processed meats, fast foods, confectionary and sugar-sweetened beverages, all commonly found in Westernized dietary patterns, is linked to an increased risk of NAFLD (320). Conversely, there is widespread agreement on the role of healthy eating patterns in protecting against NAFLD, especially lower intake of red and processed meats and increased consumption of fruits, vegetables, and whole grains (374).

Dairy products are rich in several bioactive compounds, micronutrients such as calcium, magnesium and phosphate, along with proteins such as whey, casein and milk fat globule membrane. However, concern about the effect of saturated fats in dairy products on cardio-metabolic health complicates recommendations about their consumption (224, 375). Moreover, physiochemical properties of individual dairy products vary depending on the processing methods

used (205); thus, each dairy product may have a different impact on cardio-metabolic health parameters. However, the role of dairy products in NAFLD onset remains controversial. A meta-analysis published in 2020 examined the association of food groups, including dairy products, with NAFLD. It found no significant link between total dairy product consumption and NAFLD after pooling the findings of three observational studies (376). However, the analysis only examined total dairy consumption, not individual dairy products. Another limitation was the small number of studies included in the analysis. Thus, an accurate and comprehensive estimate is needed to determine how dairy product consumption, both collectively and individually, affects NAFLD risk.

Due to the scarcity of quantitative results on the topic, we aimed to perform a systematic review and meta-analysis of observational studies focusing on the link between NAFLD and total and individual dairy product (milk, yogurt, cheese) consumption. Subgroup, meta-regression, and sensitivity analyses were performed using descriptive study characteristics as the dependent variable to identify potential causes of heterogeneity. Moreover, we evaluated the quality of meta-evidence supporting the link between dairy consumption and NAFLD using the validated NutriGrade approach (377) that adequately takes into account the inherent nature of nutrition science (e.g., reliance on observational data, diet assessment methods).

4.3 METHODS

The protocol was registered on the international prospective register of systematic reviews (PROSPERO) as CRD42022319028. This study was designed, conducted, and reported according to the meta-analysis of observational studies in epidemiology (MOOSE) (378) statement and the preferred reporting items for systematic reviews and meta-analyses (PRISMA) (379).

Data sources and search strategy

To improve the search strategy and discover all relevant studies at the time of the search, we conducted scope searches. Using MeSH terms and other related keywords, we performed a systematic search using PubMed, Web of Science, and Scopus databases to identify relevant publications prior to September 1st, 2022. We searched gray literature, particularly conference papers, using Scopus to identify additional relevant studies. For the papers included, we completed forward and backward citation checking, identified articles in relevant systematic reviews, and

consulted subject experts. In addition, we manually searched key journals deemed to contain relevant studies. No restrictions to the date of publication, language, or publication type were applied. We imported the search results into Covidence, a systematic review management software product (Veritas Health Innovation, Melbourne, Australia. Available at www.covidence.org), in which title and abstract screening along with full-text selections was completed. Duplicate records were removed automatically by the software before screening was initiated.

Study selection

The title/abstract and full text screening was performed by 2 independent authors (E.Y. and D.N.F.). We used population, exposure, comparison, outcome, and study design (PECOS) criteria to structure eligibility criteria for studies and research questions (380). The following criteria determined inclusion: studies that recruited adult individuals from any race and both sexes (population), evaluated total dairy intake and/or intake of low-fat dairy, high-fat dairy, milk, cheese, or yogurt (exposure), considered healthy participants (not NAFLD cases) and patients who were diagnosed as NAFLD or NASH with reported amounts of dairy consumption (comparison), and assessed the adjusted risk estimates (hazard ratio (HR), odds ratio (OR), or relative risk (RR) with corresponding 95% confidence interval (CI) for NAFLD/NASH in any stage (outcome, health condition of interest) in observational settings including cross-sectional, case-control and cohort studies (study design). Studies were excluded if they reported *in vitro* or *in vivo* experiments, any kind of interventional trials, systematic or narrative reviews, letters, editorials, and case reports. Moreover, we excluded studies that used the term "dairy" to name an extracted dietary pattern (e.g. through a principal component analysis). Studies focused on the relation of dairy products with the severity of already-diagnosed NAFLD were also excluded. We recorded reasons for exclusions in the Covidence software during full-text screening. The review team emailed researchers of the studies to request more information, if necessary.

Data extraction process

Two researchers (E.Y. and D.N.F.) independently extracted the following data: the first author's last name, publication year, the country where the study was conducted, study design, source(s) of funding, reported conflicts of interest for the study authors, study name, method of recruitment, range or mean age at entry, sex of the participants, number of participants, number of individuals with NAFLD, the incidence or prevalence of NAFLD, follow-up period, dietary assessment

method, the method(s) used to ascertain outcome, types and categories of dairy products, consumption frequency or amount of dairy consumption, the definition of serving size or portion size, the statistical model used, risk estimates with corresponding 95% CI from the maximally-adjusted multivariable model for each level of consumption, and covariates adjusted for in multivariate analyses. If a study reported sex-specific risk estimates with or without the total population, we extracted data for each sex in addition to the data for the whole sample.

Risk-of-bias assessment

The risk of bias (RoB) was assessed independently by two reviewers (E.Y. and D.N.F.), using the Newcastle–Ottawa Scale (NOS) (381). If relevant data was not explicitly mentioned in the included paper, information was gathered from the cited protocol, rationale, and design of study by examining the article's references. Conflicts between reviewers were resolved through discussion or by another reviewer. The NOS contains three domains with nine items, examining the selection, comparability, and exposure/outcome adapted for each study design (cohort study, case-control study, cross-sectional study) (Supplemental Appendix 3). The maximum possible score is 9. We categorized included studies according to the total scores assigned and considered scores between 0-6 as high RoB and scores between 7-10 as low RoB (381).

Data synthesis and statistical analysis

Odds ratios (ORs) were most frequently used as the risk estimates of association among the included studies. If included papers reported RRs or HRs, they were considered as ORs (382, 383). A meta-analysis was performed with a minimum of two studies. A DerSimonian–Laird random-effects model, which considers both within- and between-study variation (heterogeneity), was used to estimate the pooled OR of NAFLD for dairy products, total dairy, milk, yogurt, and cheese consumption. We used the term 'any dairy products' to refer to all types of dairy items that were included in our analysis regardless of differences in reporting between articles. We could not conduct dose-response meta-regression analyses as few studies with applicable data were available.

We used a fixed-effects model to pool the reported risk estimates for dairy sub-types, such as milk, yogurt, and cheese, when studies did not report estimates for total dairy consumption. The resulting pooled OR was used in the meta-analysis of total dairy consumption. We first combined the reported risk estimates using a fixed-effects model to obtain an overall estimate for studies that

only reported effect sizes for individual dairy foods or sub-types such as milk, yogurt, and cheese consumption, with no reporting of estimates for total dairy consumption. The pooled OR was subsequently used in the meta-analysis pertaining to the specific dairy food. It was also applied to those studies reporting high/whole-fat dairy and low/non-fat dairy or high/whole-fat milk and low/non-fat milk. We then included the pooled risk estimates in the meta-analysis.

We calculated the effect size when a study used the highest intake category as a reference using Orsini et al. (384). Studies were treated as independent when their results were stratified by sex. In addition, if results were separately provided for males and females, in addition to the total sample, we used sex-specific results when possible. If a study only reported data on a specific individual dairy product, we used the risk estimates of that individual dairy product for the 'any' dairy consumption meta-analysis and the pooled OR in the meta-analysis for the sub-type consumption.

In addition to the comparison among all subjects, we performed prior stratified analyses for the study design (cohort, cross-sectional and case-control studies), sex (male, female, and both), and individual dairy products (total dairy, milk, yogurt and cheese).

We used Cochrane's Q test in addition to I^2 for all meta-analyses performed to assess statistical heterogeneity across the studies. We considered substantial heterogeneity as $I^2 \geq 50\%$ and $p < 0.10$. To determine the source of heterogeneity across studies, we carried out subgroup analyses depending on the research design, type of dairy product, sex of participants, geographic location (Americas, Europe, or Asia), risk of bias, NAFLD diagnosis methods and dairy consumption assessment tools. Subgroup analyses were performed with ≥ 3 studies in each subgroup. Otherwise, if the number of studies in the pre-specified stratified analysis was too small to explore sources of heterogeneity, logistic meta-regression analyses were applied by considering the study characteristics, including the prevalence of NAFLD, sex and method of dairy intake assessment. Sensitivity analysis was conducted by sequentially deleting each effect size from the study to assess the possible influences of each article on the pooled effect size. If there were at least three studies, funnel plots and Egger's tests (weighted linear regression test) were used to identify the presence of publication bias (risk of bias across studies). For all statistical analyses, Stata software, version 15.0 (StataCorp), was used. *P* values of less than 0.05 were regarded as significant.

Certainty of evidence

We applied the NutriGrade scoring system to assess the quality of evidence linking exposure to various dairy products and risk of NAFLD. The NutriGrade criteria for meta-analyses of observational studies are as follows: precision, heterogeneity, directness, publication bias, funding bias, dose-response (0 to 1 point), risk of bias, and effect sizes (0 to 2 points) (377). Very low-quality evidence (0 to 3.99 points), low-quality evidence (4 to 5.99), moderate-quality evidence (6 to 7.99), and high-quality evidence (8 to 10 points) are the four categories suggested to assess the confidence of meta-analysis data using this scoring method (377).

4.4 RESULTS

Characteristics of included studies

A total of 1206 records from the three databases were identified. After removing 548 duplicate records, we screened the titles and abstracts of 658 studies, with 23 meeting the inclusion criteria for full-text review after which 12 studies were excluded (Figure 4.1). A total of 11 studies (including 15 risk estimates) that reported data on dairy consumption and NAFLD occurrence were selected for the final analysis. In detail, five studies (eight risk estimates) were included in the analysis of total dairy consumption (374, 385, 386, 387, 388), three studies (six risk estimates) in the analysis of milk (388, 389, 390), two studies (four risk estimates) in the analysis of yogurt (388, 391), three studies (five risk estimates) in the analysis of cheese (321, 388, 392), and two studies (two risk estimates) in the analysis of high-fat dairy (319, 321).

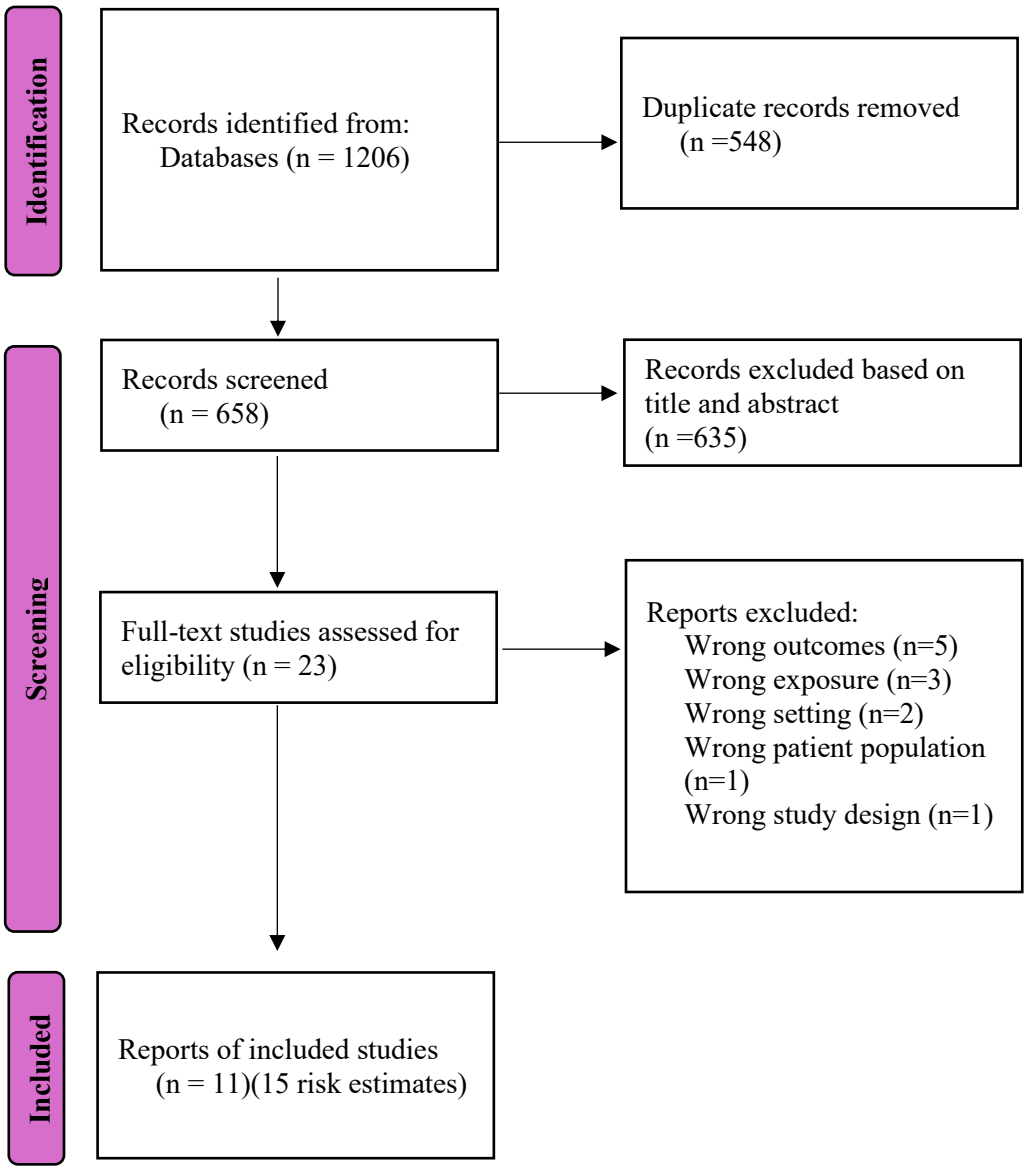


Figure 4. 1 Flowchart of study selection process.

The main characteristics of all included articles are summarized in Table 4.1. Four studies were conducted in China (374, 385, 390, 391), two in Korea (386, 388), and the others in the Netherlands (389), Germany (321), Thailand (319), Greece (392) and Iran (387). Those included comprised seven cross-sectional (319, 321, 374, 385, 387, 389, 391), three case-control (386, 390, 392) and one cohort study (388). The included studies recruited a total of 43,649 participants, ranging from 136 to 24,389, and the number of cases was 11,020 individuals, ranging from 66 to 4,658. Publication dates were between 2014 and 2021. The average age of participants across studies varied from 37.6 to 54.9 years. Nine articles provided data on both sexes; however, three studies analyzed data separately for men and women. Regarding dietary assessment, eight studies applied a food-frequency questionnaire (FFQ), two used a food record (range three days to seven days) and one used a researcher-generated diet questionnaire.

Table 4. 1 General characteristics of included studies

First author, Year	Funding	Study design	Name of the study (if available)	Country	Sample size, n	Mean age	Definition of the outcome	Diagnosis of NAFLD cases	Number of cases	Assessment of diet	Dairy items	Risk estimate (95% CI)	Model used	Covariates in Multivariable Model
Han, 2014	Ministry of Food and Drug Safety Grant	Case-control	NA	Korea	Men, 166 Women, 182	44.6	Not mentioned	Abdominal ultrasound	Men, 103 Women, 66	24-h recall and 4-day food record	Total dairy	Odds ratio	Logistic regression	Age category, current job, education attained, exercise frequency category, smoking status, and energy intake
Van Eekelen, 2019	The Netherlands Cardiovascular Research Initiative, Dutch Heart Foundation, the Nutricia Research Foundation, Unilever Research and Development Vlaardingen and Health-Holland	Cross-sectional	The Netherlands Epidemiology of Obesity (NEO)	Netherlands	Both, 1966 Men, 924 Women, 1042	Both, 55 Men, 56 Women, 55	Hepatic TG content (HTGC) of >5.56% not due to excessive alcohol consumption	Proton magnetic resonance spectroscopy	Both, 578 Men, 360 Women, 218	FFQ	Milk	Odds ratio	Logistic regression	Age, sex, smoking, education, ethnicity, physical activity in leisure time, total energy intake, and total body fat
Zhang, 2020	The National Natural Science Foundation of China, the key technologies R&D program of Tianjin, the National Science and Technology Support Program Chinese Nutrition Society Nutrition Research Foundation DSM Research Fund, the Technologies development program of Beichen District of Tianjin, the technologies project of Tianjin Binhai New Area, the Science Foundation of Tianjin Medical University), the Key Laboratory of Public Health Safety (Fudan University), Ministry of Education, and the National Training Programs of Innovation and Entrepreneurship for Undergraduates, China.	Cross-sectional	The Tianjin Chronic Low-Grade Systemic Inflammation and Health (TCLSIH) Cohort Study	China	Both, 24,389	39	Having at least two of the following three parameters and no significant alcohol intake: hyperechogenicity of liver tissue compared to the renal cortex, vascular blurring, and diffuse echogenicity of the liver	Abdominal ultrasound	4658	FFQ	Yogurt	Odds ratio	Logistic regression	Age, sex, BMI, smoking status, alcohol drinking status, education attained, working status, household income, physical activity, family history of the disease, total energy intake, carbohydrate intake, total fat intake, eicosapentaenoic acid + docosahexaenoic acid intake, soft drinks intake, vegetable intake, fruits intake, sweet foods intake, and milk intake, hypertension, diabetes, and hyperlipidemia
Watzinger, 2020	The Helmholtz Association of German Research Centers	Cross-sectional	HELENA Trial	Germany	Both, 136	50	≥ 5% liver fat content	Magnetic resonance imaging	72	FFQ	High-fat dairy and high-fat cheese	Odds ratio	Logistic regression	Age, sex, waist circumference, calorie intake, the ratio of energy intake/total energy expenditure
Hao, 2020	The National Natural Science Foundation of China	Case-control	NA	China	Both, 3506	54	Degree of liver brightening and/or	Ultrasonography	2175	FFQ	Milk	Odds ratio	Logistic regression	Age, sex, body mass index, waistline

							blurring of vessels and diaphragm ultrasonography							circumference, smoking, hyperlipidemia, hypertension, and diabetes
Lee, 2021	Ministry of Agriculture, Food and Rural Affairs	Cohort	Korean Genome and Epidemiology Study (KoGES)	Korea	Men, 2159 Women <50 y, 1555 Women ≥50y, 4160	Men, 51.3 Women <50 y, 44 Women ≥50y, 59.2	A NAFLD liver fat score > -0.640	Validated fatty liver prediction model: - 2.89 + 1.18 x metabolic syndrome (Yes: 1, No: 0) + 0.45 x diabetes mellitus (Yes: 2, No: 0) + 0.15 insulin (IU/mL) + 0.04 x aspartate aminotransferase (AST) in U/L - 0.94 x AST/alanine aminotransferase (ALT)	Men, 795 Women <50 y, 1460 Women ≥50y, 1555	FFQ	Milk, yogurt, cheese	Hazard ratio	Cox proportional hazard model	Age, BMI, physical activity, smoking status, current drinker, daily protein intake per weight, daily carbohydrate intake per weight, daily calcium intake, daily vitamin E intake, plasma glucose level, serum total cholesterol level, and serum ALT level
Charatcharoenwitthaya, 2021	The Royal College of Physicians of Thailand	Cross-sectional	NA	Thailand	Both, 252	37.6	The value of the controlled attenuation parameter (CAP) at 288 dB/m or greater	Transient elastography to compute controlled attenuation parameter (CAP)	41	7-d Food diary	High-fat dairy	Odds ratio	Logistic regression	Age, sex, healthcare profession, and daily calorie intake
Chiu, 2018	The Dalin Tzu Chi Hospital and the Buddhist Tzu Chi Medical Foundation	Cross-sectional	Tzu Chi Health Study	China	Both, 3400	54.2	Compared the liver echogenicity with that of the kidney cortex	Abdominal sonography	1911	FFQ	Total dairy	Odds ratio	Logistic regression	Age, gender, education, history of smoking, history of alcohol drinking, total energy intake, vegetarian diet, and BMI
Chan, 2015	Health and Health Services Research Fund sponsored by the Government of Hong Kong SAR and the Centre for Nutritional Studies, under the auspices of The Chinese University of Hong Kong	Cross-sectional	NA	China	Both, 797	48	Intrahepatic triglyceride content of 5% or more	Proton-magnetic resonance spectroscopy	220	FFQ	Total dairy	Odds ratio	Logistic regression	Age, sex, BMI, current smoker status, current drinker status, central obesity, triglyceride >1.7 mmol/L, reduced HDL-cholesterol, hypertension, impaired fasting glucose or diabetes, energy intake, and the PNPLA3 genotypes (CC vs. CG vs. GG

														genotypes)
Kalafati, 2019	NA	Case-control	NA	Greece	Both, 351	50.3	Based on three parameters: (a) diffuse echogenicity of the liver, (b) increased echogenicity compared to the renal cortex, (c) loss of definition of the diaphragm and blurring of the vascular margins	Abdominal ultrasound	134	FFQ	Full-fat cheese	Odds ratio	Logistic regression	Age, gender, body mass index/energy intake, physical activity, and smoking
Mansour-Ghanaei, 2019	NA	Cross-sectional	PERSIAN Guilan cohort study	Iran	Both, 630	50.0	Echogenicity increased in the liver parenchyma compared to kidney parenchyma	Abdominal ultrasound	275	A questionnaire developed by the research team	Total dairy	Odds ratio	Logistic regression	Univariate

NA, not available; FFQ, food frequency questionnaire; HELENA, healthy lifestyle in europe by nutrition in adolescence; PERSIAN, prospective epidemiological research studies in Iran; BMI, body mass index

Findings from the meta-analysis

The pooled OR from the random-effects model indicated a significant association between any dairy consumption and risk of NAFLD (OR=0.90; 95% CI: 0.83, 0.98) with substantial heterogeneity among the studies (I^2 = 67.8%, *P*-heterogeneity < 0.001) (Figure 4.2). The magnitude and direction of the pooled estimated OR were stable when individual risk estimates were excluded (Figure 4.3). In the subgroup analyses (Table 4.2), the association of any dairy intake with NAFLD occurrence significantly differed among studies in which the exposure was considered a continuous variable (one serving/d increase) and those with categorical exposure (highest vs. lowest; *P*-interaction = 0.040). There was an inverse association between any dairy consumption and the risk of NAFLD when studies compared the risk estimates in the highest to the lowest category of dairy consumption (n = 11; OR: 0.83; 95% CI: 0.74, 0.97; I^2 = 55.7%) but not among those reporting one serving/d increase of dairy consumption (n = 4; OR: 0.99; 95% CI: 0.91, 1.09; I^2 = 64.3%). However, heterogeneity remained considerable within the two subgroups. In other subgroup analyses, there were no significant interactions between the association of any dairy consumption and NAFLD occurrence. Notably, estimated heterogeneity between studies was least (I^2 = 11.9%) in an analysis that included only results from men (n=3; OR: 0.91; 95% CI: 0.85, 0.97). Assessment of the funnel plot (Figure 4.4) and Egger's tests (*P* = 0.502) confirm no publication bias concerning the relationship between any dairy consumption and NAFLD occurrence.

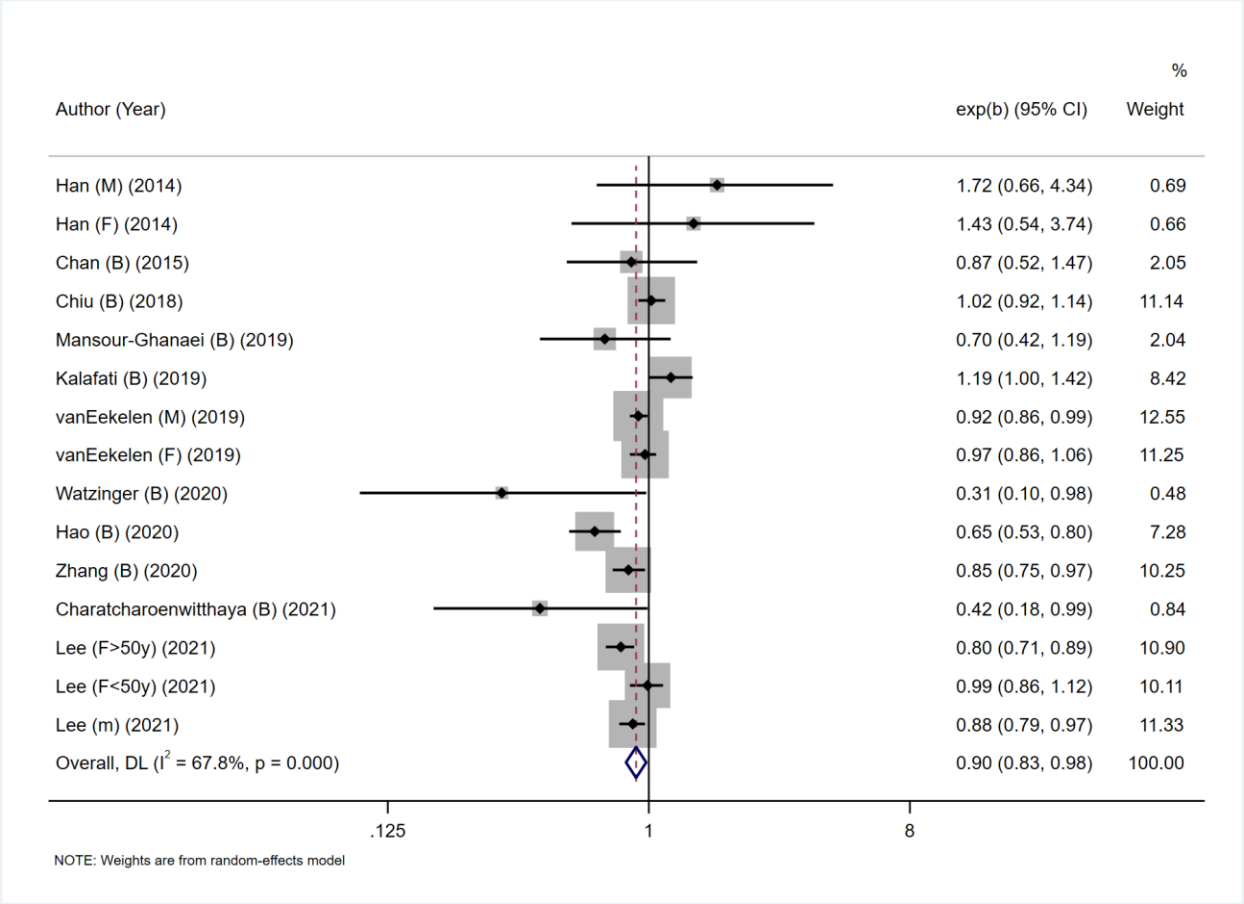


Figure 4. 2 Forest plot of the association of dairy consumption with risk of nonalcoholic fatty liver disease (NAFLD) using random-effects meta-analysis.

The square size represents the weight of each included estimate. The small diamonds indicate the single risk estimate, and black line segments indicate the 95% CI of each study. The x-axis is the odds ratio. Heterogeneity between studies is shown by I^2. The hollow diamond shows the pooled odds ratio with 95%CI. (M) male; (F) female, (B) both sexes.

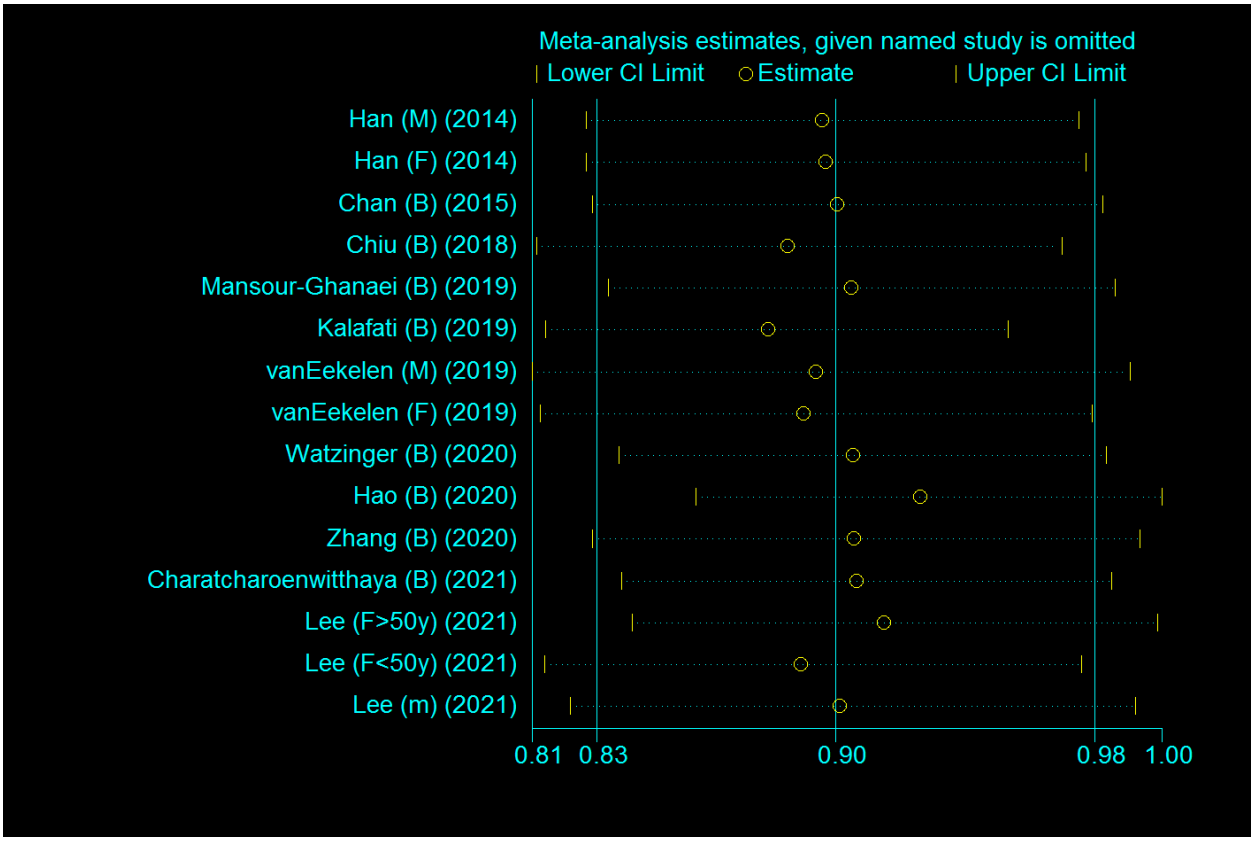


Figure 4. 3 Forest plot of sensitivity analysis for the association of dairy consumption and nonalcoholic fatty liver disease (NAFLD) risk.

The study name with corresponding risk estimate is the risk estimate that was omitted from the meta-analysis. Abbreviations: M-male, F-female, B-both sexes

Table 4. 2 Stratified meta-analysis of dairy consumption and risk of nonalcoholic fatty liver disease (NAFLD)

Stratification	**Categories**	**Risk estimates, n**	**Pooled Relative Risk (95% CI)**	**I^2 (%)**	***P*–interaction[1]**
Sex	Both	8	0.83 (0.69, 1.01)	78.3	0.977
	Male	3	0.91 (0.85, 0.97)	11.9	
	Female	4	0.92 (0.81, 1.05)	65.9	
Study design	Cross-sectional	8	0.92 (0.84, 1.00)	48.1	0.712
	Case-control	4	1.05 (0.65, 1.69)	86.2	
	Cohort	3	0.88 (0.79, 0.99)	65.4	
Risk of Bias	Low risk of bias	10	0.91 (0.84, 0.97)	56.0	0.908
	High risk of bias	5	0.96 (0.64, 1.43)	82.6	
Study location	Asia	11	0.86 (0.78, 0.96)	64.5	0.260
	Europe	4	0.98 (0.85, 1.13)	73.0	
Dietary assessment	FFQ	11	0.91 (0.84, 0.98)	72.8	0.738
	Other	4	0.87 (0.49, 1.56)	52.8	
Exposure comparison	Highest vs. lowest	11	0.83 (0.74, 0.97)	55.7	0.040
	Per 1-serving increase	4	0.99 (0.91, 1.09)	64.3	

[1] Meta-regression was used to calculate P-interaction

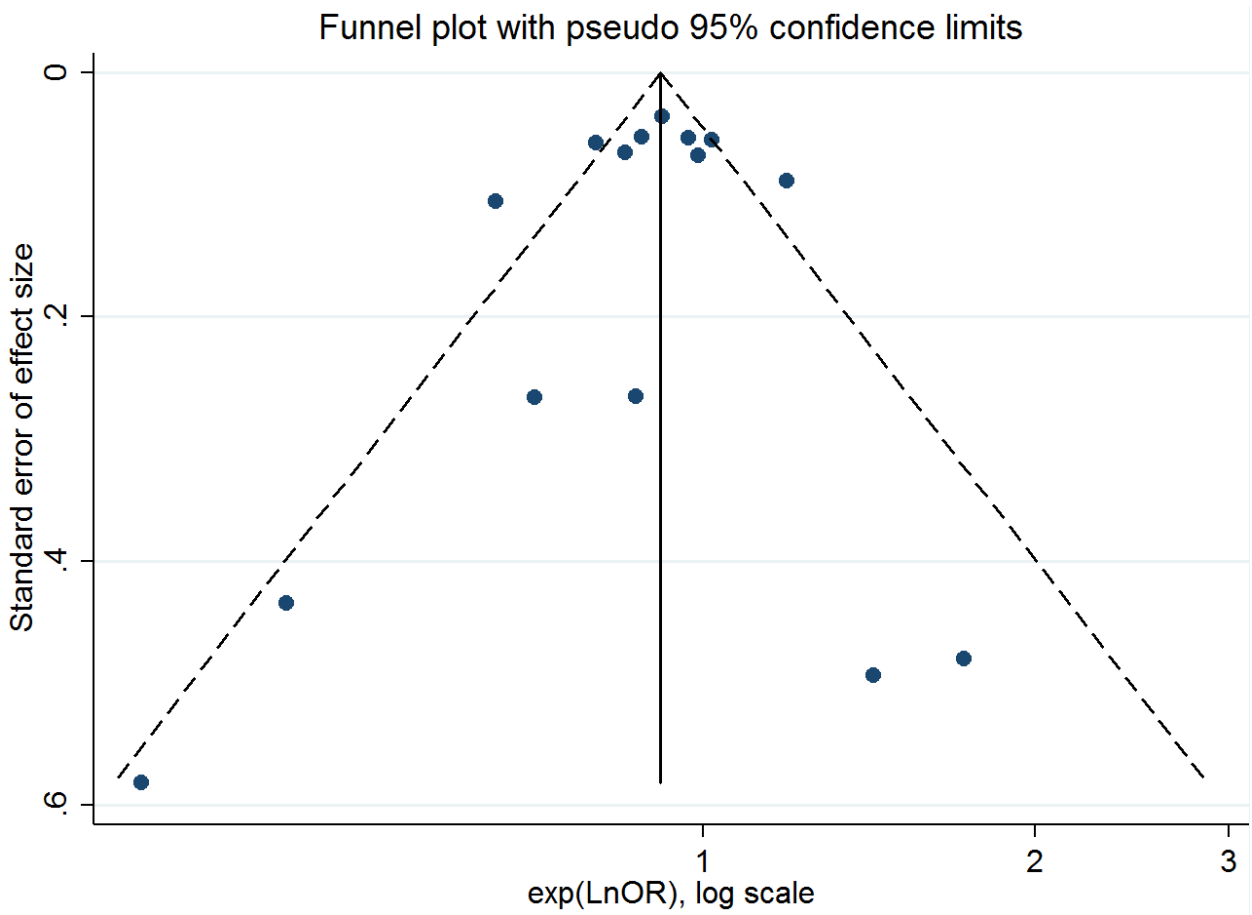


Figure 4. 4 Funnel plot to assess the presence of publication bias in the association of dairy consumption and risk of nonalcoholic fatty liver disease (NAFLD)

Eight risk estimates obtained from five publications, including 13,049 total participants and 3,370 NAFLD cases, all conducted in Asian countries, were included in the association of total dairy consumption and NAFLD occurrence. The pooled OR for total dairy consumption, according to the random-effects model, was 0.92 (95% CI: 0.83, 1.01), with moderate evidence of heterogeneity (I^2 = 52.9%) (Figure 4.5). Pooled ORs did not change significantly after considering confounding variables in the meta-regression analysis (Table 4.3). Strikingly, sensitivity analysis indicated that excluding the study conducted by Chiu et al. 2018 (385) influenced the association of total dairy consumption and NAFLD occurrence, resulting in a significant inverse association (OR: 0.88; 95% CI: 0.80, 0.98; Figure 4.6). The funnel plot (Figure 4.7) and Egger's tests (P = 0.175) indicated no evidence of publication bias.

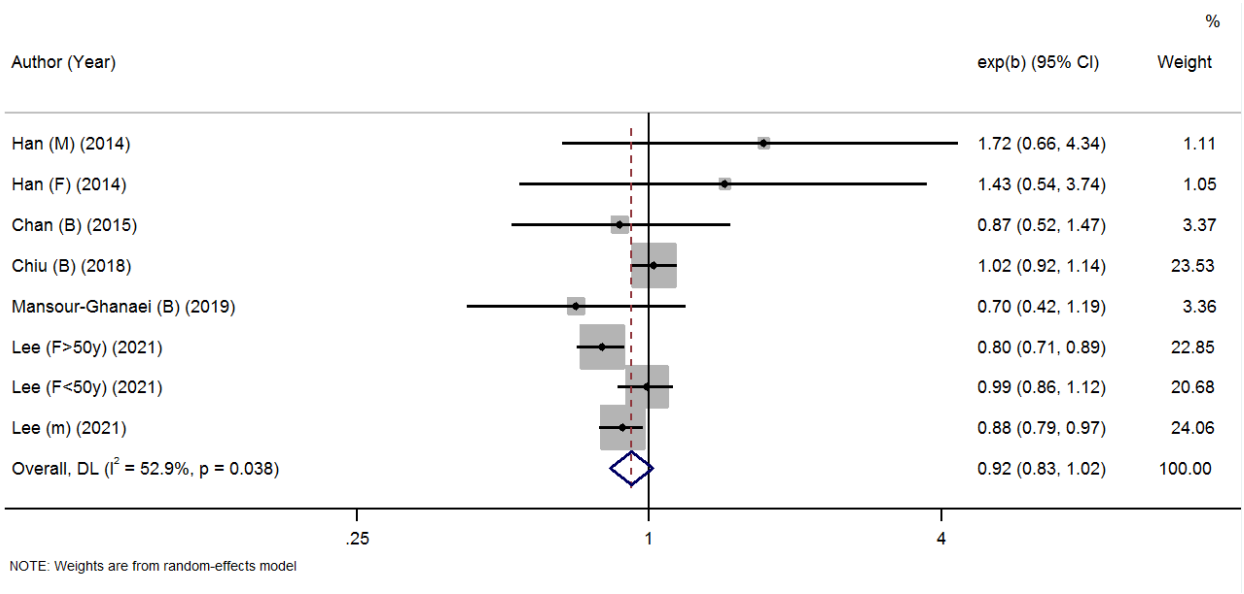
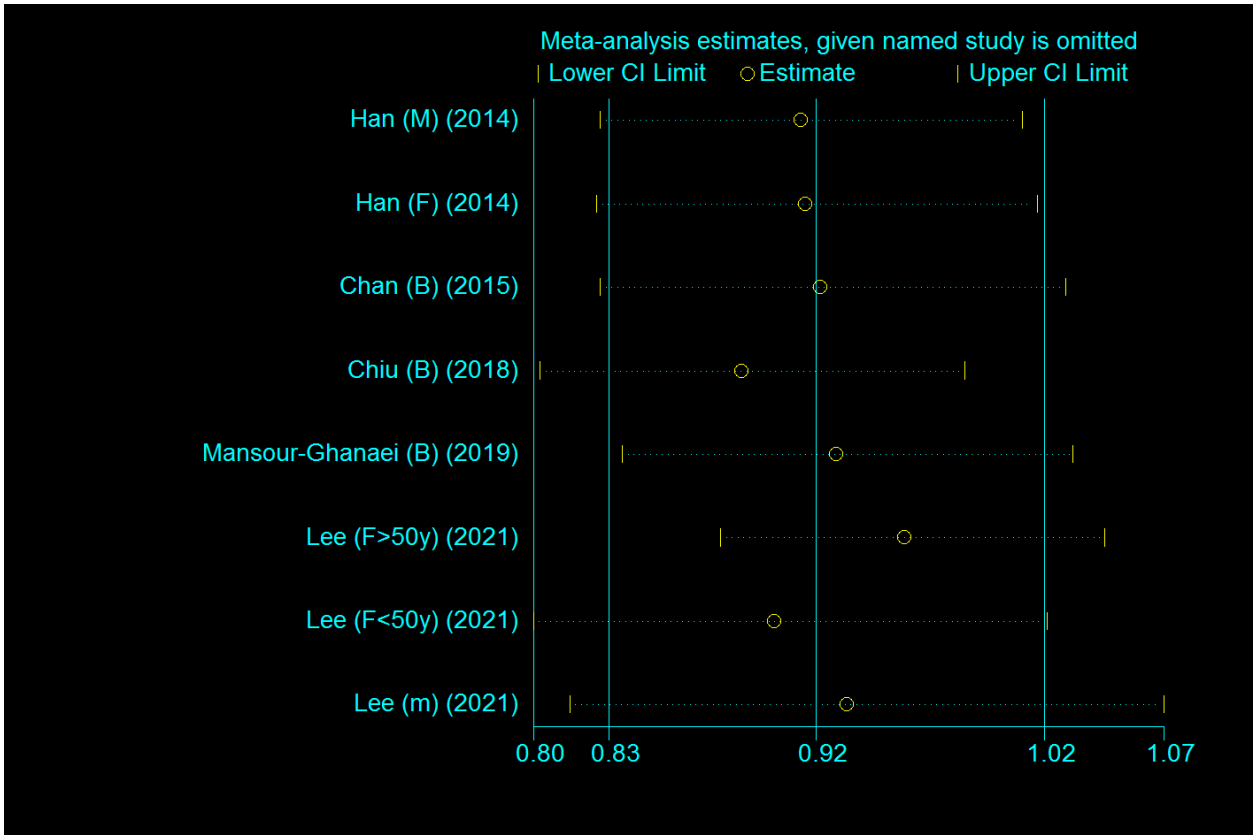


Figure 4. 5 Forest plot of the association of total dairy consumption with risk of nonalcoholic fatty liver disease (NAFLD) using random-effects meta-analysis.

The squares size represent the weights of included estimate. The small diamonds indicate the single risk estimate, and black line segments indicate the 95% CI of each study. The x-axis is the odds ratio. Heterogeneity between studies is shown by I^2. The hollow diamond shows the pooled odds ratio with 95% CI. M), male; (F), female, (B), both sexes.



Study omitted	Estimate	[95% Conf.	Interval]
Han (M) (2014)	.9114418	.8261137	1.0055833
Han (F) (2014)	.91340661	.82457292	1.0118107
Chan (B) (2015)	.9198851	.82618791	1.0242083
Chiu (B) (2018)	.8862685	.80052006	.98120201
Mansour-Ghanaei (B) (2019)	.92635566	.83542824	1.0271796
Lee (F>50y) (2021)	.9554435	.87737006	1.0404644
Lee (F<50y) (2021)	.90030301	.79771024	1.01609
Lee (m) (2021)	.9308877	.81308836	1.0657536
Combined	.91772314	.82970243	1.0150817

Figure 4. 6 Forest plot of sensitivity analysis for the association of total dairy consumption and nonalcoholic fatty liver disease (NAFLD) risk

The study name with corresponding risk estimate is the risk estimate that was omitted from the meta-analysis. Abbreviations: M-male, F-female, B-both sexes

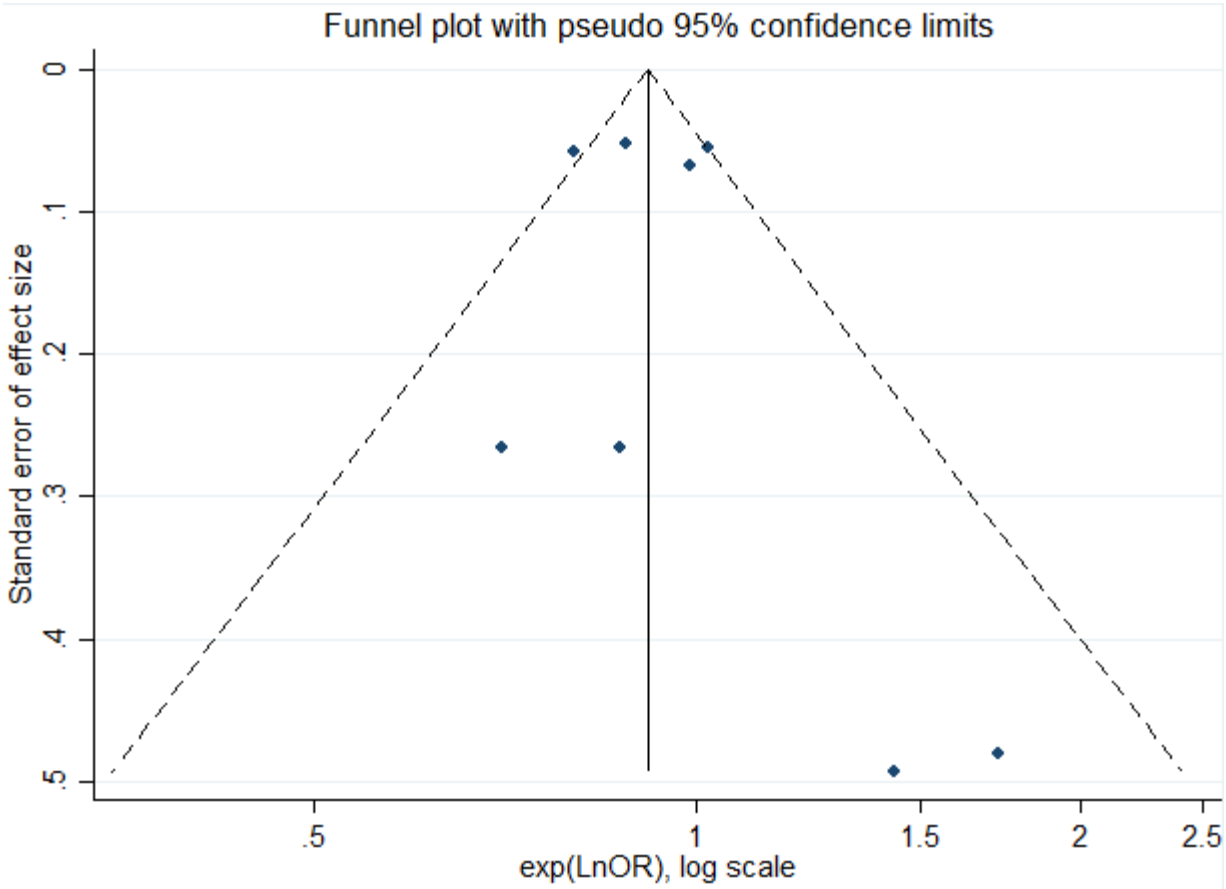


Figure 4. 7 Funnel plot to assess the presence of publication bias in the association of dairy consumption and risk of nonalcoholic fatty liver disease (NAFLD).

Table 4. 3 Meta-regression analysis to control covariates odds ratio (95% confidence interval) for the association of total dairy consumption and nonalcoholic fatty liver disease (NAFLD) of the studies included in the meta-analysis

Covariate	**Pooled odds ratios (95% CI)**	**R^2 (%)**	**I^2 res (%)**	***P*–interaction[1]**
Sex	1.02 (0.86, 1.23)	-20.8	51.2	0.735
Study design	0.95 (0.83, 1.08)	20.4	41.5	0.383
Risk of Bias	1.05 (0.60, 1.86)	-8.6	59.0	0.824
Dietary assessment	1.05 (0.59, 1.86)	-8.6	59.5	0.824
Exposure comparison[2]	1.18 (0.88, 1.50)	28.8	36.3	0.244

[1] Meta-regression was used to calculate P-interaction.

[2] Studies that report a risk estimate calculated based on highest vs. lowest or per 1-serving increase of exposur

There were six risk estimations from three studies, including 13,346 total participants and 3,548 NAFLD cases, for the meta-analysis of the relationship between milk consumption and NAFLD occurrence. Pooling ORs revealed that milk consumption was inversely associated with NAFLD occurrence (OR: 0.86; 95% CI: 0.78, 0.95), with moderate heterogeneity among included publications (I^2 = 65.7%) (Figure 4.8). Sensitivity analysis indicated no single risk estimate changed the pooled estimate when omitted (Figure 4.9). When risk estimates were adjusted for confounding variables, including sex, study design, risk of bias, and exposure, the association did not change (Table 4.4).

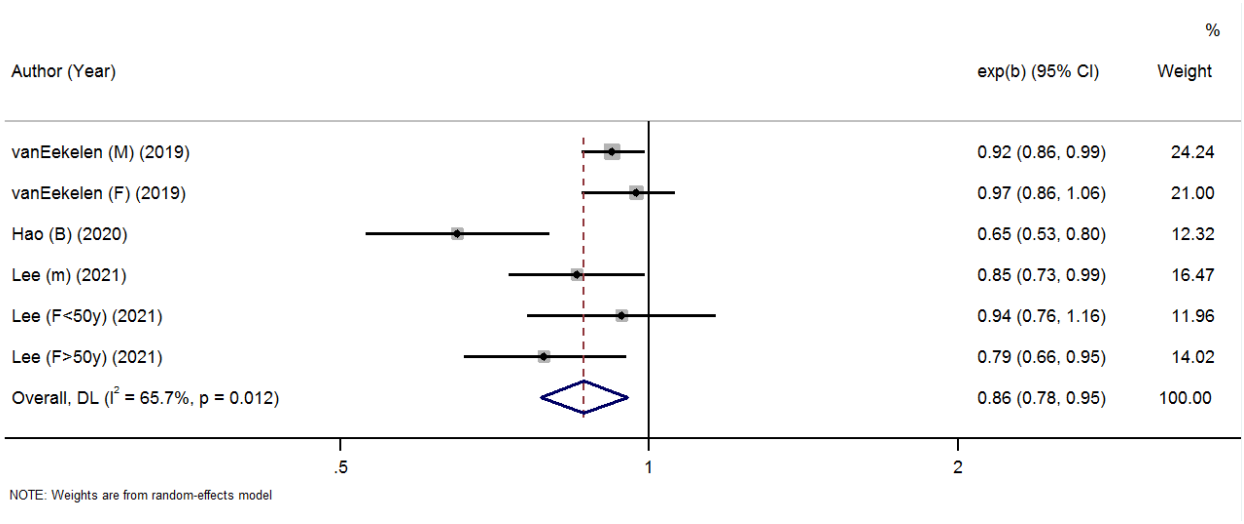
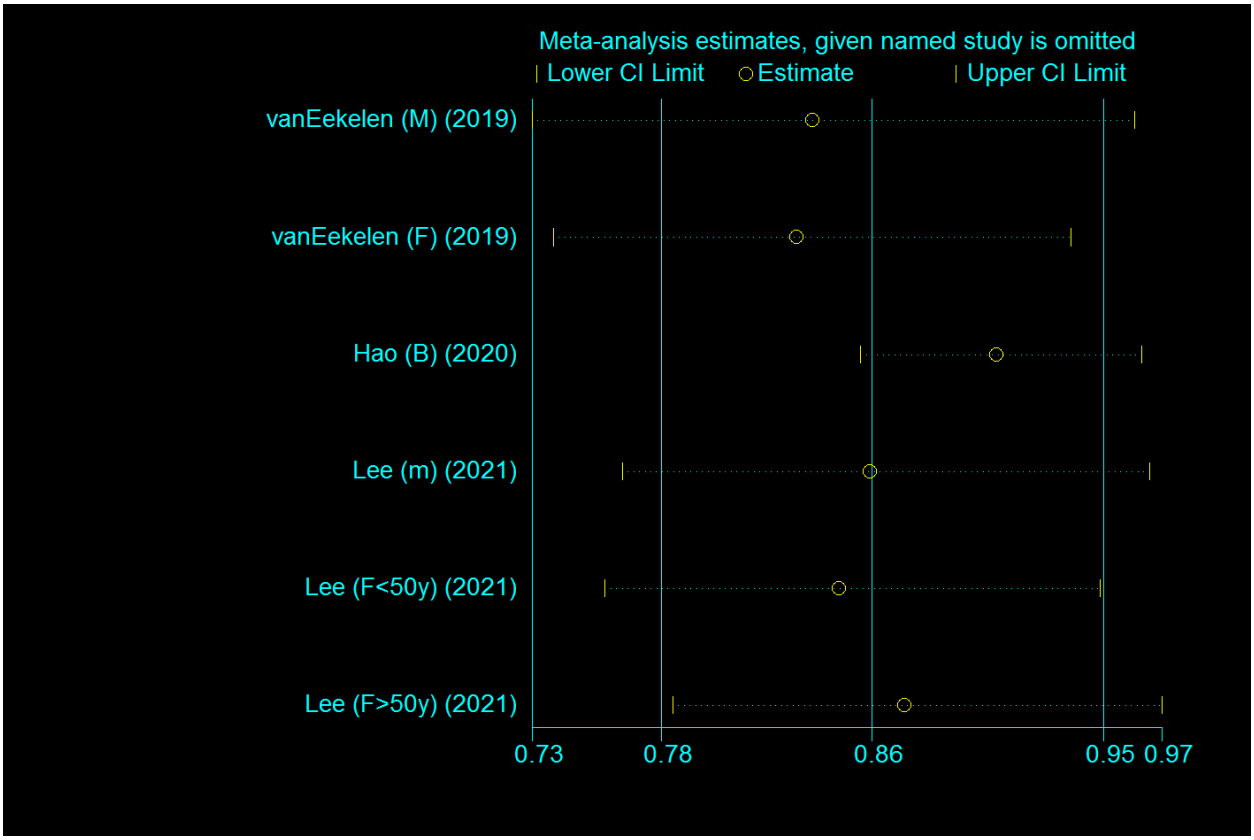


Figure 4. 8 Forest plot of the association of milk consumption with risk of nonalcoholic fatty liver disease (NAFLD) using random-effects meta-analysis.

The squares size represent the weights of included estimate. The small diamonds indicate the single risk estimate, and black line segments indicate the 95% CI of each study. The x-axis is the odds ratio. Heterogeneity between studies is shown by I^2. The hollow diamond shows the pooled odds ratio with 95% CI. (M), male; (F), female, (B), both sexes.



Study omitted	Estimate	[95% Conf.	Interval]
vanEekelen (M) (2019)	.84094644	.73458213	.96271175
vanEekelen (F) (2019)	.83487546	.7425124	.9387278
Hao (B) (2020)	.91072208	.85899353	.96556574
Lee (m) (2021)	.86278826	.76867616	.96842295
Lee (F<50y) (2021)	.85077918	.76217401	.94968504
Lee (F>50y) (2021)	.87560081	.78780913	.97317576
Combined	.86339229	.78370841	.95117806

Figure 4. 9 Forest plot of sensitivity analysis for the association of milk consumption and nonalcoholic fatty liver disease (NAFLD) risk.

The study name with corresponding risk estimate is the risk estimate that was omitted from the meta-analysis. Abbreviations: M-male, F-female, B-both sexes

Table 4. 4 Meta-regression analysis to control covariates for pooled odds ratio (95% confidence interval) for the association of milk consumption and nonalcoholic fatty liver disease (NAFLD) of the studies included in the meta-analysis

Covariate	Pooled odds ratio (95% CI)	R^2 (%)	I^2 res (%)	*P* –interaction[1]
Sex	0.89 (0.76, 1.10)	11.7	65.7	0.176
Study design	0.95 (0.80, 1.12)	-2.65	62.1	0.413
Risk of Bias	0.71 (0.51, 0.98)	100	14.5	0.824
Exposure comparison[2]	1.16 (0.92, 1.47)	77.9	46.1	0.143

[1] Meta-regression was used to calculate P-interaction

[2] Studies that report a risk estimate calculated based on highest vs. lowest or per 1-serving increase of exposure

The meta-analysis examining the association of yogurt consumption and NAFLD development included four risk estimates extracted from two studies. Yogurt consumption was associated with a 12% lower risk of NAFLD development with no heterogeneity among the studies (OR: 0.88; 95% CI: 0.82, 0.96; I^2 = 0.00 %) (Figure 4.10). There was no significant association between cheese intake and NAFLD development (pooled OR: 1.01; 95% CI: 0.82, 1.25) with moderate heterogeneity (I^2 = 65.5%, P = 0.024) in three studies including five risk estimates (Figure 4.11). The pooled OR for high-fat dairy consumption was 0.38 (95% CI: 0.19, 0.75) with no significant heterogeneity among the studies (I^2 = 00.0%, P = 0.678) (Figure 4.12).

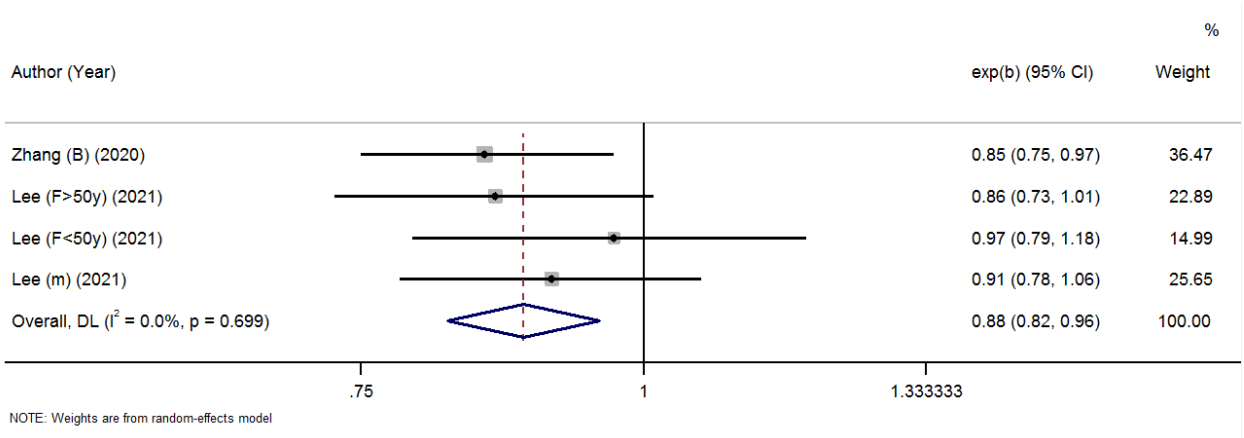


Figure 4. 10 Forest plot of the association of yogurt consumption with risk of nonalcoholic fatty liver disease (NAFLD) using random-effects meta-analysis.

The squares size represent the weights of included estimate. The small diamonds indicate the single risk estimate, and black line segments indicate the 95% CI of each study. The x-axis is the odds

ratio. Heterogeneity between studies is shown by I^2. The hollow diamond shows the pooled odds ratio with 95% CI. M), male; (F), female, (B), both sexes.

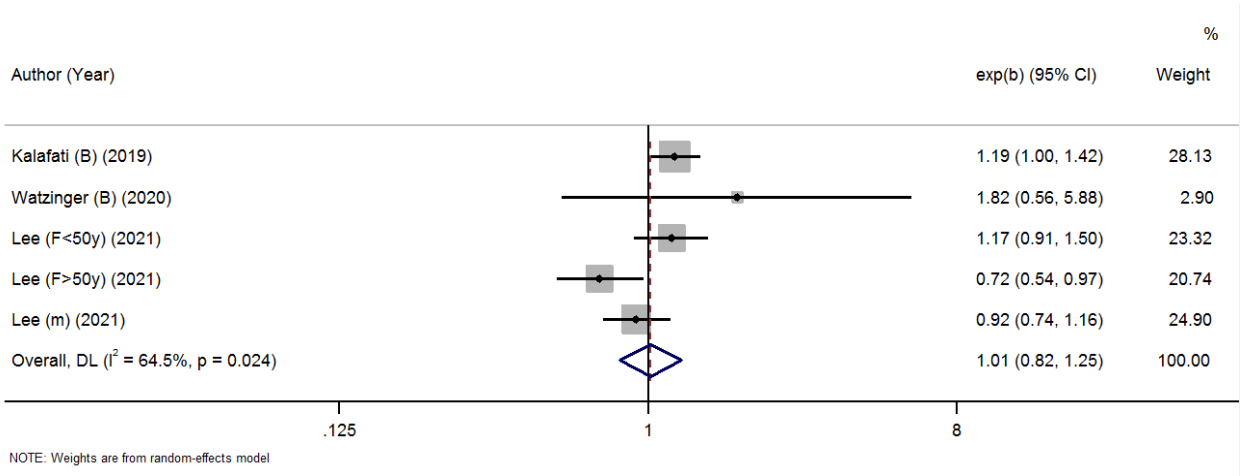


Figure 4. 11 Forest plot of the association of cheese consumption with risk of nonalcoholic fatty liver disease (NAFLD) using random-effects meta-analysis.

The squares size represent the weights of included estimate. The small diamonds indicate the single risk estimate, and black line segments indicate the 95% CI of each study. The x-axis is the odds ratio. Heterogeneity between studies is shown by I^2. The hollow diamond shows the pooled odds ratio with 95% CI. M), male; (F), female, (B), both sexes.

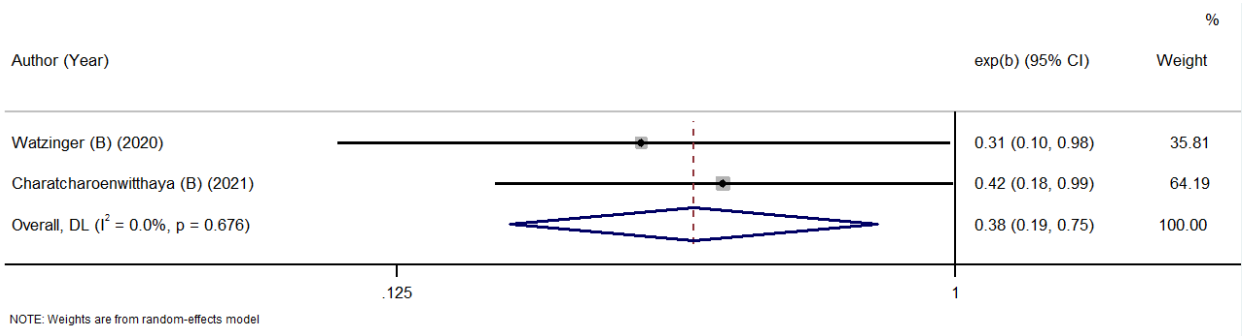


Figure 4. 12 Forest plot of the association of high-fat dairy consumption with risk of nonalcoholic fatty liver disease (NAFLD) using random-effects meta-analysis.

The squares size represent the weights of included estimate. The small diamonds indicate the single risk estimate, and black line segments indicate the 95% CI of each study. The x-axis is the odds ratio. Heterogeneity between studies is shown by I^2. The hollow diamond shows the pooled odds ratio with 95% CI. M), male; (F), female, (B), both sexes.

The assessment of ROB using the NOS is presented in Table 4.5. According to the evaluation, seven studies out of 11 were rated 7-9 points, indicating low ROB, and four studies were rated 0-6 points, indicating high ROB. The quality of the evidence supporting the inverse association of any dairy consumption with NAFLD was graded as low by the NutriGrade scoring system, whereas the quality was classified as very low for the rest of the analysis on dairy subtypes. Due to a lack of robust relationships in the meta-analysis (i.e., effect size item) and a lack of dose-response connections, the quality of evidence for the link between dairy consumption and NAFLD occurrence was low.

Table 4. 5 Newcastle-Ottawa scale score for each included study based on the study design

Author, year	Selection				Comparability[2]		Exposure/Outcome			Total
	Representativeness of sample	Sample size	Non-respondents	Ascertainment of exposure	Control for primary confounders	Control for secondary confounders	Assessment of the outcome	Statistical test		
Cross-sectional										
Van Eekelen, 2019	1	1	0	2	1	1	2	1		8
Zhang, 2020	0	1	1	2	1	1	2	1		9
Watzinger, 2020	1	0	1	2	1	1	0	1		7
Charatcharoenwitthaya, 2021	0	1	0	2	1	1	2	1		7
Chiu, 2018	0	1	0	2	1	1	2	1		8
Chan, 2015	1	1	0	2	1	1	2	1		9
Mansour-Ghanaei, 2019	0	1	0	0	0	0	2	1		4
	Is the case definition adequate?	**Representativeness of the cases**	**Selection of controls**	**Definition of controls**	**Control for primary confounders**	**Control for secondary confounders**	**Ascertainment of exposure**	**Same method of ascertainment for cases and controls**	**Non-response rate**	
Case-control study										
Han, 2014	1	0	0	1	1	1	1	1	0	6

Hao, 2020	1	0	0	1	1	1	1	1	0	6
Kalafati, 2019	1	0	0	1	1	1	1	1	0	6
	Representativeness of the exposed cohort	**Selection of the non-exposed cohort**	**Ascertainment of exposure**	**Outcome of interest present at start of the study**	**Control for primary confounders**	**Control for secondary confounders**	**Assessment of outcome**	**Duration of follow-up**	**Adequacy of follow-up**	
Cohort										
Lee, 2021	1	1	1	1	1	1	1	1	1	9

4.5 DISCUSSION

In the current meta-analysis, we summarize epidemiologic evidence from 11 observational studies with a total sample of 43,649 individuals from across the globe. Pooled risk estimates for any dairy consumption and NAFLD occurrence reveal modest, yet consistent inverse associations. However, there is moderate to low heterogeneity in the observed association. With the exception of the plausible conceivable effect modification caused by exposure comparison (one-serving increase vs. highest-lowest categories) from the original research, we cannot pinpoint a clear source of between-study heterogeneity in this respect. According to sensitivity analyses, no single study significantly impacts the pooled results. There is no evidence of an association between the other exposures assessed in this systematic review and NAFLD risk.

Regarding individual dairy products, we observe that milk and yogurt consumption are associated with a modestly lower occurrence of NAFLD. However, we do not observe an association between cheese consumption and NAFLD. Across the included studies, the overall risk of bias is low. As the most recent and detailed meta-analysis on dairy consumption and NAFLD occurrence, this study provides a comprehensive overview of the possible advantages and lack of evidence of risks associated with dairy intake in the context of NAFLD prevention. However, the number of pooled studies is small and it is important to recognize that this meta-analysis on dairy product consumption and NAFLD relied on observational data of low quality, according to NutriGrade.

Thus far, there is only one other meta-analysis investigating the association of total dairy consumption with NAFLD risk (376). Contrary to our findings, the pooled risk estimate of three cross-sectional studies shows a null association between dairy product consumption and the likelihood of NAFLD (OR = 0.95; 95% CI: 0.82, 1.10) (376). In the present study, higher dairy consumption is associated with a 10% (95% CI: 0.83, 0.98) reduced risk of NAFLD. In the subgroup analysis, we report that pooling risk estimates from the cross-sectional studies (n = 8) yields no significant association between consumption of any dairy products and the risk of NAFLD (OR = 0.92; 95% CI: 0.84, 1.00). Notably, only one of the included case-control studies indicates that dairy product consumption increases the risk of NAFLD (OR = 1.19; 95% CI: 1.00, 1.42) (392); after pooling the OR from four publications, any dairy products consumption has no association with the risk of NAFLD (OR = 1.05; 95% CI: 0.65, 1.69). Having only three studies

included in the abovementioned meta-analysis (376) might explain some discrepancies, as the current work includes more than double the number of studies. Multiple meta-analyses investigating the association of dairy consumption with metabolic health conclude that dairy products generally prevent or lead to improvements in cardio-metabolic health (234, 285, 291, 393, 394). Our results are in line with a recent umbrella review that synthesizing all known meta-analyses on the link between dairy consumption and health outcomes and indicating consistent and plausible evidence of a lower risk of metabolic syndrome, elevated blood pressure, T2D, stroke and CVD for those with higher dairy consumption (393). Furthermore, milk consumption is consistently shown to decrease the risk of metabolic syndrome, elevated blood pressure, and T2D (393).

Whether dairy products are metabolically healthy foods has long been contested (332). This meta-analysis focuses on NAFLD as part of an effort to expand our understanding of the impact of dairy intake on human metabolic health and lends credence to the hypothesis that consuming dairy products reduces the risk of NAFLD. However, dairy products have varying nutritional values and physical states depending on the processing methods each product undergoes (224). Thus, each dairy product may not have the same impact on liver function and structure. We report that milk consumption is associated with a 14% lower risk of NAFLD. However, yogurt consumption has less pronounced effects than milk, whereas no benefit is observed with cheese intake.

Due to the high amount of saturated fat and cholesterol in dairy products, dietary guidelines recommend restricted dairy intake to lower the risk of cardiovascular disease (199) because intake of saturated fatty acids triggers lipotoxic processes that proactively stimulate the activation of NAFLD-related pathways (395). We find no evidence that total or individual dairy products increase the risk of NAFLD; conversely, a higher intake of dairy consumption comprised of low-fat and high-fat dairy foods is associated with a lower risk of NAFLD. This is important to highlight as debate continues about the role of dairy products in diets that emphasize low intakes of red and processed meats and high intakes of minimally processed plant foods (375). The impact of the dairy matrix is one possible explanation for lack of negative or even beneficial relationship between intake of dairy products and risk of NAFLD (205, 224). Indeed, the dairy matrix contributes to disparate effects of eating dairy fat from hard cheese (producing a benefit) compared with butter on plasma cholesterol (225).

Dairy consumption improves the blood lipid profile by lowering triglyceride and total cholesterol, and raising HDL cholesterol, which are strongly linked to fat accumulation in the liver (396). Changes in lipid profiles coupled with increased fecal fat excretion lead to less availability of fat for expansion in the body, which might account for decreased liver fat accumulation (397, 398). Intervention with nonfat dry milk reduces weight gain and adiposity despite equal net digestible energy compared to controls. There is a significant improvement in glucose tolerance, reduced blood insulin and significantly lower liver steatosis (399).

There is evidence that dairy components improve liver function, although the exact mechanisms remain unclear. Dairy proteins, including whey and casein, elicit insulinotropic effects and positively influence lipid metabolism (400, 401). Whey protein induces a potent response by increasing the activity of the incretin hormones gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1) (400). These hormones are induced by amino acids such as valine, leucine, and isoleucine, all of which are branched-chain amino acids (BCAAs), and bioactive peptides that are produced following the digestion of whey protein (401). They may upregulate the activity of the mammalian target of rapamycin (mTOR) pathway and increase the release of β-cell insulin, improving lipid metabolism in the liver (402). Additionally, increased satiety in response to higher whey protein intake is directly linked with reduced energy intake, lower weight gain and decreased fat accumulation in the liver (403).

Strengths and limitations

There are several advantages to this meta-analysis. First, to the best of our knowledge, this is the first meta-analysis of dairy consumption and NAFLD occurrence that comprehensively synthesized all available observational studies, indicating broadly consistent findings despite studies using several analytical methods, populations and dairy subgroups. Furthermore, we did not consider any restrictions on the date of publication, language, or geographical region in the inclusion criteria.

When interpreting our findings, there are some limitations to consider. First, although higher dairy consumption is associated with a lower risk of NAFLD, the strength of this conclusion is limited by the wide range of individuals' racial and socioeconomic backgrounds as well as their eating patterns, as reflected in significant statistical heterogeneity between included studies. Notably, the heterogeneity found between studies was not fully explained after subgroup analyses to consider

study characteristics such as prevalence of NAFLD, sex and method of dairy intake assessment. Second, while the majority of the included studies in the meta-analysis provide risk estimates that were adjusted for the most likely confounders, unmeasured or residual confounding variables cannot be solved completely because it was not adjusted consistently across studies. The association between fatty liver and insulin resistance is widely reported in the literature (404, 405). Only a few of the studies presented evaluate carbohydrate consumption, physical activity, and comorbidities, and all of these factors may interfere with the presented results and conclusions of the present study (406). Furthermore, although age is a co-variable in the regression models, there is a vast disparity in age and male/female proportion among the studies presented in this meta-analysis. Thus, we further performed a stratified analysis to examine the effects of potential confounding factors. Third, the magnitude of the association between dairy consumption and the occurrence of NAFLD is not robust. As a result, more research is needed before conclusive evidence on the relationship between dairy consumption and NAFLD occurrence can be formed. Fourth, most studies assess diet using self-reported questionnaires, mostly FFQs, and a few use interviews to estimate dairy product consumption leading to potential measurement errors and recall bias. Fifth, the findings may be skewed away from the null due to non-differential misclassification of dairy product consumption categories. Various approaches to categorizing dairy products may lead to conflicting results since dietary evaluations across studies are based on different databases and FFQs. Sixth, dose-response analysis is not possible due to variations in exposure estimates. Finally, the low certainty of evidence reduces the certainty of the conclusion. Further well-designed observational studies are warranted as the number of eligible studies investigating the association of dairy products with different fat content and dairy subtypes, especially cheese, in this review is low.

Conclusions

In the present systematic review and meta-analysis of observational studies, we find an inverse association between consumption of dairy products and its subtypes, including milk and yogurt, and NAFLD occurrence. We did not find a significant relationship between cheese consumption and NAFLD development. The results for meta-analyses are mostly consistent across multiple participant and study characteristics. However, the findings of the current meta-analysis should be interpreted with caution due to the low certainty of the evidence observed. Large-scale prospective cohort studies are necessary to confirm the study conclusions further.

Protocol amendment

While our PROSPERO registration indicated that we would assess the certainty of evidence using GRADE, we amended our protocol to instead use NutriGrade due to its ability to evaluate the quality of evidence for nutritional outcomes specifically. This change was made prior to conducting the systematic review and meta-analysis.

5 DIFFERENTIAL EFFECTS OF MILK, YOGURT, AND CHEESE ON ENERGY HOMEOSTASIS AND BROWN ADIPOSE TISSUE PHENOTYPE IN HIGH-FAT DIET-INDUCED OBESE MICE

Differential Effects of Milk, Yogurt, and Cheese on Energy Homeostasis and Brown Adipose Tissue Phenotype in High-Fat Diet-Induced Obese Mice

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5.1 ABSTRACT

Aim: We hypothesized that milk, yogurt, and cheese have differential impacts on energy expenditure (EE) and obesity in mice fed a high-fat diet (HFD).

Methods: C57BL/6 mice (n=16 per group) were fed a HFD or HFD supplemented with fat-free milk (MILK), fat-free plain yogurt (YOG), or reduced-fat cheese (CHE; 19 kcal% fat), each provided at 10% of the daily energy intake, for 8 weeks. EE and respiratory quotient (RQ) were quantified using a metabolic chamber. Metabolic pathways related to BAT mitochondrial function and uncoupling protein 1 (UCP1) abundance were assessed. Serum lipidomics profiles were analyzed to identify potential mediators of the observed effects.

Results: MILK supplementation lowered weight gain and fat accumulation and enhanced EE and BAT thermogenesis, perhaps via the SIRT1-PPARγ-PGC1α axis in BAT. This led to elevated UCP1 abundance and enhanced the abundance of Hormone-sensitive lipase (HSL). MILK also altered serum lipid species, indicating enhanced energy use, and promoted BAT thermogenesis

and mitochondrial function pathways. YOG exhibited a similar pattern but a lower magnitude of effects than MILK on reducing weight gain and fat mass, increasing EE, and BAT thermogenic proteins. Both MILK and YOG showed the relative increased in serum PC 15:0_15:0 and LPC 15:0. In contrast, CHE reduced weight gain and increased EE without impacting BAT thermogenesis proteins or serum lipid species.

Conclusion: Our study shows that MILK, YOG, and CHE reduce weight in mice on a high-fat diet by increasing EE. MILK also up-regulates BAT thermogenesis, while YOG additionally alters lipids involved in fat metabolism and inflammation. CHE does not affect BAT thermogenesis.

Keywords: diabetes, dairy products, thermogenesis, mitochondrial metabolism, brown adipose tissue

5.2 INTRODUCTION

The worldwide prevalence of obesity has increased more than twofold in recent decades, and concurrently, the rates of related noncommunicable diseases, such as type 2 diabetes (T2D), metabolic dysfunction-associated steatotic liver disease (MASLD), and cardiovascular diseases, have risen (407). Obesity is the accumulation of excessive fat leading to impaired health and results from a complex combination of genetic and environmental variables (408). Importantly, in the process of obesity, there is an imbalance in energy homeostasis, in which the intake of excess calories exceeds the expenditure of energy (409). Adherence to the Western diet, which is characterized by higher consumption of ultra-processed products, saturated fats, and simple sugars, is linked to greater energy consumption, explaining weight gain and an increased risk of overweight and obesity (198). While healthy eating remains the cornerstone for the treatment and prevention of obesity and associated diseases (409, 410, 411).

Dairy products are a component of healthy diet patterns identified in dietary guidelines (199). Notably, 3 servings of low-fat dairy items per day are recommended in the 2020–2025 Dietary Guidelines for Americans (412). Several systematic reviews and meta-analyses of observational studies indicate a reduction in T2D incidence associated with dairy product intake, mostly attributable to yogurt and low-fat dairy products, resulting in a resurgence of interest in dairy products for the prevention of T2D (234, 286, 289, 342). Based on these findings, the American Heart Association (413) and the American Diabetes Association (414) now recommend adding 2–3 servings per day of dairy products, including milk, yogurt, and cheese (all low or non-fat), as

part of a balanced diet to reduce the risk of cardiovascular disease and T2D. Regarding weight gain, a recent meta-analysis discovered total dairy, milk, and yogurt intake is linearly associated with a lower risk of overweight or obesity (234). Adding 1 serving of dairy products into a diet improves body composition, alongside a notable 5–10% reduction in fat mass and a corresponding weight loss (415, 416, 417). The favorable association of dairy consumption with the incidence of T2D among individuals with overweight and obesity or prediabetes (302, 418, 419) suggests dairy product consumption alleviates health impairments associated with Westernized diets, such as weight gain and impaired glucose tolerance.

Variations in the manufacturing processes, including microbial fermentation, impact nutrient bioavailability and product functioning, causing disparities in bioactive content and health consequences across dairy products. Animal studies explored the potential anti-obesogenic and anti-diabetic properties of dairy products or dairy-derived components (e.g., whey protein and dairy-enriched fatty acids) to identify the underlying cellular pathways and metabolic processes in response to dairy consumption. These studies are particularly relevant when a Western-style background diet is utilized (273). Rodent studies provide evidence that dairy component feeding yields beneficial metabolic effects such as reduced fat mass, improved nutrient metabolism and mitochondrial function, enhanced thermogenesis, and reduced inflammation, thus mitigating the negative effects of high-fat diets (253, 266, 420, 421).

In this context, current literature provides limited information on the distinct influence of commercially available low- or non-fat dairy products, such as fat-free milk, fat-free yogurt, and reduced-fat cheese, on energy homeostasis and obesity-associated metabolic complications. This underlines the need for systematic investigations into the individual roles of these dairy products. Therefore, this study aimed to address this gap by investigating the effects of fat-free milk, fat-free yogurt, and reduced-fat cheese on energy homeostasis in high-fat diet-induced obese mice. We thus hypothesized that, due to fermentation and probiotic effects, yogurt and cheese would have a more significant mitigating effect than milk on energy balance and metabolic health in obese mice.

5.3 METHODS

Six-week-old C57BL/6 mice were purchased from Charles River Canada (St. Constant, QC, Canada) and acclimated to the animal facility with free access to water and a standard laboratory diet. Mice were housed 4 per cage in a controlled environment with a temperature of 23 ± 1 °C

and humidity of 40–60% on a reverse 12-hour light/dark cycle. After 7 days, mice were randomly divided into 2 groups and then given free access to either a low-fat diet (LFD, n = 16; 10% of calories derived from fat; 3.82 kcal/gm; Research Diets, New Brunswick, NJ, D12450H) or a high-fat diet (HFD, n = 64; 45% of calories derived from fat, 4.73 kcal/gm; Research Diets, D12451), with protein accounting for 20% of the total energy of both diets.

The animal experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta (AUP00003066) following guidelines issued by the Canadian Council on Animal Care. Reporting followed the ARRIVE guidelines.

Sample size calculation

According to our pilot study (4 mice/group), investigating the effect of milk consumption on body weight (BW), we estimated that the standard deviations (S1 and S2) of BW in the HFD control and milk-feed mice were 1.11 and 2.05, respectively, considering an effect size of 7%. We set a two-sided α of 0.05, a power of 80%, and calculated a sample size of 8 mice per group according to the following formula:

$$n = \frac{2(z_{\alpha/2} + z_{\beta})^2 \sigma^2}{\delta^2}$$

Diets and study plan

The amount of each dairy product provided was representative of 2 serving equivalents per day recommended for human adults by the Dietary Guidelines for Americans (412), the American Heart Association (AHA) (413), and the American Diabetes Association (ADA) (414). These guidelines provide daily serving size recommendations for dairy products based on an individual's calorie intake of 2000 kcal. Assuming that all dairy consumption comes from low/non-fat items, dairy products would supply almost 10% of total energy. Given that the energy requirement of mice is approximately 12 kcal/d, we calculated the amount of fat-free milk, fat-free yogurt, and reduced-fat cheese necessary to provide 10% of total energy intake in mice. Then, on 5 of 7 days of the week, each mouse in the treatment groups received 2 serving equivalents per day of one of the dairy products.

Mice on the LFD served as a healthy reference group and remained on LFD throughout the entire experiment, whereas HFD mice after 1 week were randomly divided into a main comparison

negative control group (n = 16; HFD) and 3 treatment groups (n = 16 per group) which was ad libitum access to HFD supplemented with 1 of 3 dairy products, either 3.0 ml of fat-free milk (MILK; Dairyland, Saputo Dairy Products, Canada), 2.1 ml of fat-free plain yogurt (YOG; ASTRO® Original Balkan Plain, Lactalis Group, Canada), and 360 mg of reduced-fat (19% fat) cheddar cheese (CHE; Armstrong Old Light Cheddar Cheese, Saputo Dairy Products, Canada). None of the products contained added sugar. Due to the inherent differences in the appearance of milk, yogurt, and cheese, it was not possible to blind the providers to the dietary intervention.

Feeding the dairy foods was accomplished by individually placing each mouse into a 4-chamber cage with access to the food for 2 h each day at the beginning of the dark cycle, when they were most active and likely to eat, after which they were returned to their original cage. The HFD and LFD control groups were provided with half a plain Cheerio to control for handling. All mice had ad libitum access to food pellets and water in their home cages throughout the whole experiment. Food intake was meticulously tracked once a week throughout the study. The estimated energy intake was calculated by multiplying the number of grams eaten each week by the energy density of the diet.

The mice were euthanized using CO_2 after an 8-week intervention. Tissues were weighed and harvested for further analysis, and serum was isolated from blood collected from the caudal vena cava. For each diet, cages were randomly assigned to either a fasted or fasted-refed situation. In the fasting state, tissues and serum from n = 8 mice in each study group were collected between 9:30 AM and 12:30 PM after 12 h of fasting. The fasted-refed state was induced by fasting the mice for 12 hours, followed by a 4-hour exposure to their respective diets, and then euthanasia between 1 PM and 4 PM in a refed state. All the samples from the cohort were collected at the same time of day to reduce variability.

Body weight and body composition measurement

Body weight (BW) was measured once weekly. The fat and lean mass of conscious, live mice was measured using an Echo Medical Systems' Echo-MRI™ Whole Body Composition Analyzer EF-014 (EchoMRI; Echo Medical Systems LLC, Houston, TX, USA). The mice were restrained and the total fat and lean mass were recorded within one minute. Triplicate measurements were taken and the average was considered for each mouse's fat and lean mass.

Metabolic rate and physical activity

After 7 weeks of dairy intervention, metabolic chambers (Oxymax (CLAMS); Columbus Instruments, Columbus, OH) were used to measure the feeding pattern, spontaneous activity, total energy expenditure (EE), respiratory exchange ratio (from VCO_2 and VO_2), and nutrient oxidation of individually-housed mice during 24 h ad libitum feeding. Randomly, 8 mice were selected from each study group and placed in the chambers for a 24 h acclimation period. LFD mice were provided their usual low-fat diet whereas all mice in the HFD ± dairy intervention were provided the high-fat diet (without any dairy). The temperature in the metabolic cages was maintained at 25-26 °C. The respiratory exchange ratio (RER) was calculated by dividing VCO_2 by VO_2, with 100% carbohydrate oxidation resulting in a value of 1 and 100% fat oxidation resulting in a value of 0.7. Energy expenditure (EE) was calculated by the formula:

$EE = VO_2 \times [3.815 + (1.232 \times RER)]$.

Carbohydrate and lipid oxidation rates were calculated using the stoichiometric equations of Frayn (422), with the assumption that the urinary nitrogen excretion rate was negligible.

Rate of glucose oxidation (g/min)=$4.55VCO_2$ (l/min) - $3.21VO_2$ (l/min)

Rate of lipid oxidation (g/min)=1.67 (VO_2 (l/min) -VCO_2 (l/min))

Total activity was determined by combining the number of infrared beam breaks along the X- and Y-axes, and ambulatory activity was calculated by combining consecutive X- and Y-axes beam breaks occurring in a single series. Linked load cells were used to determine food intake from suspended feeder baskets. Total BW was included as a covariate and adjusted in the models used to analyze the data obtained from the CLAMS experiments.

Tissue sample collection

After euthanizing the animals with CO_2 at the indicated time points, the entire liver, epididymal white adipose tissue and interscapular brown adipose tissue (BAT) were dissected out and weighed. Tissues were snap-frozen in liquid nitrogen and subsequently stored at −80 °C. Collected blood was centrifuged at 5,000g for 30 min at 4 °C. Serum was collected and frozen at −80 °C.

Western blotting for protein abundance measurement

BAT tissue lysates were prepared following the Removal of Excess Lipids (RELi) protocol, as described by Marin et al. (2019). Briefly, approximately 50 mg of frozen BAT was placed in a tube with RIPA buffer (50 mmol/L Tris HCL pH:8.0, 150 mmol/L NaCl, 0.1 % Triton X-100, 0.5

% sodium deoxycholate, 0.1 % SDS) and protease plus phosphatase inhibitors (2 µg/mL aprotinin (Calbiochem), 5 mmol/L sodium fluoride, 5 mmol/L sodium orthovanadate, and protease inhibitor cocktail (FastPrep®-24, MP Biomedicals)). and metal beads. Using a tissue lyser, samples were disrupted at 40 Hz for 30 seconds, then cooled. This process was repeated 3 times, and then samples were left on ice for 1 h. Subsequently, the samples were centrifuged at 4°C for 15 min at 15,000 × g to separate the supernatant from the lipid layer. This step was repeated 3 times for efficient lipid clearance. Prior to storage at −80°C, protein quantification of the samples was conducted using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA, 23225). Proteins were diluted with Laemmli protein sample buffer (4X; Bio-Rad, 1610747) for SDS-PAGE and boiled for 5 min in the presence of 2-mercaptoethanol. Total protein (30 µg) was loaded and electrophoresed in a 15-well 4–12% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). Proteins were then transferred to a nitrocellulose membrane. Membranes were stained with Ponceau S stain for 5 min and imaged using a ChemiDoc imager (Bio-Rad). Membranes were then de-stained using phosphate-buffered saline (PBS)-Tween (PBS with 0.05% Tween-20) buffer for 5 min, repeated 4 times. For blocking, a 5% bovine serum albumin (BSA) solution in PBS-Tween was used at room temperature for 2 h. Primary antibody incubation at a 1:1,000 or 1:500 dilution was conducted at 4 °C overnight. Antibodies for total OXPHOS Cocktail (Abcam, ab110411), UCP1 (Sigma, U6382), CPT1α (Cell Signaling, 97361), PPARγ (Santa Cruz, sc-7273), CREB-1 (Santa Cruz, sc-377154), PGC-1α (Invitrogen, PA5-72948), ATGL (Cell Signaling, 2439), HSL (Cell Signaling, 4107), AMPK β (Cell Signaling, 23021), PPARα (Santa Cruz, sc-398394), FGF21 (Santa Cruz, sc-81946), and Sirt1 (MilliporeSigma, 07-131) were used. After incubation with primary antibodies, membranes were washed 3 times for 10 min in PBS-Tween. Incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (Sigma-Aldrich) at a 1:5,000 dilution occurred at room temperature for 1 h. Blots were developed using enhanced chemiluminescent (ECL) detection reagents (SuperSignal West Pico PLUS, Thermo Scientific, 34580) and imaged in the ChemiDoc (Bio-Rad) imager. Protein abundance was quantified using Image Lab (Bio-Rad) software.

Mass spectrometry analysis of lipid metabolites

For each treatment group of 8 mice, serum samples from 2 mice were randomly pooled together, resulting in 4 distinct samples per group that were representative of the entire group. These pooled samples were then subjected to lipidomic analysis by The Metabolomics Innovation Centre (TMIC, University of Alberta, Canada) using an untargeted LC-MS approach based on the In-depth Global Lipidomics method (423, 424, 425). Lipid extraction was performed using a modified Folch liquid-liquid extraction protocol. The serum sample was mixed with internal standard solution (NovaMT LipidRep (Nova Medical Testing Inc., Edmonton, AB, Canada), a mixture composed of 15 deuterated lipid standards belonging to different lipid classes). Lipids were then extracted with dichloromethane and methanol, and a clean-up step was performed with water. Samples were equilibrated at room temperature for 10 min and centrifuged at 16,000 g for 10 min at 4°C. The organic layer was evaporated through the smooth flow of nitrogen. The dried residues were reconstituted in NovaMT MixB and diluted with NovaMT MixA, and injected into a ThermoVanquish UHPLC (ThermoFisher Scientific, Edmonton, Canada) coupled to a Bruker Impact II QTOF Mass Spectrometer (Bruker Corporation, Billerica, MA, USA). The analyses were performed in both positive and negative ionization modes with a Waters Acquity CSH C18 column (1.7 µm particles). A pooled mixture of all samples was used as a quality control (QC) sample and injected before and after each experimental sample to ensure technical stability. A total of 16 experimental samples and 13 QC samples were analyzed in each ionization mode. The LC-MS data were processed independently for each ionization mode using NovaMT LipidScreener 4.0, which aligned the chromatograms and combined them into a single feature-intensity table.

A 3-tier approach was utilized to identify the detected peaks based on MS/MS spectral similarity. Tier 1 features had a high confidence identification with a MS/MS match score ≥500, a precursor m/z error ≤ 5 mDa, and an isotope pattern match (mSigma) score ≤ 50. Tier 2 features had a moderate confidence identification with a MS/MS match score between 100 and 500. Tier 3 features had a low confidence identification with a mass match m/z error ≤5 mDa. The MS/MS match score was calculated by comparing the acquired spectrum with the spectra available in the databases in terms of m/z values and relative intensities of precursor and fragment ions. The identified lipid species were normalized by the most similar internal standard and the median intensity ratio. The data were then imported into MetaboAnalyst 5.0 (426) for statistical analysis.

Data processing and analysis of lipid metabolites

Features that were not detected in ≥80% of injections in at least 1 sample group or QCs were filtered out. Missing values were imputed using the following criteria: (1) the median intensity of the sample group for features detected in at least 75% of injections within the group (MILK, YOG, CHE, HFD, or QCs); (2) the minimum intensity within the group for features detected in at least 50% of injections; or (3) the global minimum for all sample and QC injections for features detected in less than 50% of injections within the group. The identified lipid species were normalized by the most similar internal standard and the median intensity ratio. Non-informative features (e.g., internal standards and common contaminants) were also removed during data processing. The analysis process was sequentially performed, from alignment, peak picking then identification of lipids. Metabolites were identified by referring to the Lipid Maps Database (www.lipidmaps.org) and the Human Metabolome Database (www.hmdb.ca). For multivariate statistics, features with low reproducibility in QC experiments (determined by high interquartile range to median ratio (IQR/median) for QCs) and features with near-constant values between the groups (determined by low IQR/median for all samples) were removed after uploading the dataset to MetaboAnalyst. The dataset was also auto-scaled. No other filtering, normalization, transformation, or scaling methods were applied before multivariate statistical analysis. The data were analyzed by unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA) to estimate the significant differences and the predictive ability of lipidomics profiles between different groups. The lipid species were further analyzed by supervised multivariate models using sparse partial least squares discriminant analysis (sPLS-DA) to better discriminate the 4 groups by reducing the number of variables in the data and producing a robust and easy-to-interpret model. Lipid molecules whose concentration changes contributed most to the separation of the different experimental groups were identified and their significance was determined by analysis of variance (ANOVA). Heat map graphical representations of differences between the different treatment groups were generated. Volcano plots were used to visually examine the difference in lipid species in each dairy group compared to HFD. Lipid species with significant concentration differences (P values <0.05) between each dairy product treatment compared with HFD groups were selected for functional enrichment analysis of the lipidomics data using the Lipid Ontology (LION) enrichment analysis web application (LION/web, www.lipidontology.com).

Statistical analysis

Data are presented as the mean value ± standard error of the mean (SEM) unless indicated otherwise, and a significance level of 0.05 (alpha) was considered the threshold for statistical significance. Depending on the parameter, GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), R (4.3.3, https://www.r-project.org/), or SPSS 17 (IBM Corp., Armonk, N.Y., USA) were used for statistical analyses.

The normality of the data was assessed using the Shapiro-Wilk test, descriptive statistics, and the values of skewness and kurtosis. Except if stated otherwise, the statistical strategy was to first identify differences in the parameters of diet-induced obesity between the low-fat diet (LFD) group and the high-fat diet (HFD) group using an independent, 2-tailed t-test. The LFD group served as a reference group to show the development of obesity in the HFD group after 8 weeks of intervention. For statistical analysis involving multiple groups, a two-way ANOVA followed by Tukey's post hoc test was performed to assess the effect of the milk, yogurt, and cheese treatment impact on the HFD effect, and to test if there was a significant difference between treatment groups.

To address the variability in animal weights and facilitate a more accurate comparison of EE across groups, a linear regression analysis was employed. For this analysis, the treatment group was considered a fixed factor and animal weight was incorporated as a covariate. The model was adjusted for the influence of varying weights on EE measurements. The differences in the weight-adjusted EE among the groups were evaluated using a one-way ANOVA followed by Tukey's post hoc test.

5.4 RESULTS

Dairy products decrease body weight gain and attenuate fat mass expansion.

First, we evaluated the effect of dairy intake on the development of obesity. At the end of the study, mice fed the HFD diet had increased BW, with an increased percent fat mass compared to those fed the LFD (Figure S1A-D). Every day, MILK (3 ml), YOG (2.1 ml), and CHE (0.36 g) were added to the HFD to provide two servings per day, which is equivalent to 10% of the total daily energy intake. To estimate the nutrients digested by each mouse in every experimental group, the average pellet intake for each mouse per day was calculated and combined with the daily desired amount of MILK, YOG, and CHE provided (Table 1).

The BW in HFD mice and MILK, YOG, and CHE groups had similar BW at weeks -1 and 0 before adding dairy products to their diet (Fig. 5.2A). Interestingly, the mice fed HFD supplemented with MILK, YOG, or CHE had attenuated BW gain (-25, -22, and -14% compared with HFD at week 8, respectively; Fig. 5.2A, B), largely due to a reduced fat mass accumulation (-32, -31, and -21% at week 8, respectively; Fig. 5.2C). Consequently, the percentage of lean mass was higher for MILK and YOG (Fig. 1D). The reduction in fat mass was accompanied by smaller epididymal white adipose tissue (eWAT) in the MILK and YOG compared with mice in HFD (Fig. 5.2E). Mice consuming the HFD and MILK, YOG, and CHE ate similar pelleted food (gram per day) during the study (Fig. 5.2F, G). The small decrement in energy from HFD pellets seen in the dairy-fed groups was compensated for calories from the dairy products (Fig. 5.2H). Thus, the differences in BW and fat mass gain are likely not due to altered energy intake, as these showed no significant differences between the groups.

Table 5. 1 Nutritional composition and intake of high-fat diets with dairy product supplementation

	HFD	Interventions		
		HFD+Milk	HFD+Yogurt	HFD+Cheese
Dairy (/d)	-	3 ml	2.1 ml	0.36 g
Pelleted chow (g/d)	2.49	2.19	2.26	2.21
Macronutrient intake*				
Protein (g)	0.60	0.63	0.64	0.64
Carbohydrate (g)	1.02	1.05	1.06	0.93
Fat (g)	0.60	0.52	0.54	0.59
Saturated fatty acid (g)	0.2	0.2	0.2	0.2
Monounsaturated fatty acid (g)	0.2	0.2	0.2	0.2
Polyunsaturated fatty acid (g)	0.1	0.1	0.1	0.1
Energy (kcal)	11.8	11.4	11.8	11.5
Ingredients*				
Casein (g)	0.6	0.6	0.6	0.6
Whey (g)	-	0.03	0.02	0.00
L-Cystine (mg)	8.7	8.3	9.6	7.8
Sucrose (g)	0.5	0.5	0.5	0.5
Maltodextrin (g)	0.3	0.3	0.3	0.3
Corn Starch (g)	0.2	0.2	0.2	0.2
Lactose (g)	-	0.2	0.1	0.0
Fiber (g)	0.1	0.1	0.1	0.1
Lard (g)	0.5	0.5	0.5	0.5
Soybean Oil (g)	0.1	0.1	0.1	0.1
Calcium (mg)	53.5	50.7	51.7	50.8
Sodium (mg)	7.5	6.7	7.9	9.2
Potassium (mg)	47.8	46.7	47.5	42.8
Choline (mg)	5.7	5.6	5.6	5.2

*Each week of the experiment, the pellet intake (g) per cage (4 mice per cage) was measured, and then the average macronutrient consumption for each mouse for a day was estimated. The nutrient values represent the combined contributions from the high-fat diet (HFD) pellet diet and the dairy products provided as interventions. Similarly, the intake of ingredients from the pelleted high-fat diet and dairy products was estimated per mouse per day.

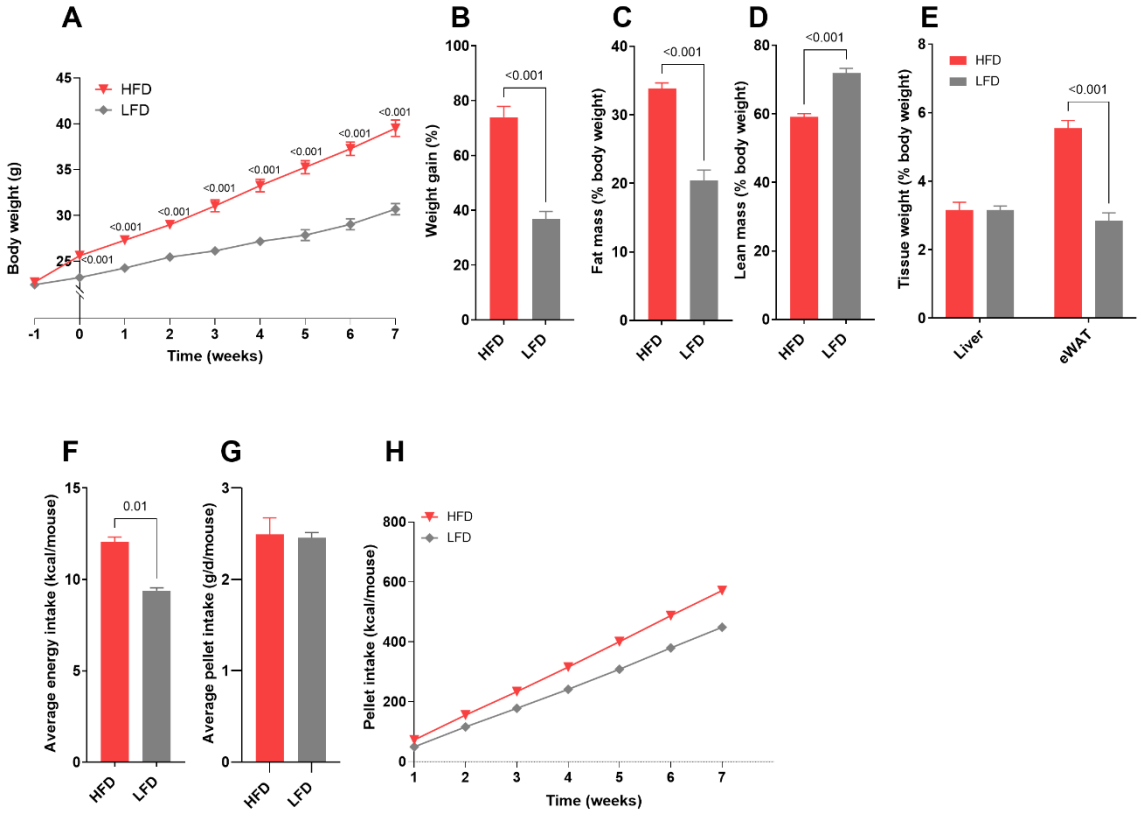


Figure 5. 1 Body weight, body composition, and food intake of mice fed HFD vs LFD for 8 weeks (n=16 per group)

(A) Body weight; (B) Weight gain as a percentage from baseline after 8 weeks; (C,D) Body fat mass and lean mass were assessed using EchoMRI technology and presented as a percentage of body weight; (E) Liver and epididymal white adipose tissues (eWAT) were harvested and weighed during necropsies; (F,G) Average energy consumption and daily intake of food pellets per mouse estimated from group-housed mice (4 mice/cage); (H) Cumulative food intake of mouse during 8 weeks of intervention. Data are expressed as the mean±SEM. Statistical analyses performed with (A, H) two-way repeated measures analysis of variance (ANOVA) with Tukey's post hoc test and (B-G) unpaired Student's t-test.

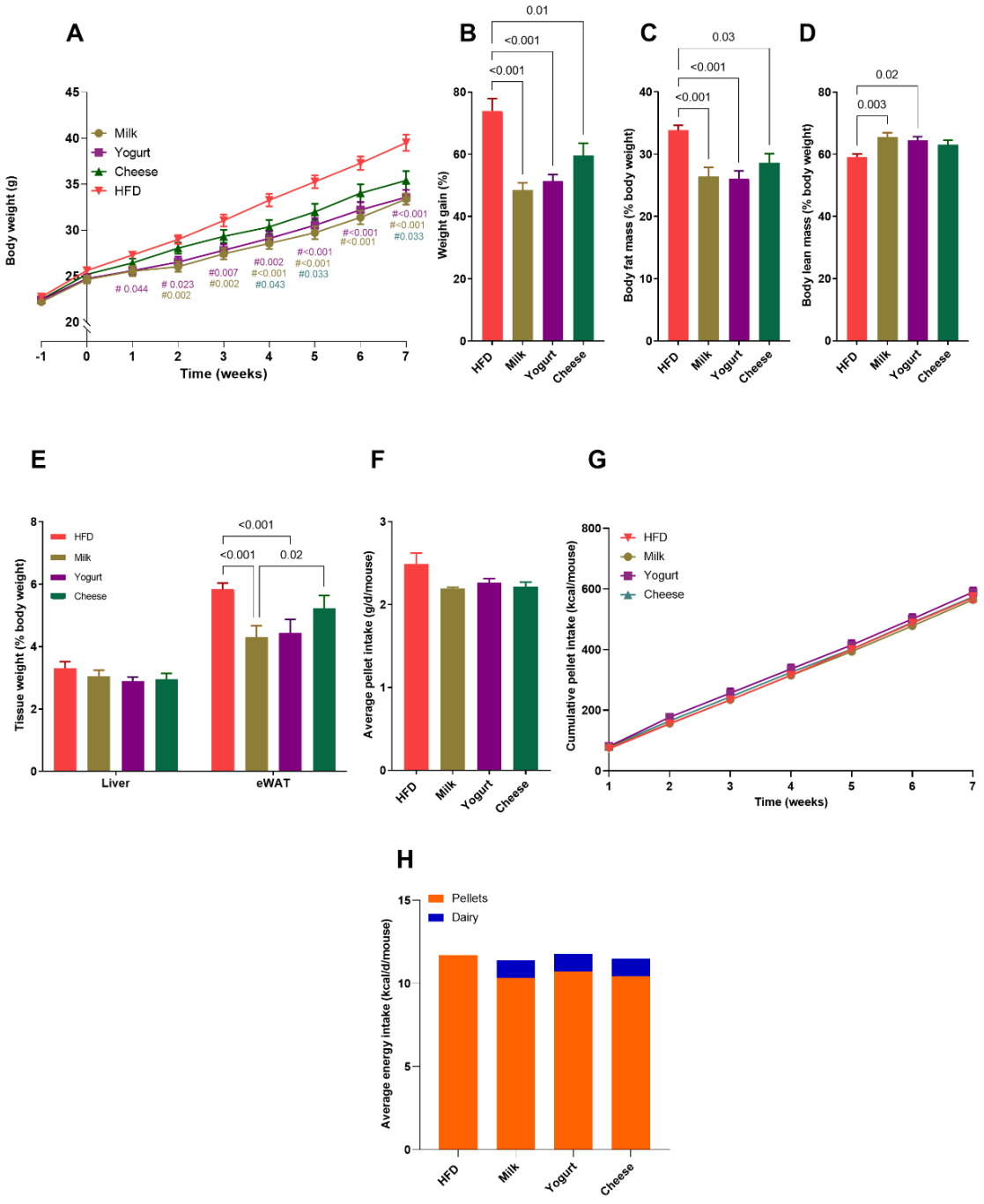


Figure 5. 2 Body weight, body composition, and average food intake of mice fed HFD with or without dairy products for 8 weeks (n=16 per group).

(A) Weekly body weight; (B) Weight gain as a percentage of baseline body weight; Body composition showing (C) Percentage of fat mass and (D) Percentage of lean mass; (E) Weight of liver and epididymal white adipose tissue (eWAT); (F) Average daily intake of food pellets for each mouse estimated from group-housed mice (4 mice/cage); (G) Cumulative food intake of mouse during 8 weeks of intervention; (H) Average energy intake per mouse from food pellets ±

dairy products. Data are expressed as mean±SEM. Statistical analyses in (**A, G)** were performed with two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical analyses in (**B, C, D, E, and F)** were performed with one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test.

Adding dairy products to HFD promoted energy metabolism.

To understand the net energy deficit and consequent reduction in body mass, despite similar energy intake, we measured energy expenditure by indirect calorimetry after 7 weeks of intervention. We also monitored activity and measured energy intake. Compared with LFD, HFD mice had significantly suppressed EE, VO_2, and RER (Fig. 5.3A-F), predominantly in the dark phase, as well as higher fat oxidation and lower carbohydrate utilization (Fig. 5.3G-J).

Mice in the MILK, YOG and CHE groups showed an overall 11.9%, 9.2%, and 10.3% increment in EE, respectively, compared with HFD mice despite not being provided with dairy products while in the CLAMS apparatus (Fig. 5.4A-C). This effect was more marked during the dark (active) hours than during the light hours and while food intake was greatest (Fig. 5.4C, D). In linear regression analyses, we observed a strong correlation between BW and EE in HFD and CHE groups. This was an expected outcome. However, in MILK and YOG, there was no correlation between BW and EE indicating that EE in these groups of mice was independent of their BW (Fig. 5.4B). Mice in the dairy groups produced more CO_2 than the HFD controls (Fig. 5.4E, F). The RER, which reflects substrate use, was increased in MILK, suggesting increased carbohydrate utilization (Fig. 5.4G). When analyzing light and dark hours separately, the RER in the MILK consistently remained high; however, in the light phase, the YOG also exhibited significantly higher RER compared with HFD controls (Fig. 5.4H, I). This suggests that under HFD conditions, MILK and YOG promote the utilization of glucose rather than lipids. To establish whether changes in substrate oxidation might contribute to the dairy-induced decrease in adiposity, we calculated the rates of both lipid and carbohydrate oxidation (Fig. 5.4J), observing that MILK had greater magnitude increases in carbohydrate oxidation than YOG and CHE. Changes in physical activity did not explain the increase in EE observed in the dairy-fed groups, as locomotor activity was similar in all groups of intervention (Fig. 5.4K). The energy intake of the HFD controls and the dairy-treated mice was similar (Fig. 5.4L). Notably, pelleted HFD intake as measured by CLAMS over 24-h showed a strong correlation with manual food intake measurements taken weekly throughout the study ($r = 0.81$, $P<0.001$). The results of energy balance experiments indicate that adding dairy to a HFD reduces obesity by increasing energy expenditure (MILK = YOG > CHE).

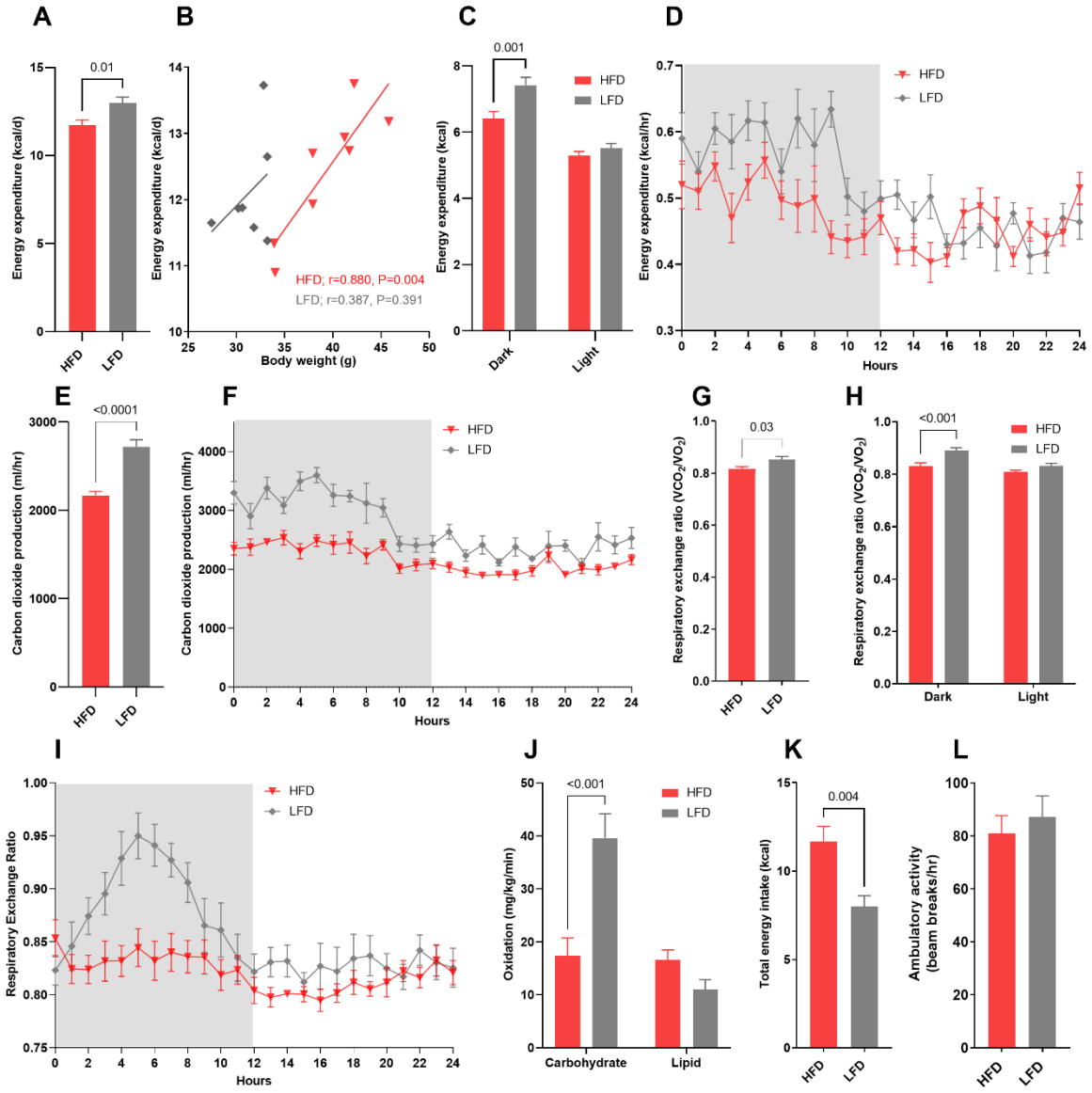


Figure 5. 3 Energy expenditure, oxygen consumption, and substrate utilization of mice following HFD vs LFD feeding for 8 weeks (n = 8 per group).

(A) Body weight-adjusted total energy expenditure (EE) over a 24-h period; (B) Energy expenditure vs body weight plotted as a linear regression; (C) Body weight-adjusted EE during light and dark periods; (D) Average hourly body weight-adjusted EE during 12-h dark (grey)/12-h light (white) periods; (E) Whole-body carbon dioxide production (VCO_2) monitored continuously over a 24-h period; (F) Average hourly VCO_2 during 12-h dark (grey)/12-h light (white) periods); (G) Respiratory exchange ratio (RER) over a 24-h period; (H), Average RER during dark and light periods; (I) Average hourly RER during 12-h dark (grey)/12-h light (white) periods; (J), Average lipid and carbohydrate oxidation rate; (K) Caloric intake from food pellets over 24h; (L) Total ambulatory activity over a 24-h period. Results are shown as mean ± SEM. Statistical analyses in (A, C, E, G, H, J, K, and L) were performed with a two-tailed, unpaired

Student's t-test. Statistical analyses in (B) were performed using the Pearson correlation coefficient.

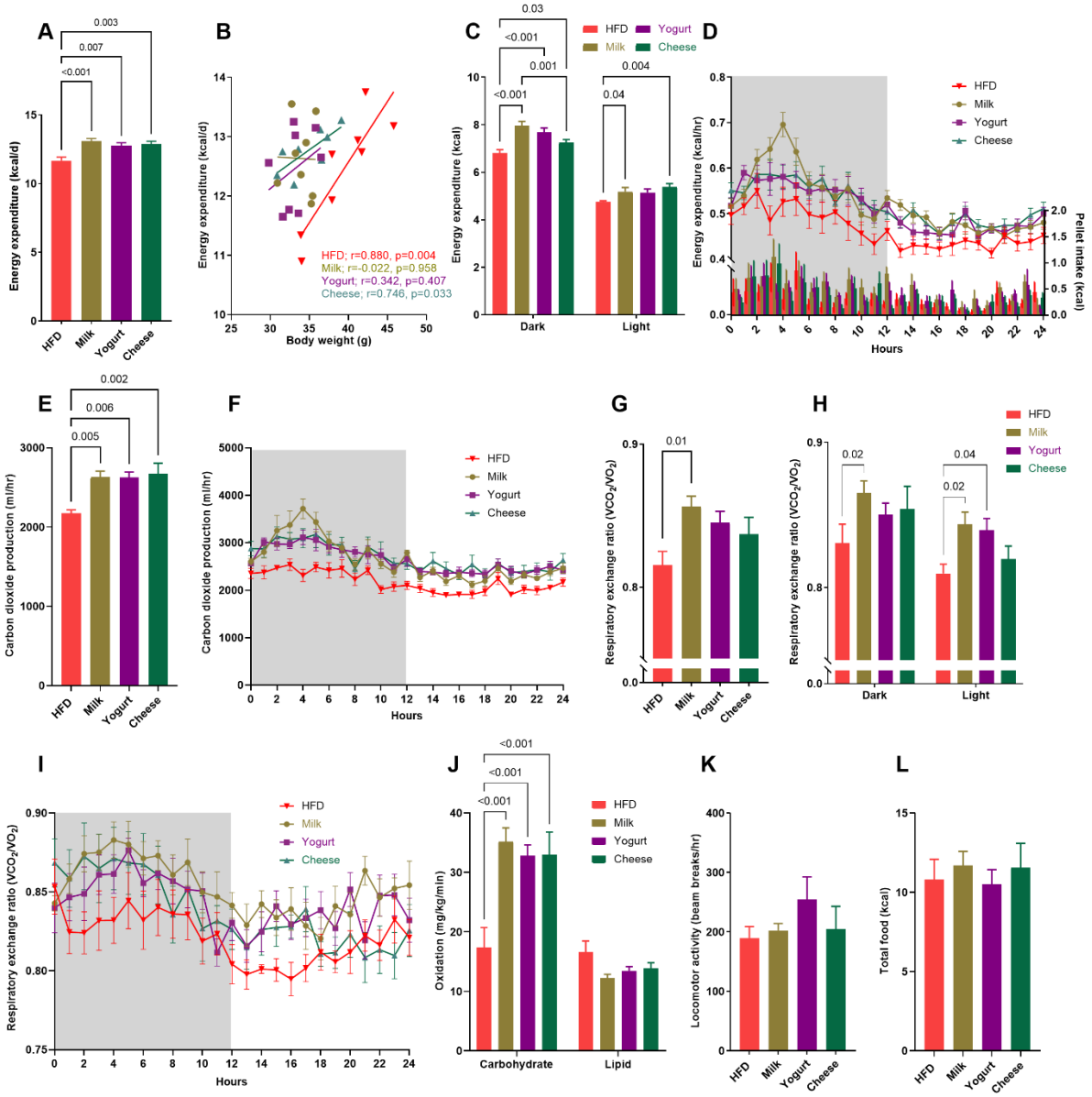


Figure 5. 4 Energy expenditure, oxygen consumption, and substrate utilization of mice fed HFD with or without dairy products for 8 weeks (n=8 per group).

(A) Body weight-adjusted total energy expenditure (EE) over an 24-h period; (B) Energy expenditure versus body weight in each group plotted as a linear regression; (C) Body weight-adjusted EE during dark and light periods; (D) Average hourly body weight-adjusted EE and HFD pellet consumption during 12-h dark (grey)/12-h light (white) periods; (E) Whole-body carbon dioxide production (VCO_2) monitored continuously over a 24-h period; (F) Average hourly VCO2 during 12-h dark (grey)/12-h light (white) periods); (G) Respiratory exchange ratio (RER) over 24 h; (H), Average RER during light and dark periods; (I) Average hourly RER during 12-h dark (grey)/12-h light (white) periods; (J) Average lipid and carbohydrate oxidation rate; (K) Total locomotor activity over a 24-h period (L); Caloric intake from food pellets over a 24-h period.

Results are shown as mean ± SEM. Statistical analyses (in A, C, E, F, H, and J) were performed with one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Statistical analyses (in B) were performed using the Pearson correlation coefficient.

Adding dairy products to HFD upregulates proteins involved in BAT activaty.

The lower weight gain and fat mass and the increased energy expenditure in the treatment group, even in the absence of dairy consumption for 2 days, suggest a persistent increase in diet-induced adaptive thermogenesis in dairy-fed mice, which may be partly explained by the enhanced thermogenic activity of BAT. To test this hypothesis, we measured the abundance of thermogenesis-related proteins in BAT and found that UCP1 protein was consistently higher in both fasted and refed HFD than LFD mice (Fig. 5.5). MILK further increased UCP1 protein in BAT (Fig. 5.6A), in both the fasting and refed states compared with mice fed with a HFD. Furthermore, significant enhancement of UCP1 was observed in YOG compared with HFD mice in the refed state (Fig. 5.6A). To assess the impact of the interventions on mitochondrial oxidative capacity, we examined the proteins of the respiratory chain (OXPHOS) complexes in BAT. Overall, there were no notable differences in OXPHOS subunit content between the HFD and LFD groups (Fig. 5.6B). Additionally, the general protein abundance pattern of OXPHOS complexes was similar between the dairy groups and HFD controls; however, MILK exhibited a notable increase in Complex II (Fig. 5.6B), which plays a crucial role in the final step of electron transfer in the respiratory chain.

Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α, peroxisome proliferator-activated receptor gamma (PPARγ), sirtuin 1 (SIRT1), cAMP response element-binding protein (CREB) and AMP-activated protein kinase (AMPK) are key regulatory factors involved in BAT function. PGC1α and PPARγ in refed HFD mice were lower compared with the LFD group (Fig 5.5C,D), but significantly higher in the MILK group in comparison to the HFD group in both fasted state and refed states, respectively (Fig. 5.6C,D). PGC1α was also higher in YOG in the fasted state compared to HFD (Fig 5.6C). Furthermore, the abundance of SIRT1 in the refed state was decreased in HFD mice compared to LFD mice (Fig. 5.5E); however, the addition of MILK to the HFD diet restored SIRT1 levels, surpassing those in the HFD group (Fig. 5.6E). No significant differences were observed between the treatment groups and the HFD group regarding CREB (Fig. 5.6D). We observed a notable reduction in AMPKβ in the HFD group compared to the LFD group in the fasted state (Fig 5.5G). Mice receiving HFD supplemented with MILK or YOG exhibited a noteworthy increase in AMPKβ, compensating for the reduction observed in the HFD group (Fig 5.6G). We measured CPT1α, involved in fatty acid oxidation, to investigate the regulation of substrate utilization in BAT but it was not significantly affected by

the interventions or the HFD diet (Fig. 5.6H). MILK significantly increased total HSL in BAT in the fasting state (Fig 5.6I). HSL contributes to hydrolysis of triacylglycerols and may indicate increased capacity for fat mobilization. Another lipolysis enzyme, ATGL showed an increasing trend in the MILK ($p=0.09$) and YOG ($p=0.12$) groups during fasting (Fig 5.6J). Among the dairy products tested, MILK had the most beneficial effects on the thermogenic and mitochondrial oxidative capacity of BAT, as evidenced by the increased levels of UCP1, AMPK, PPARγ, SIRT1, and Complex II. YOG consumption increased UCP1, AMPK, and PGC1α but CHE was less effective in changing important BAT proteins.

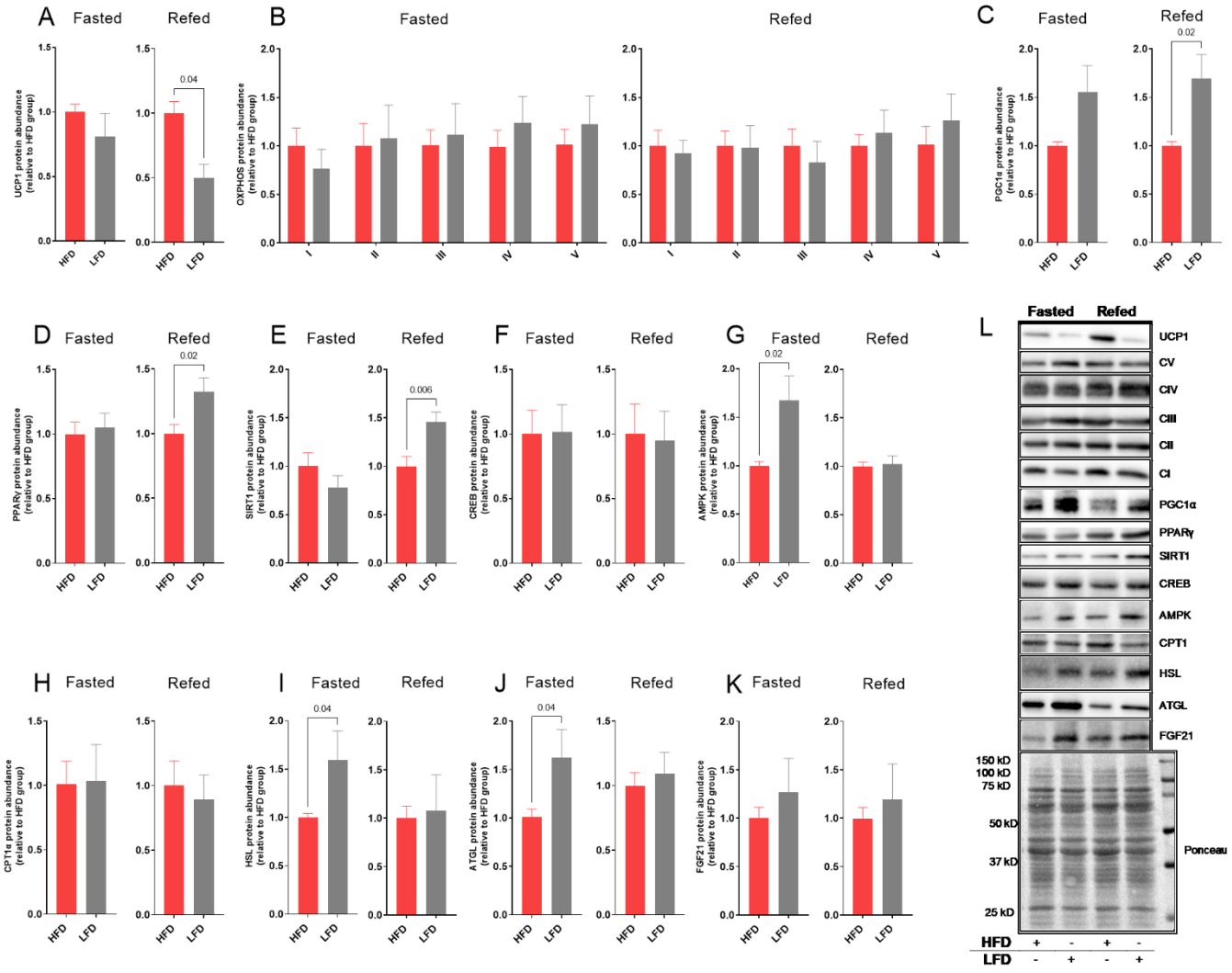


Figure 5. 5 The relative abundance of proteins associated with adaptive thermogenesis in brown adipose tissue of mice fed HFD and LFD for 8 weeks.

Before collecting the tissues, mice were fasted for 12-h, or were refed with HFD for 4-h immediately before sample collection after 12-h of fasting. Western blots were run using n = 6-8/group and normalized to total protein (Ponceau S). (A) UCP1; (B) oxidative phosphorylation (OXPHOS) complex; (C) PGC1α; (D) PPARγ; (E) SIRT1; (F) CREB; (G) total AMPK; (H) CPT1A; (I) total HSL; (J) total ATGL; (K) FGF21. (L) Representative Western blot images (corresponding Ponceau S stain shown below the blots). Data were analyzed using an unpaired Student's *t*-test and presented as mean ± SEM relative to the HFD group.

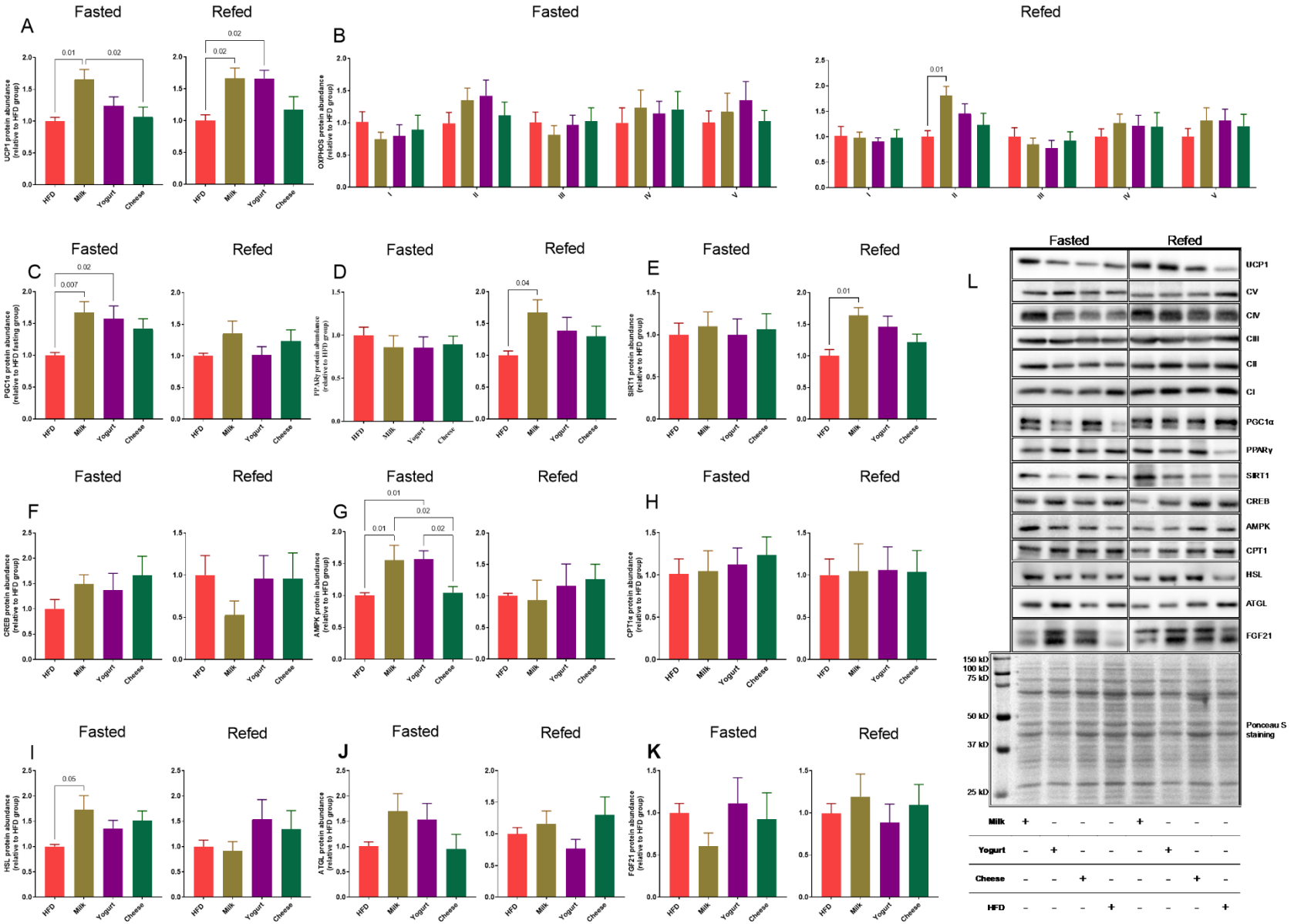

A
Fasted
Refed
UCP1 protein abundance (relative to HFD group)
HFD
Milk
Yogurt
Cheese
B
Fasted
Refed
OXPHOS protein abundance (relative to HFD group)
I
II
III
IV
V
C
PGC1α protein abundance (relative to HFD fasting group)
D
PPARγ protein abundance (relative to HFD group)
E
SIRT1 protein abundance (relative to HFD group)
F
CREB protein abundance (relative to HFD group)
G
AMPK protein abundance (relative to HFD group)
H
CPT1a protein abundance (relative to HFD group)
I
HSL protein abundance (relative to HFD group)
J
ATGL protein abundance (relative to HFD group)
K
FGF21 protein abundance (relative to HFD group)
L
UCP1
CV
CIV
CIII
CII
CI
PGC1α
PPARγ
SIRT1
CREB
AMPK
CPT1
HSL
ATGL
FGF21
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
Ponceau S staining
Milk
Yogurt
Cheese
HFD

Figure 5. 6 The relative abundance of proteins associated with adaptive thermogenesis in brown adipose tissue of mice fed HFD with or without dairy products for 8 weeks.

Mice were fasted for 12h or were refed with HFD for 4h immediately before sample collection after 12h of fasting. Western blots were run using n = 6-8 /group and normalized to total protein (Ponceau S). (A) UCP1; (B) oxidative phosphorylation (OXPHOS) complex; (C) PGC1α; (D) PPARγ; (E) SIRT1; (F) CREB; (G) total AMPK; (H) CPT1; (I) total HSL; (J) total ATGL; (K) FGF21. (L) Representative Western blot images are shown (corresponding ponceau S stain is below the blot). Data were analyzed using one-way ANOVA with Tukey's post hoc analysis and are presented as means ± SEM relative to the HFD group.

Dairy products change serum lipid profile.

Based on previous studies (427, 428), we hypothesized that the beneficial effects of dairy products are not due to specific bioactive molecules in these products, but to new metabolites produced by the modulation of metabolic pathways by these dairy products. Therefore, we obtained a 12-h fasted lipid profile to capture the specific lipid changes associated with each intervention, and to explore their putative influence on metabolic pathways.

A total of 5186 lipidomic features detected in all samples were identified. To explore variations in lipid composition among treatment groups, we employed an unsupervised PCA approach, maximizing variance to generate unsupervised loading scores (Fig. 5.8A). The PCA did not show a distinct separation among the 4 treatment groups, indicated by substantial overlap of their confidence areas (colored circles in the plots). We performed a PLS-DA analysis on the concentrations of lipid species to investigate the differences among the 4 groups (Fig. 5.8B). The PLS-DA model explained 62.5% of the total variance. The score plot showed a separation of the 4 groups, thus indicating the lipid profiles were distinct among the experimental conditions. To enhance discrimination, we implemented supervised Sparse Partial Least Squares Discriminant Analysis (sPLS-DA). This approach allows variable selection of the most predictive or discriminative features in the data, thus aiding in sample classification. The sPLS-DA model consisted of two components that explained 76.3% of the total variance and demonstrated distinct separations between groups, with the HFD group clearly segregated from the MILK and YOG groups (Fig. 5.7A). However, the separation between the HFD and CHE groups was less pronounced, suggesting no substantial modification of the lipidomic profile. Variable Importance for Prediction (VIP) scores were calculated from the PLS-DA, highlighting the top 15 major lipids influencing separation along components 1 and 2 (Fig. 5.7 C), which were diverse and not limited to a specific lipid class. Furthermore, we identified specific lipid species that significantly differentiated experimental groups based on ANOVA testing. We identified PG 40:4, LPC 15:0, PC 15:0_15:0, and Cer 37:6; O5 in the plasma as the main contributors to metabolic differences between groups in both components. The resulting heatmap reflects the top 20 lipid species with the lowest q-values in the ANOVA test (Fig. 5.7B). Clustering of individual lipid species indicated three principal clusters, each displaying a dissimilar, intervention-dependent trend. The first leaf comprised 2 distinguished sub-clusters, including 9 lipid species significantly more abundant in

MILK and YOG compared with HFD. The second group consisted of 16 lipid species showing markedly lower abundances in MILK and YOG compared to HFD and CHE.

Volcano plot analysis was employed to discern differential lipid molecules between treatment groups and HFD. Utilizing criteria of fold-change ≥ 1 and $p < 0.05$, 121 (positive) and 92 (negative) significant differences in serum lipid compounds between MILK and HFD were identified (Fig. 5.7C), along with 63 (positive) and 58 (negative) lipids in the comparison between YOG and the HFD group (Fig. 5.7D), and 87 (positive) and 74 (negative) lipids in the CHE and HFD group (Fig. 5.7E, F). A Venn diagram was utilized to delineate commonly differential lipid metabolites among the three pairwise treatment groups with HFD. Notably, the Venn diagrams (Fig. 5.7G) revealed 23 core lipid molecules differing between dairy products plus HFD and HFD-fed mice across all 3 intervention groups. Also, a ANOVA test followed by Tukey's test was performed on the main 16 lipid species that contributed the lowest q-value (with $q < 0.05$) to test for significant differences between the 3 conditions (Fig 5.8). These findings collectively provide evidence that the supplementation of MILK, YOG, and CHE can induce alterations in the serum lipid profiles of mice on a HFD, even after overnight fasting and indicates a distinct influence of dairy product supplementation on the lipidomic landscape.

Applying lipid ontology (LION) enrichment to significantly altered lipids (unadjusted $p < 0.05$), distinct lipid pathways that were modulated by dairy products compared to the HFD group were identified (Fig 5.7H), thus allowing the association of lipid species with specific biological features and functions. The lipid enrichment analysis between the MILK and HFD revealed downregulation of sphingolipids, phosphosphingolipids, golgi apparatus lipids, and endosome/lysosome-associated lipids, coupled with upregulation of the metabolism of several groups of medium and long-chain mono-unsaturated and saturated fatty acids including fatty acids with 15 carbons, C15:0, and positive intrinsic curvature (Fig. 5.8 D). Comparative enrichment analysis between the YOG and HFD groups revealed downregulation of sphingolipids, phosphosphingolipids, glycerophosphoethanolamines, membrane components, simple glc series (a ceramide with a single glucose or galactose residue), ceramide phosphoinositols, and headgroups with positive charge/zwitter-ion. Concurrently, upregulation was observed in lipid storage, lipid droplet, glycerolipids, triacylglycerols, and headgroups with a neutral charge (Fig. 5.8 E). The enrichment analysis demonstrated that only lipid species containing fatty acids with 18 carbons were considerably higher following the CHE intervention compared to the HFD (Fig. 5.8 F).

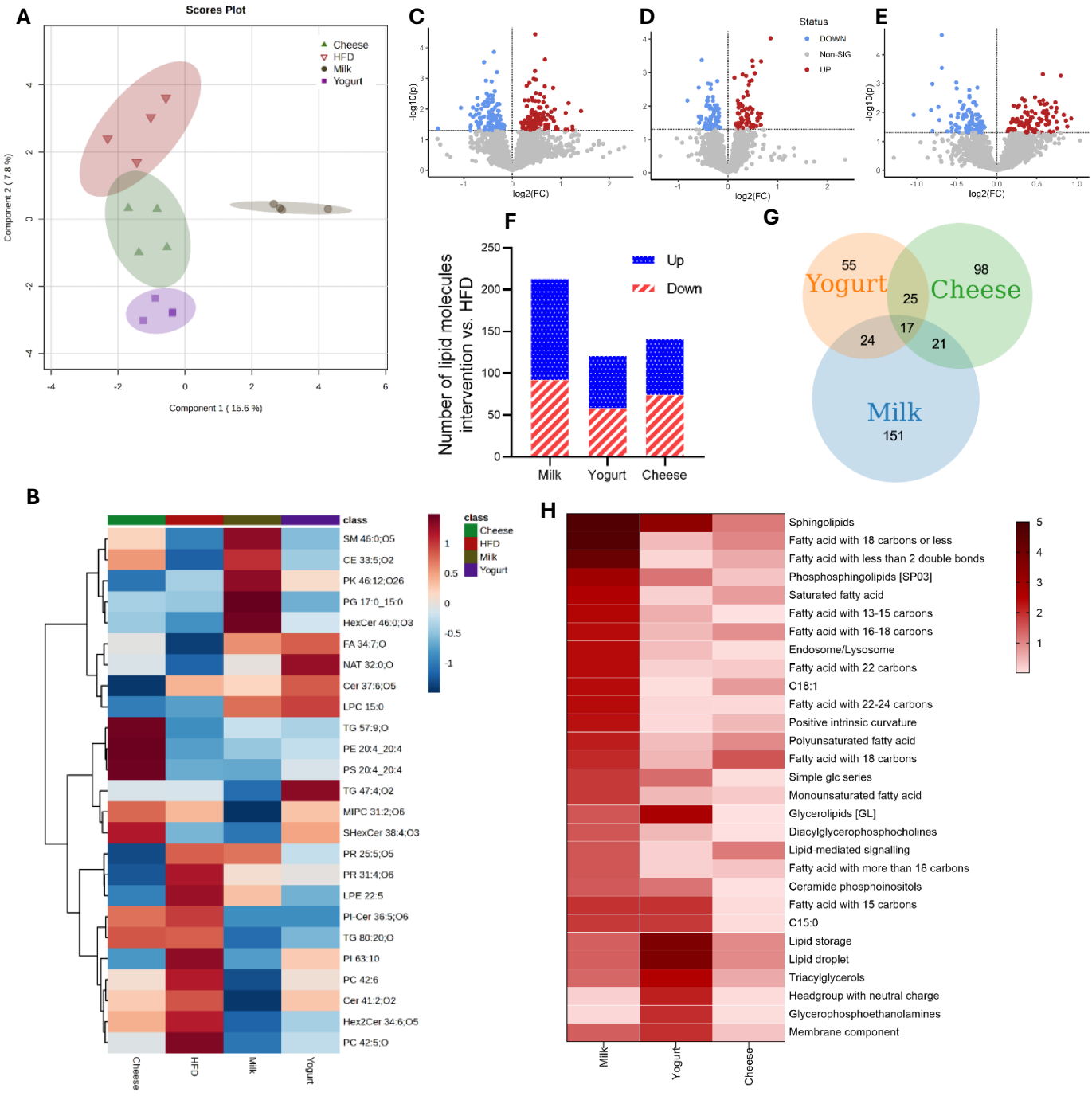
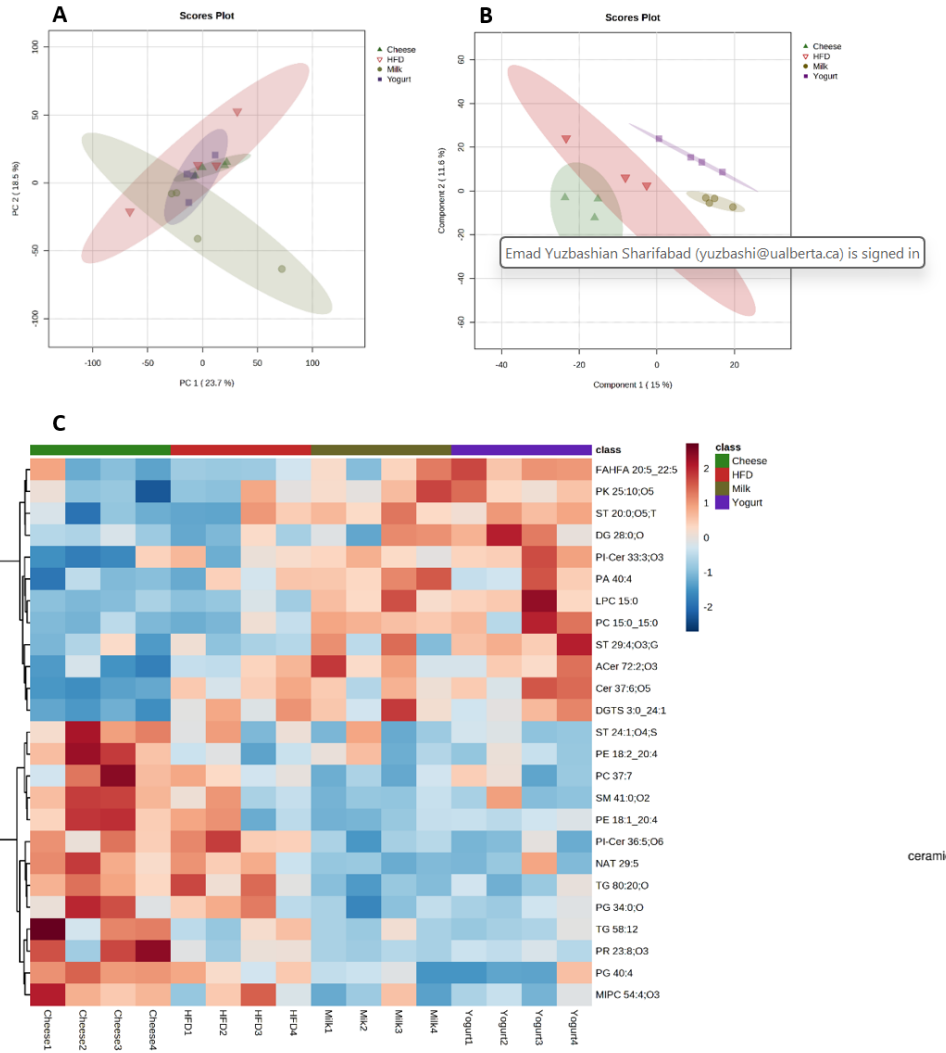


Figure 5. 7 Lipidomics analysis of plasma.

(A) Sparse partial least squares—discriminant analysis (sPLS–DA) score plots colored by sample group using intervention as a grouping variable (B) Heatmap of ANOVA test results to identify the top 25 highly significant differences in lipid composition amongst the 4 groups; (C) Volcano plot of lipid species comparing MILK and HFD, (D) YOG and HFD, (E) CHE and HFD; (F) The total number of serum lipid species differences in mice treated with milk, yogurt, and cheese

compared with HFD; (G) Venn diagram depicting the distribution of lipid species with a significant difference ($p < 0.05$) compared with HFD; (H) LIONweb enrichment analysis of significantly different ($p<0.05$) lipid species compared with HFD based on data ranking mode.


A
Scores Plot
Cheese
HFD
Milk
Yogurt
PC 1 (23.7 %)
PC 2 (18.5 %)
B
Scores Plot
Component 1 (15 %)
Component 2 (11.6 %)
Emad Yuzbashian Sharifabad (yuzbashi@ualberta.ca) is signed in
C
class
FAHFA 20:5_22:5
PK 25:10;O5
ST 20:0;O5;T
DG 28:0;O
PI-Cer 33:3;O3
PA 40:4
LPC 15:0
PC 15:0_15:0
ST 29:4;O3;G
ACer 72:2;O3
Cer 37:6;O5
DGTS 3:0_24:1
ST 24:1;O4;S
PE 18:2_20:4
PC 37:7
SM 41:0;O2
PE 18:1_20:4
PI-Cer 36:5;O6
NAT 29:5
TG 80:20;O
PG 34:0;O
TG 58:12
PR 23:8;O3
PG 40:4
MIPC 54:4;O3
Cheese1
Cheese2
Cheese3
Cheese4
HFD1
HFD2
HFD3
HFD4
Milk1
Milk2
Milk3
Milk4
Yogurt1
Yogurt2
Yogurt3
Yogurt4
D
fatty acid with 18 carbons or less
fatty acid with less than 2 double bonds
saturated fatty acid
fatty acid with 13-15 carbons
fatty acid with 22 carbons
fatty acid with 16-18 carbons
C18:1
fatty acid with 22-24 carbons
positive intrinsic curvature
C22:0
polyunsaturated fatty acid
fatty acid with 18 carbons
fatty acid with 15 carbons
monounsaturated fatty acid
C15:0
lipid-mediated signalling
glycerolipids [GL]
fatty acid with more than 18 carbons
triradylglycerols [GL03]
lipid storage
lipid droplet
fatty acid with more than 3 double bonds
C18:2
triacylglycerols [GL0301]
glycerophosphates [GP10]
negative intrinsic curvature
fatty acid with 3-5 double bonds
diacylglycerols [GL0201]
C22:5
sphingolipids [SP]
phosphosphingolipids [SP03]
golgi apparatus
endosome/lysosome
simple glc series [SP0501]
diacylglycerophosphocholines [GP0101]
plasma membrane
ceramide phosphoinositols [SP0303]
ceramide phosphocholines (sphingomyelins) [SP0301]
membrane component
above average lateral diffusion
up
down
-LOG(FDR q-value)

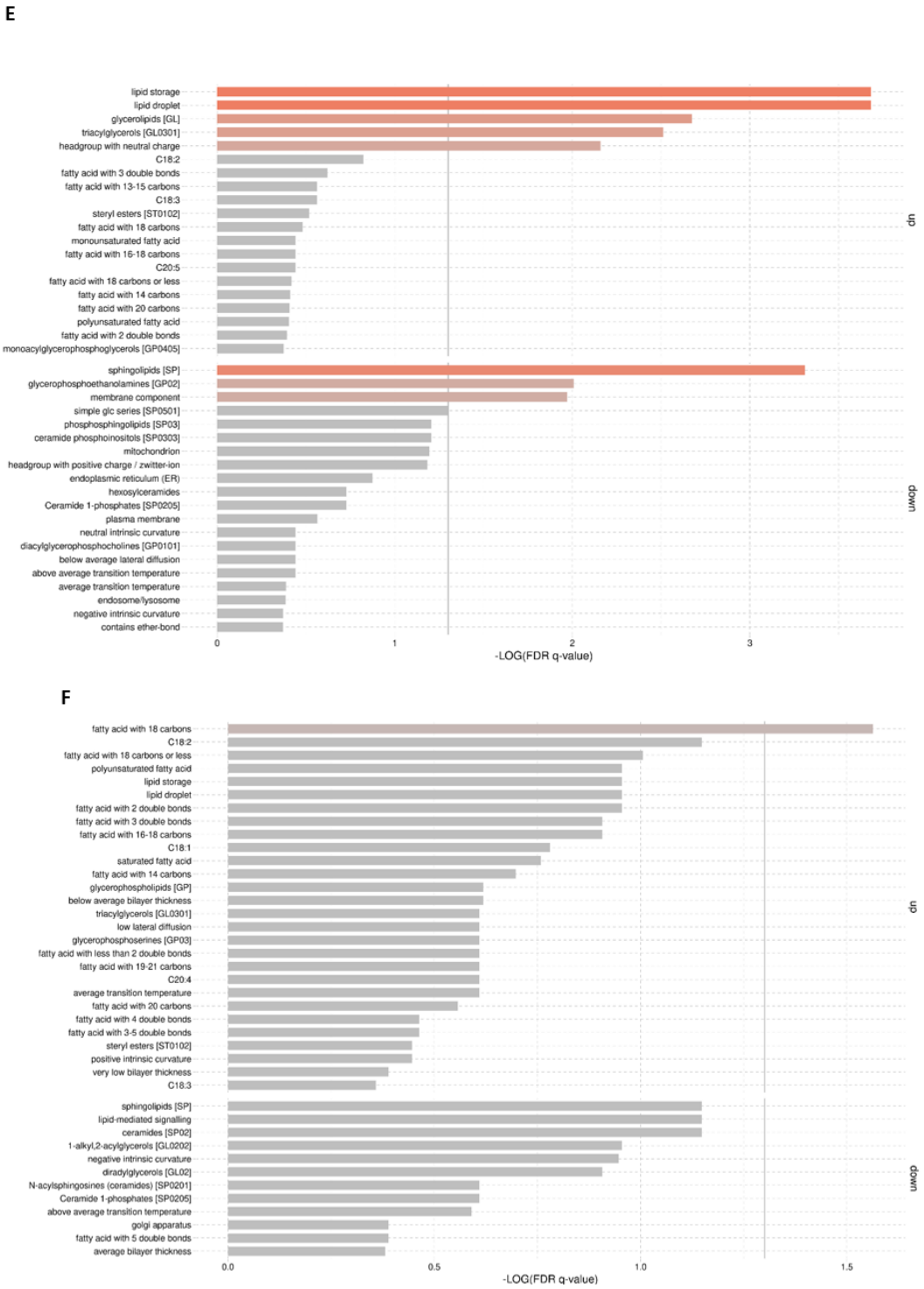


Figure 5. 8 Visualization of the mouse serum lipidomics dataset.

(A) Principal component analysis (PCA) plot; (B) Partial least squares-discriminant analysis (PLS-DA) plot; (C) Heatmap of 25 lipid species selected by the highest PLS-DA VIP. Lipid ontology enrichment analysis (LION) was performed using the ranking mode, in which input

lipids are ranked by numeric values and compared between two groups which included (D) Milk vs HFD; (E) Yogurt vs HFD; (F) Cheese vs HFD. The gray vertical lines imply the cut-off value of significant enrichments ($p < 0.05$).

5.5 DISCUSSION

This study investigated the effects of fat-free milk, fat-free plain yogurt, and reduced fat (19%) cheddar cheese on weight gain and fat mass accumulation in diet-induced obese mice. Contrary to our hypothesis, MILK was the most effective in reducing weight gain and fat mass, followed by YOG, while CHE had the least effect. We discovered that all dairy products increased EE, but only MILK boosted BAT thermogenesis by activating UCP1 through SIRT1-PPARγ-PGC-1α axis pathways. Lipidomics analysis showed potential mechanisms of the benefits of MILK and revealed some common and some unique pathways with YOG; however, the serum lipid profile of CHE was like HFD. Therefore, MILK, YOG, and CHE influence energy homeostasis in mice fed with HFD via different pathways.

In our preclinical mouse obesity model, we found that MILK, YOG, and CHE reduced body weight gain by -25%, -22%, and -14%, respectively, compared to HFD after an 8-week intervention. Few intervention studies in humans have compared the effects of different dairy products on weight gain and body composition (264, 312). The conventional yogurt intervention in obese women with MASLD had favorable impacts on fat mass and waist circumference compared to those who consumed milk (312). Conversely, males with abdominal obesity showed no noteworthy distinctions in the effects between fermented and non-fermented dairy products, including milk, yogurt, heat-treated yogurt, and acidified milk, on the cardiometabolic-related outcomes (264). Animal studies also support the anti-obesity effects of milk and yogurt. Skimmed milk powder provided in a high-fat, high-sucrose (HFHS) diet reduced weight gain as much as exercise training (-50%) (256) and, in pair-fed rats, skimmed milk powder lowered weight gain (-5%) and fat mass (317). In mice, whole milk added to a HFD decreased eWAT weight and weight gain (429). Yogurt also effectively suppressed HFD-induced abdominal fat, body weight, and fat index in HFD-induced obese rats (430, 431). However, cheese intake had a small or neutral effect on weight gain (283, 432).

We identified enhanced EE (MILK = YOG > CHE), particularly in the dark phase when the mice are more active and consume more food. We assumed that the higher EE after food consumption might be attributed to the higher diet-induced thermogenesis has been extensively explored, revealing its significance in regulating energy balance and metabolism (433). Elevated UCP1 content of BAT also further supports this hypothesis, suggesting BAT as a potential target to

combat obesity and metabolic disorders through the adaptive thermogenesis of food consumption (145). While MILK, YOG, and CHE yield comparable results regarding increased EE, the mechanisms regulating these alterations in fat mass and body weight appear to differ. Among the MILK group, the observed increase in EE, alongside enhanced RER and glucose utilization without changes in food intake or physical activity, in addition to slightly different macronutrient proportions compared to the HFD alone, suggesting that bioactive compounds rather than macronutrients might be more potent to be responsible for the anti-obesity activity of dairy foods. Milk and its derivatives contain a range of natural bioactive molecules such as casein hydrolysate (434), whey hydrolysate (400), Miltin (435), milk fat globule membrane (MFGM) (253), polar lipids (436), and oligosaccharides (437) derived from milk fats, carbohydrates, proteins which their influence on BAT activation was reported (438). However, the lipidomic changes in response to milk and yogurt consumption revealed an increase in certain lipid molecules of PC, PE, and LPC classes, some of which are among the polar lipid compounds of the MFGM (439). The browning effect of MFGM compounds was related to the PC content in an in vitro study of 3T3-L1 adipocytes, indicating that PC treatment dramatically up-regulated the expression of genes and proteins unique to brown fat, including UCP1, PGC-1α, PRDM16, PPARα, and other indicators of beige cells (440), suggesting a potential function for PC in the activation of energy expenditure. The presence of specific lipids like PC 15:0_15:0 and LPC 15:0 in the serum of MILK and YOG groups could play a key role in promoting BAT activity and thermogenesis; therefore, further research into the potential of these lipids as dietary mediators for metabolic health is warranted.

To address underlying mechanisms contributing to increased EE elicited by MILK, we demonstrated that in BAT, the SIRT1-PPARγ-PGC-1α axis stimulates diet-induced adaptive thermogenesis through upregulation of UCP-1 expression (441). Beyond the effects on activation of BAT by MILK and YOG, other indirect effects are also plausible; for example, links to the elevation of glucagon-like peptide-1 (GLP-1) (442) and adiponectin (443) levels by MILK and YOG may contribute to BAT thermogenesis and beige fat development, aligning with our findings of AMPK–SIRT-1–PGC1-α pathway involvement in BAT activation. Few studies have examined how milk affects EE and BAT activation. Milk by-products may directly regulate BAT function without altering sympathetic nerve activity or adipocyte differentiation (444). Li et al. reported an enhancement of BAT activity evidenced by increased UCP1 protein and gene expression following an increase in PGC-1α and PPARα in mice fed with HFD. Furthermore, they showed that when

3T3-L1 pre-adipocytes were treated with MFGM, it stimulated the conversion of brown-like adipocytes by upregulating the expression of UCP1 and other thermogenic genes, as well as beige cell markers (440). In children, thermal imaging showed that semi-skimmed milk raised body temperature in the supraclavicular region, where BAT is located in humans, indicating its activation (445, 446). The potential of MILK feeding to activate SIRT1 and its downstream targets such as PGC-1α, was highlighted in an *ex vivo* study, indicating stimulation of mitochondrial biogenesis and oxidative capacity (447). However, we did not directly measure BAT activity or the whole-body temperature, which would provide more direct evidence for the thermogenic effect of dairy consumption. Furthermore, the study lacks histological analysis to provide phenotypic support for our immunoblotting findings due to insufficient BAT availability.

Untargeted lipidomics analysis of fasting serum indicated that the effects of dairy interventions extend beyond their molecular contents to encompass a myriad of factors and metabolites, which were not limited to the postprandial state but also traceable during periods of fasting. A human study revealed that fasting serum metabolome changes metabolome following a 28-day intervention with milk (448). Similarly, in the present study, MILK markedly changed serum lipidome after a 12-hour fast, showing systemic intervention effects on lipid metabolism that may also reflect how milk influences BAT metabolism. MILK lowered sphingolipids, phosphosphingolipids, and Golgi/endosome/lysosome lipids, which are indicators of reduced lipid synthesis, trafficking, and storage (449, 450, 451). Concurrently, increased oxidation of medium and long-chain saturated and unsaturated fatty acids supported the observation of higher EE, which might be attributed to higher energy demand for BAT activation. MILK also elicited positive intrinsic curvature in serum lipids, which is linked to improving membrane stability and fluidity (452). This contrasts with the negative intrinsic curvature nature of sphingolipids and phosphosphingolipids, which make membranes rigid and ordered (452).

The health benefits of consuming yogurt, including weight control, have received considerable attention in recent years, as shown by reviews on this topic (453, 454), and are consistent across meta-analyses of prospective cohort studies (234, 289, 455). Furthermore, an RCT found that consuming yogurt reduced indices of adiposity, such as BMI and waist circumference (415). Animal studies also investigated the potential impact of yogurt consumption on obesity phenotypes (266, 271, 273, 456, 457). Tang et al. reported a reduction in body weight and fat index in mice fed a HFD supplemented with yogurt (266), while Daniel et al. found a significant yet slightly

lower body weight in obese mice (273). However, these studies did not measure EE as a plausible metabolic response or explore the possible physiological and underlying mechanisms responsible for the positive impacts of yogurt. We observed that YOG reduced weight gain, fat mass, and eWAT in HFD-induced obese mice via increased EE. Further, YOG induced thermogenic pathways in BAT, exemplified by significantly increased UCP1, PGC1α, and AMPK and a trend to raising FGF21 ($p=0.07$). FGF21 is a hormone that stimulates BAT thermogenesis and glucose uptake (458).

Although the physiological and BAT-specific effects of YOG were similar to MILK, we noted that YOG had distinct effects on the serum lipidomic profiles. YOG lowered lipid classes and subclasses related to inflammation and oxidative stress but raised lipids related to energy storage and utilization (451, 452). Therefore, these changes in lipid molecules by YOG may explain why the YOG group had higher circulating lipid storage and droplet species, glycerolipids, and triacylglycerols, which are the primary forms of both stored and mobilized lipids in the body. Therefore, the changes in the lipidomic profiles of the YOG may reflect a shift in the lipid flux and utilization in BAT and possibly other WAT in response to yogurt consumption. This may also affect systemic lipid homeostasis and inflammation in HFD mice.

In the current study, CHE showed lower weight gain and higher EE than HFD mice but did not affect indicators of BAT thermogenesis. The literature on cheese and weight loss is inconsistent. Some studies report beneficial effects on weight loss, while others report neutral effects (283, 432). Interestingly, the CHE had a pattern of lipid species profile that was more similar to the HFD than the other dairy groups, suggesting that metabolic changes in response to cheese consumption cannot be reflected in lipid profiles. This may indicate the distinct physicochemical characteristics of cheese versus yogurt and milk, which can be because of varying fermentation and ripening processes. Therefore, although cheese contains relatively higher MFGM than milk and yogurt, its unique properties and a complex network of various components within its matrix may mask its impact to be mirrored in lipidomics data. Furthermore, we hypothesize that increased EE in CHE is mediated via targeting pathways in tissues other than BAT. The higher EE in CHE may be due to the browning of subcutaneous fat, which turns white adipocytes into beige adipocytes with BAT-like features (459). We did not examine the effects of dairy product consumption on other tissues or organs that are involved in energy homeostasis, such as the liver, muscle, or brain. Importantly, the subcutaneous adipose tissue of mice was not collected at the time of euthanasia,

so we could not test if dairy products can also initiate the browning of WAT in addition to BAT activation.

It is important to note that the findings of this study should be translated to humans with caution, given it was conducted on mice, which do not fully represent the physiological and metabolic responses in humans, especially with respect to BAT. Our findings highlight the role of BAT in the anti-obesity effects of dairy products and call for further human studies and exploration of each dairy product's specific role in combating obesity and metabolic disorders.

5.6 CONCLUSION

Our study revealed that MILK, YOG, and CHE mitigate weight gain and fat mass accumulation in diet-induced obese mice. While all dairy products increased EE, MILK uniquely enhanced BAT thermogenic potential via the SIRT1-PPARγ-PGC-1α pathway. Serum lipidomics analysis identified an increase in PC 15:0_15:0 and LPC 15:0 in response to MILK and YOG, which may mediate effects on BAT. CHE, although effective in weight control and raising EE, lacks an impact on BAT thermogenesis.

6 DIFFERENTIAL EFFECTS OF MILK, YOGURT, AND CHEESE ON INSULIN SENSITIVITY, HEPATIC FUNCTION, AND GUT MICROBIOTA IN DIET-INDUCED OBESE MICE

A Comparison of the Effects of Milk, Yogurt, and Cheese on Insulin Sensitivity, Hepatic Function, and Gut Microbiota in Diet-Induced Obese Mice

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6.1 ABSTRACT

Background and aim: The prevalence of obesity and associated metabolic disorders, including insulin resistance (IR) and metabolic-associated steatotic liver disease (MASLD), is a major global health problem primarily linked to overnutrition. Recent meta-analyses indicate that dairy consumption may mitigate IR and fatty liver, particularly low-fat dairy products. However, the underlying molecular mechanisms remain elusive. Herein, we compare the effects of different dairy subgroups on markers of the obese phenotype, glucose homeostasis, hepatic steatosis, and gut microbiota composition in high-fat diet (HFD)-induced obese mice.

Methods: C57BL/6 mice (n = 16/group) were fed a high-fat diet (HFD, 45% fat) or HFD with supplementation of either fat-free milk (MILK), fat-free yogurt (YOG), or reduced-fat (19%) cheddar cheese (CHE) at 10% of the total energy of the diet for 8 weeks. Body weight, fat mass, fasting blood glucose (FBG), plasma insulin, and the homeostasis model assessment (HOMA-IR)

were measured. Liver tissues from fasted mice were analyzed for lipid content, lipid droplet size, enzymes involved in lipid and carbohydrate metabolism, and lipidomics. Gut microbiota composition was assessed via 16S rRNA sequencing from fecal samples.

Results: Mice on the HFD developed notable obesity, IR, and hepatic steatosis. MILK, YOG, and CHE significantly reduced weight gain, fat mass, and FBG. Only MILK significantly reduced fasting insulin and HOMA-IR. YOG and MILK decreased hepatic triglyceride (TG) content and lipid droplet size, while CHE had no effect. MILK and YOG induced higher phosphorylation of AMP-activated protein kinase (AMPK) and down-regulated *de novo* lipogenesis enzymes via regulating phosphorylated acetyl–coenzyme A (CoA) carboxylase (pACC)/ACC. In the MILK group, the abundance of sirtuin 1 (SIRT1), peroxisome proliferator-activated receptors (PPAR)-α, and carnitine palmitoyl transferase (CPT)-1α was increased, whereas in YOG, only peroxisome proliferator-activated receptor γ coactivator (PGC)-1α and CPT-1α were elevated. Both MILK and YOG increased AKT phosphorylation (pAKT/AKT) and decreased hepatic gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6 phosphatase (G6PC). Liver lipidomics in the MILK and YOG groups revealed unique profiles dominated by decreased pro-inflammatory lipid species, including diacylglycerols (DG) and ceramides. Gut microbiota analysis indicated increased beneficial bacteria such as *Streptococcus* in the YOG and *Anaerotignum* in the MILK groups. Only a mild effect of CHE was seen, enriching the beneficial bacteria like *Sporofaciens* and *Streptococcus* without altering the liver lipid profile.

Conclusion: Intake of fat-free milk or yogurt significantly improved the adverse effects of HFD, including obesity, IR, and hepatic steatosis, with a superior effect in the milk group. Most importantly, these improvements might be related to increased energy expenditure, liver de novo lipogenesis and gluconeogenesis suppression, and enhanced liver lipid oxidation. Thus, dairy, specifically low-fat milk and yogurt, could be beneficial in preventing HFD-induced MASLD.

6.2 INTRODUCTION

The incidence of metabolic dysfunction-associated steatotic liver disease (MASLD), characterized by the accumulation of triglycerides (TG) in liver cells in the absence of excess alcohol intake, is rising rapidly (460), with the worldwide prevalence estimated to be 32% according to the latest meta-analysis (461). Because of the strong correlation that obesity and insulin resistance (IR) have

with MASLD, it is considered the hepatic manifestation of obesity-induced metabolic dysfunction (462). Diet modification is among the top strategies for preventing and treating MASLD (463). Suboptimal nutrition, like Western diet patterns containing high amounts of processed food, red meat, and refined grains, is associated with an 56% increased risk of developing MASLD (464) and contributes to the development of hyperglycemia, IR, inflammation, and gut microbiome dysbiosis (465). On the other hand, adherence to a prudent dietary pattern rich in fruits, vegetables, nuts, whole grains, legumes, and low-fat dairy products decreases the likelihood of MALSD (464). In this context, the lack of pharmaceutical treatments for MASLD until recently and the important role of an optimal diet in its prevention underline the importance of population-based planning focused on the contributions of foods from specific food groups to metabolic health.

The dairy food group contains nutrient-dense foods supplying high-quality protein and many micronutrients. They are considered as foundational foods included in healthy dietary patterns (199). Dairy foods, especially low-fat products, are associated with a lower risk of obesity-related metabolic dysfunction, including metabolic syndrome (287), IR (307), and type 2 diabetes (342). A recent meta-analysis of observational studies indicates that higher total dairy consumption is associated with a 10% lower risk of MASLD. However, there are insufficient studies meeting the inclusion criteria that consider the fat content of dairy products to pool their findings (466). Data from the UK Biobank, a large prospective cohort study with 11 years of follow-up, indicates that low-fat dairy products are associated with less risk of incident MASLD than high-fat dairy products (-22% vs. -3%) (467). Dairy products, including milk, yogurt, and cheese, differ in their nutritional composition and physical properties based on the processing techniques used, and this affects their digestion, absorption, and effects on metabolism (233); thus, the effects of dairy products on individuals' metabolic health may vary.

Regarding milk, observational studies link higher consumption with a lower risk of MAFLD (466, 468). However, findings from interventional trials are conflicting (311, 312, 469), and suggest that the beneficial effect of liquid milk on metabolic health depends on the fat content. Thus, low-fat or skimmed milk intake yields greater improvement in metabolic parameters than whole milk (316, 317, 318). Yogurt, a semi-solid probiotic-containing food, is produced by fermenting milk with certain lactic acid bacteria, such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. bulgaricus (470). A solid milk derivative, cheese, is also made by fermenting milk with lactic acid bacteria, specifically *Lactococcus lactis* and *Lactococcus cremoris*, with an additional

ripening step (471). Since gut dysbiosis is linked to the development of diet-induced MASLD (472), these natural, milk-derived probiotic sources in the human diet are assumed add health-related value to the dairy nutrient matrix, potentially preventing or delaying MASLD development by modifying the gut microbiome structure or function (473). Our group's previous study in obese mice found that whole milk (3.25% milk fat) but not yogurt (2.9% milk fat) or regular-fat cheese (31% milk fat) reduced hepatic steatosis (329). We speculate that the null effect of yogurt and cheese could be related to the high-fat content.

Thus, we hypothesize that both milk and fermented dairy product consumption will ameliorate the detrimental metabolic effects of a HFD, leading to protection against hepatic steatosis through both shared and unique mechanisms. In addition, consuming fermented dairy products will result in greater improvements to metabolic risk indicators and a greater decrease in liver fat than milk. This study aims to determine the influence and underlying mechanisms of fat-free milk, fat-free plain yogurt, and reduced-fat cheddar cheese on obesity phenotype markers, glucose homeostasis, and hepatic steatosis in mice fed with a HFD to guide future research into their potential use in the prevention of MASLD.

6.3 METHODS

Experimental procedures

All procedures involving animals were approved by the University of Alberta's Animal Care and Use Committee (AUP00003066), following guidelines issued by the Canadian Council on Animal Care. Experiments were reported following ARRIVE guidelines. Eighty male C57Bl/6 mice were obtained at 6 weeks of age from Charles River, Canada (St. Constant, QC, Canada) and were maintained at 23 ± 1°C in a humidity-controlled room (50 ± 10%) under an alternate 12:12 light-dark cycle (lights on at 10:00 pm). Following a week of acclimatization and free access to standard chow and water, the mice were weighed and randomly assigned into either a low-fat diet (LFD; n = 16) consisting of 10 kcal% fat from soybean oil and lard with digestible energy of 3.82 kcal/g (D12450H Research Diets, New Brunswick, NJ, USA) or a high-fat diet (HFD; n = 64) consisting of 45 kcal% fat from soybean oil and lard with digestible energy of 4.73 kcal/g (D12451 Research Diets) for 7 days. Both diets had the same amount of sucrose and protein. While mice in the LFD group remained on the same diet throughout the study, mice in HFD groups were re-randomized

into 1 of 4 groups (n=16 per group) as shown in Figure 1: (1) HFD, (2) HFD + 3.0 ml of fat-free milk (MILK; Dairyland, Saputo Dairy Products, Montreal, Canada), (3) HFD + 2.1 ml of fat-free plain yogurt (YOG; ASTRO® Original Balkan Plain, Lactalis Group, Toronto, Ontario, Canada), and (4) HFD + 360 mg of reduced-fat (19% fat) cheddar cheese (CHE; Armstrong Old Light Cheddar Cheese, Saputo Dairy Products). Mice were housed 4 per cage by diet group and followed for 56 days (8 weeks), with the provision of dairy products on 5 out of 7 days each week. Our previous manuscript detailed the study design, including the sample size, dairy dosage calculation, and the feeding method (Chapter 5). Some of the phenotype data from these mice has been presented elsewhere (Chapter 5).

Body weight and food intake measurements were recorded weekly, and dietary intake and energy intake were calculated. Indirect calorimetry (using Oxymax (CLAMS); Columbus Instruments, Columbus, OH, USA) and body composition analysis (using EchoMRI; Echo Medical Systems LLC, Houston, TX, USA) were performed during days 50 to 55 of the intervention, as elaborated previously (Chapter 5). Blood glucose was measured from the tail vein using a glucometer (Contour®Next, Mississauga, ON, Canada) at baseline (day 0) and day 56 after 12-h of fasting. At the end of week 8, to evaluate metabolic markers in the fasting and refed states, all mice were subjected to a 12-h fast or a 12-h fast followed by a 4-h refeeding period with their corresponding background diet prior to euthanasia with CO_2. Blood was collected and centrifuged (5,000g, 4 °C, 30 min), and the serum was stored at −80 °C until analysis. The entire liver was removed and weighed, and then samples were divided into fixatives for histopathology analysis or frozen in liquid nitrogen and stored at −80°C for enzyme and metabolite analyses.

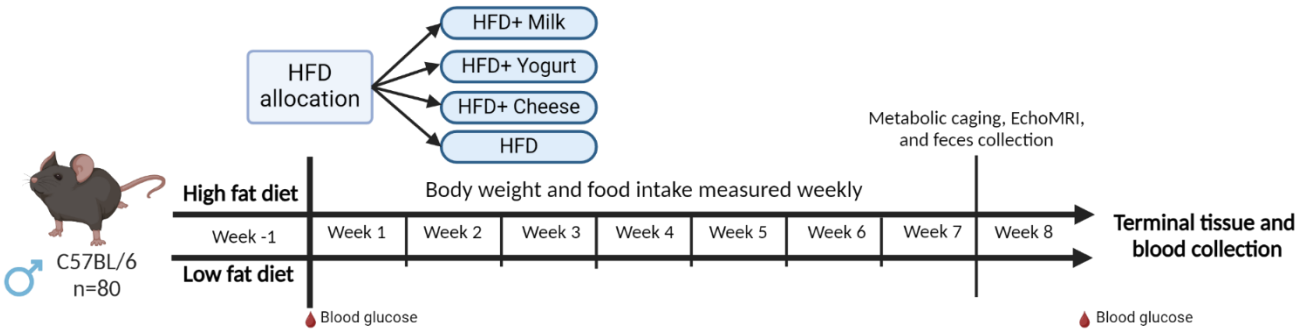

Figure 6. 1 Study design and experimental timeline for assessing the effects of milk, yogurt, and cheese on metabolic health in HFD-fed C57BL/6 mice

Biochemical analyses of liver and serum

Lipids were extracted from thawed liver tissue using a modified version of the Folch method (474) as described previously (475). The dried extract was then resuspended in H_2O and kept at -80 °C until further assessment. Serum and liver total cholesterol (TC) and triacylglycerol (TG) content were measured spectrophotometrically using commercial kits (InfinityTM, Thermo Scientific, Waltham, MA, USA). Liver TG and TC content were normalized to the total protein content of the liver quantified using Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA).

The serum insulin concentration of 12-h fasted mice was measured using a mouse insulin ELISA (ALPCO, Salem, NH, USA). IR was estimated with the homeostatic model assessment-insulin resistance (HOMA-IR) using the following formula: HOMA-IR = [fasting plasma insulin (µU/mL) × fasting blood glucose (mmol/L)/22.5].

Histological Staining

The liver samples for histology were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 µm thickness. Following deparaffinization, tissue sections were used for hematoxylin and eosin (H&E) staining using standard protocols. For each mouse, a minimum of 12 photomicrographic images were taken by one researcher at 20X magnification using light microscopy (Axio Observer A1 with AxioCam HRc, Germany). The lipid droplet area of images was manually quantified using ImageJ (National Institutes of Health, USA) (476). The researcher

was blinded during image capturing and quantification. Representative images were selected for presentation according to the quantification data.

Immunoblot Analysis

To investigate hepatic enzyme abundance and phosphorylation, 50 mg of frozen liver tissue was homogenized in RIPA lysis buffer. Then, homogenates were centrifuged, and the supernatant was collected. Total protein concentrations were determined by BCA. Samples (2 µg/µL) were prepared in Laemmli protein sample buffer (4X; Bio-Rad, Hercules, CA, USA) in the presence of 2-mercaptoethanol. Sample proteins were separated on 8% to 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk or 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-0.05% Tween for 1 h. Subsequently, membranes were exposed to specific primary antibodies (Table S1) at a dilution of 1:1000 or 1:500 based on the manufacturer's recommendation in 2.5% BSA overnight at 4 °C and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Table 6.1) at dilution of 1:5000 in 2.5% BSA at room temperature for 1 h. Protein bands were imaged using electrochemiluminescence (ThermoFisher Scientific, Rockford, IL, USA) and analyzed using Image Lab (Bio-Rad version 6.1.0) software.

Table 6. 1 List of antibodies

Antibodies	Source	Identifier
Primary		
Acetyl-CoA carboxylase	Cell Signaling	3676
Phospho-acetyl-CoA carboxylase	Cell Signaling	11818
Acetyl-CoA synthetase	Cell Signaling	3658
Fatty acid synthase	Cell Signaling	3180
ATP-citrate lyase	Cell Signaling	4332
Phospho-ATP-citrate lyase	Cell Signaling	4331
Long-chain acyl-CoA synthetase	Cell Signaling	9189
Carnitine palmitoyltransferase-1α	Cell Signaling	97361
Sirtuin 1	Cell Signaling	2310
Adipose triglyceride lipase	Cell Signaling	2439
AKT	Cell Signaling	9273S
Phospho-AKT	Cell Signaling	4060S
5' AMP-activated protein kinase-α	Cell Signaling	2603S
Phospho-5' AMP-activated protein kinase-α	Cell Signaling	2531S
Cluster of differentiation 36	Santa Cruz	7309
Microsomal triglyceride transfer protein	Santa Cruz	515742
Fibroblast growth factor 21	Santa Cruz	81946
Peroxisome proliferator-activated receptor-α	Santa Cruz	398394
Peroxisome proliferator-activated receptor -γ	Santa Cruz	2435
Peroxisome proliferator-activated receptor gamma coactivator-1α	Santa Cruz	518025
OXPHOS (complexes I to V)	Abcam	110413
Glucose 6-phosphatase	Proteintech	22169-1-AP
Phosphoenolpyruvate carboxykinase	Cayman	10004943
β-actin	Sigma Aldrich	A5441
Anti-mouse IgG-peroxidase	Sigma Aldrich	A4416
Anti-rabbit IgG-peroxidase	Thermo Fisher Scientific	31460

Lipidomics

The Metabolomics Innovation Centre (TMIC, University of Alberta, Canada) conducted a comprehensive analysis of liver lipid extracts from 4 randomly selected samples, each pooled from 2 mice per treatment group. Untargeted LC-MS and LC-MS/MS methods were used for in-depth global lipidomics analysis (423, 424, 425). A detailed description of the procedures used by TMIC is described previously (Chapter 5). Identified features were normalized using internal standards and the median intensity ratio. Statistical analysis was performed in MetaboAnalyst 6.0

(https://www.metaboanalyst.ca/). The pathway enrichment analysis was conducted in the Lipid Ontology (LION) enrichment analysis web application (LION/web, www.lipidontology.com) by inputting the compound details of altered lipid species (P values <0.05) for each dairy product group compared with the HFD group.

Gut Microbiota Analysis

Fecal samples were collected in the morning of day 49 and immediately frozen at −80 °C until 16S rRNA amplicon gene sequencing was performed. For every 100 mg feces sample, 700 µl of Zymo lysis solution (Zymo Research Corp, Irvine, USA) and 100 µl of Roth zirconia/silica beads (0.1 mm in diameter) were added. Bacteria were lysed by mechanical disruption 3 times for 5 minutes each, with 5 minutes on ice in between, using a Mini-BeadBeater-96 (BioSpec, Bartlesville, OK, USA). Subsequently, the homogenate was centrifuged, and 200 µl of supernatant was transferred into a 96-deep well plate (Nunc, ThermoFisher Scientific, Rochester, NY, USA) for purification to thoroughly remove debris, particles, and beads. DNA was extracted using the Zymo Research 96-well DNA Extraction Kit using 40 µl of beads on a Tecan Fluent in accordance with the manufacturer's instructions. The 16S rRNA gene amplification of the V4 region (forward: CCTACGGGNGGCWGCAG, reverse: GACTACHVGGGTATCTAATCC) was carried out according to an established protocol (477, 478). DNA was standardized to 25 ng/µl and used for sequencing polymerase chain reaction (PCR), including unique 12-base Golary barcodes with specific primers (Sigma-Aldrich, Saint Louis, MI, USA). For every sample, PCR was carried out in triplicate using Q5 polymerase (New England Biolabs, Ipswich, MA, USA), using the following conditions: 30 s initial denaturation at 98°C, followed by 25 cycles of 10 s at 98°C, 20 s at 55°C, and 20 s at 72°C. PCR amplicons were sequenced using 300 bp paired-end sequencing (PE300) on an Illumina MiSeq Sequencing Platform 300PE after pooling and standardization to 10 nmol/L. The Usearch v11 software program examined microbiome data to compile, filter, and cluster the generated reads. Using QIIME v1.8.0 (479), sequences were filtered for low-quality reads and binned based on sample-specific barcodes. fastq_maxdiffs 30 and -fastq_mergepairs were used for the merging process. Fastq_filter (-fastq_maxee 1) was used to do quality filtering with a minimum read length of 300 bp and a minimum of 1,000 reads per sample. By using operational taxonomic units (OTUs) selection, the reads were clustered into 97% identity threshold OTUs, and representative sequences were found using the UPARSE algorithm of Usearch (480).

Representative sequences for each OTU were aligned and taxonomically assigned using the GreenGenes2 database (481).

The processed data was then uploaded into Microbiome Analyst (482) using a low count filter with a prevalence threshold of 10% and a low variance filter with a threshold of 10%. The number of observed OTUs, Shannon index, and Simpson's reciprocal indices were used to assess alpha-diversity. The Bray-Curtis dissimilarity, weighted UniFrac distances, and unweighted UniFrac distances were used to measure β-diversity, and principal coordinate analysis (PCoA) was used to show the data, and the dissimilarities were assessed using PERMANOVA. To evaluate the comparative abundance of different taxa between groups, DESeq2 was used at the genus level.

Statistical analysis

Data was analyzed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), R (4.3.3, https://www.r-project.org/), or SPSS 17 (IBM Corp., Armonk, N.Y., USA). Significance for statistical tests was set to $P < 0.05$ (two-sided), unless stated otherwise. Mean ± SEM was used present all parameters except lipidomics and microbiome data, which were presented as relative fold changes. The Student's t-test compared parameters between LFD and HFD groups. One-way ANOVA followed by Tukey's post hoc test was used to determine differences between MILK, YOG, CHE, and HFD effects.

6.4 RESULTS

HFD-induced obesity and development of MASLD

Table 6.2 compares the adiposity indices and biochemical parameters between HFD and LFD groups. HFD for 8 weeks resulted in notable obesity, specifically, increased body weight gain and lower lean-to-fat mass ratio following increased energy intake, dysglycemia (higher FBG and HOMA-IR), and hyperlipidemia (elevated serum TG and TC in the refed state). In addition, 8 weeks of HFD elicited significantly higher liver TG and TC accumulation than LFD in both fasted and refed states (Fig. 6.2A, B). This was confirmed by histology, which indicated a right shift toward a bigger size distribution of lipid droplets in HFD mice (Fig. 6.2A, B). These results

together demonstrated that the MASLD model is appropriately induced by 8 weeks of feeding C57BL/6 mice with HFD.

Table 6. 2 Comparison of the effect of high-fat and low-fat diets on body weight, calorie intake, and biochemical markers[1].

	LFD	HFD	p-value[2]
Final body weight (g)	30.7 (0.6)	39.5 (0.9)	<0.001
Body weight gain (g)	7.5 (0.6)	13.9 (0.9)	<0.001
Final fat body mass (g)	6.0 (0.5)	12.6 (0.5)	<0.001
Final lean body mass (g)	20.7 (0.2)	21.9 (0.3)	0.002
Lean-to-fat mass ratio	3.6 (0.3)	1.7 (0.3)	<0.001
Average energy intake (kcal/day/mouse)[3]	9.4 (0.1)	12.6 (0.2)	0.010
Energy expenditure (kcal/d)[4]	13.0 (0.3)	11.6 (0.2)	0.010
Initial fasting blood glucose (mmol/L)	7.4 (0.4)	8.1 (0.4)	0.023
Final fasting blood glucose (mmol/L)	7.5 (0.2)	10.6 (0.3)	<0.001
Final serum insulin (pmol/L; n=8)	60.3 (29.7)	158.5 (31.5)	<0.001
HOMA-IR (n=8)	1.5 (0.4)	3.4 (0.7)	0.04
Fasting serum triglyceride (mg/dL; n=8)[5]	116.8 (24.7)	169.2 (16.3)	0.098
Refed serum triglyceride (mg/dL; n=8)[6]	122.9 (15.3)	308.5 (41.9)	<0.001
Fasting serum cholesterol (mg/dL; n=8)	14.3 (1.5)	23.2 (3.3)	0.029
Refed serum cholesterol (mg/dL; n=8)	14.8 (2.4)	23.1 (2.7)	0.033

[1]Values are expressed as mean (standard error of the mean) for n=16 mice unless otherwise indicated.
[2]Compared using the unpaired Student's t-test.
[3] The pellet intake (g) per cage (4 mice per cage) was measured each week of the experiment, and then the average caloric intake for each mouse for a day was estimated using the energy density of each diet.
[4] Energy expenditure was adjusted for body weight using the residual model (as described in Chapter 5)
[5] Measured in 12-h fasted serum collected at euthanasia
[6] Measured in serum collected at euthanasia after refeeding for 4 h, following 12 h of fasting.

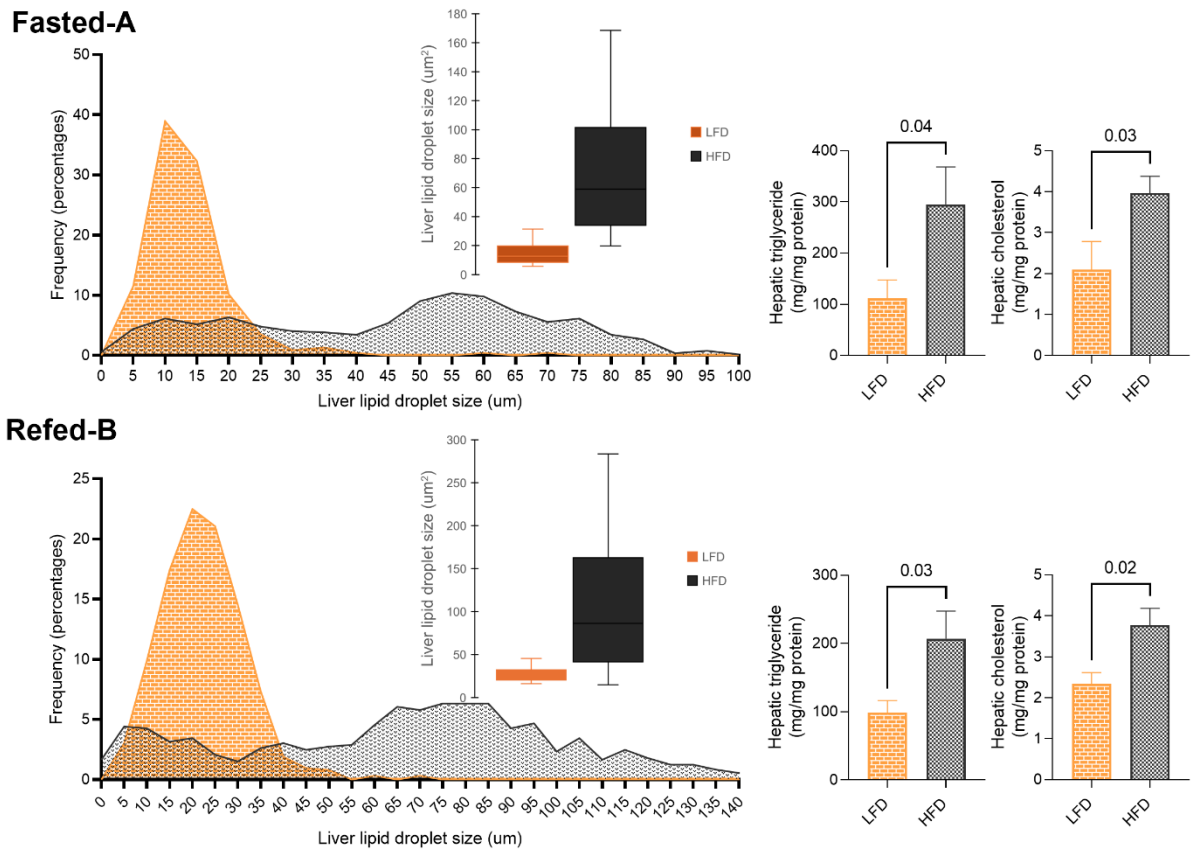

Figure 6. 2 Effects of high-fat diet (HFD) versus low-fat diet (LFD) on hepatic lipid droplet size and hepatic lipid content in C57BL/6 mice.

(A) Frequency distribution of liver lipid droplet size and hepatic triglyceride and cholesterol content in the fasted state. (B) Frequency distribution of liver lipid droplet size and hepatic triglyceride and cholesterol content in the refed state. The p-values indicate significant differences between groups.

Effect of dairy product consumption on obesity phenotypes and glucose and lipid parameters

The overall characteristics of the mice are summarized in Table 6.3, with the HFD supplemented with MILK, YOG and CHE compared with HFD alone. At day 0, there was no significant difference in body weight between the groups fed a HFD. At day 56, MILK, YOG, and CHE mice had lower body weight, weight gain, and fat mass than those in HFD. The lean tissue mass in the MILK and YOG was slightly reduced, while the lean mass in the CHE was similar to the HFD, resulting in MILK and YOG having significantly higher lean-to-fat mass ratios than HFD. Although the average daily energy intake did not differ among the groups, MILK, YOG, and CHE had higher energy expenditure (adjusted for body weight) compared with the HFD group. The FBG at the beginning of the study was similar across all groups, but it was significantly lower in the MILK, YOG, and CHE than in the HFD group at the end of the experiment. Compared to the HFD, mice in MILK had significantly lower serum fasting insulin and HOMA-IR, indicating higher insulin sensitivity. There was a nonsignificant trend for decreased fasting insulin and HOMA-IR in the YOG and CHE groups compared with HFD ($p<0.1$). While fasting, TG was not different between groups, but mice in MILK and YOG had lower serum TG than HFD under the refed condition. No differences in serum TC were observed.

Table 6. 3 Effects of milk, yogurt, and cheese on body weight, calorie intake, and biochemical markers[1,2].

	Groups (n=16)			
	HFD	MILK	YOG	CHE
Initial body weight (g)	25.6 (0.3)[a]	24.6 (0.4)[a]	24.8 (0.4)[a]	25.1 (0.3)[a]
Final body weight (g)	39.5 (0.9)[a]	33.4 (0.3)[b]	33.5 (0.8)[b]	35.4 (1.0)[b]
Body weight gain (g)	13.9 (0.9)[a]	8.5 (0.9)[b]	9.2 (0.7)[b]	10.3 (1.2)[b]
Final fat body mass (g)	12.6 (0.5)[a]	8.5 (0.6)[b]	8.7 (0.6)[b]	9.9 (0.7)[b]
Final lean body mass (g)	21.9 (1.1)[a]	20.6 (0.7)[b]	20.8 (1.2)[b]	21.2 (0.9)[a,b]
Lean-to-fat mass ratio	1.7 (0.3)[a]	2.7 (1.1)[b]	2.6 (0.7)[b]	2.3 (0.8)[a,b]
Average energy intake (kcal/day/mouse)[3]	12.6 (0.2)[a]	12.0 (0.1)[a]	12.3 (0.1)[a]	11.9 (0.3)[a]
Energy expenditure (kcal/d)[4]	11.6 (0.2)[a]	13.1 (0.2)[b]	12.8 (0.2)[b]	12.9(0.2)[b]
Initial fasting blood glucose (mmol/L)	8.1 (0.4)[a]	8.2 (0.3)[a]	8.0 (0.4)[a]	7.5 (0.3)[a]
Final fasting blood glucose (mmol/L)	10.6 (0.3)[a]	9.3 (0.3)[b]	9.2 (0.2)[b]	9.4 (0.4)[b]
Final serum insulin (pmol/L; n=8)	158.5 (31.5)[a]	61.9 (6.9)[b]	94.7 (18.8)[a,b]	94.7 (17.0)[a,b]
HOMA-IR (n=8)	3.4 (0.7)[a]	1.3 (0.2)[b]	2.0 (0.3)[a,b]	2.0 (0.4)[a,b]
Fasting serum triglyceride (mg/dL; n=8)[5]	169.2 (16.3)[a]	157.4 (23.8)[a]	144.4 (16.3)[a]	149.6 (11.6)[a]
Refed serum triglycerides (mg/dL; n=8)[6]	308.5 (41.9)[a]	191.1 (14.3)[b]	208.6 (16.6)[b]	240 (19.6)[a,b]
Fasting serum cholesterol (mg/dL; n=8)	23.2 (3.3)[a]	23.9(1.3)[a]	22.9 (1.2)[a]	23.1 (1.8)[a]
Refed serum cholesterol (mg/dL; n=8)	23.1 (2.7)[a]	20.4 (1.6)[a]	25.5 (1.8)[a]	20.4 (1.2)[a]

[1]Values are expressed as mean (standard error of the mean).
[2]Different superscript letters within a row indicate a significant difference ($p < 0.05$) between groups according to Tukey's post-hoc test following ANOVA.
[3] The pellet intake (g) per cage (4 mice per cage) was measured each week of the experiment, and then the average caloric intake for each mouse for a day was estimated using the energy density of each diet.
[4] Energy expenditure was adjusted for body weight using the residual model
[5] Measured in 12-h fasted serum collected at euthanasia
[6] Measured in serum collected at euthanasia after refeeding for 4 h, following 12 h of fasting

Effect of dairy product consumption on accumulation of fat and lipid metabolism in the liver

Consistent with reduced adiposity indices in dairy-fed groups, visual examination of the liver histology samples (Fig. 6.3A) revealed smaller lipid droplets than in HFD livers. MILK had the most notable decrease in hepatic lipid droplet size, followed by YOG and CHE, as shown in the size-frequency distribution in both fasted (Fig. 6.3B) and refed states (Fig. 6.4A). Liver TG content of MILK and YOG was about one-third that of HFD in the fasting state (Fig. 6.3C), while in the refed state, MILK and YOG showed a trend ($p<0.1$) to decreased liver TG content compared to the HFD group (Fig. 6.4B). No significant differences were found in hepatic TC content (Fig. 6.3D) between the groups.

To investigate potential explanations for decreased lipid droplet size and hepatic TG in dairy-treated mice, immunoblotting was conducted on tissues collected in both the refed and fasted states to assess the abundance of regulatory proteins involved in hepatic lipid metabolism. The most consistent results were observed in the fasting state (Fig. 6.3E-K). A significant increase in the phosphorylated AMP-activated protein kinase-α (pAMPK)/AMPK ratio was found in MILK and YOG (Fig. 6.3E). Greater sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor-α (PPAR-α) proteins (Fig. 6.3F, G) were also detected in the MILK group. Increased peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) (Fig. 6.3H) was seen in YOG when compared with the HFD control group. Regarding lipid oxidation enzymes, although long-chain acyl-CoA synthetase-1 (ACSL-1) was similar regardless of the intervention (Fig. 6.3I), carnitine palmitoyltransferase-1α (CPT-1α) was almost 2-fold increased in all 3 intervention groups, compared with mice on HFD (Fig. 6.3J). Some effects of dairy were also seen in tissue from refed mice. YOG increased AMPK phosphorylation (pAMPK/AMPK) 1.8-fold in refed mice (Fig. 6.4D), while the MILK group showed an increase in ATGL, which catalyzes lipolysis (Fig. 6.4K), but no other differences were detected.

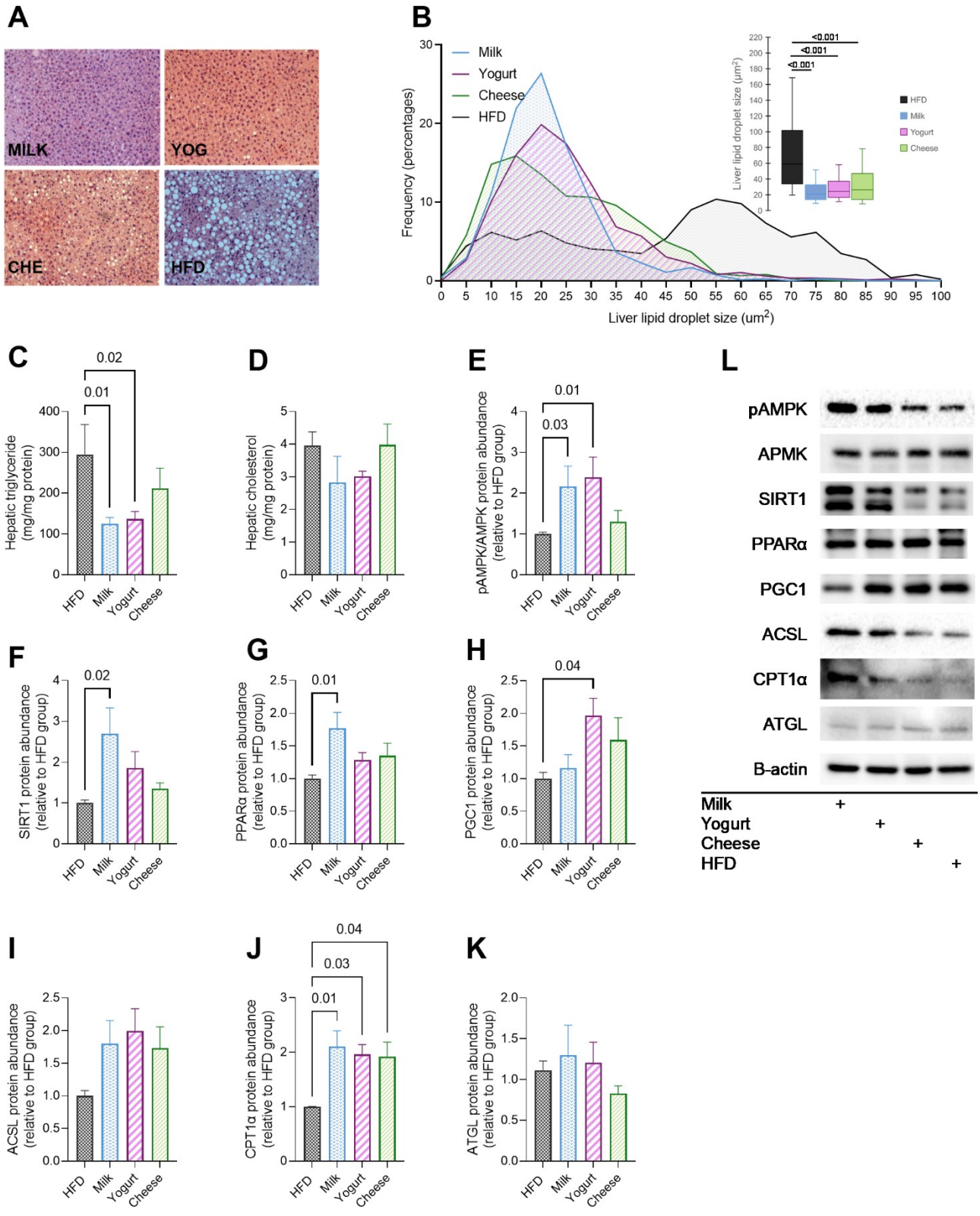
A
MILK
YOG
CHE
HFD
B
Milk
Yogurt
Cheese
HFD
Frequency (percentages)
Liver lipid droplet size (um2)
<0.001
<0.001
<0.001
Liver lipid droplet size (μm2)
C
Hepatic triglyceride (mg/mg protein)
0.02
0.01
D
Hepatic cholesterol (mg/mg protein)
E
pAMPK/AMPK protein abundance (relative to HFD group)
0.01
0.03
L
pAMPK
APMK
SIRT1
PPARα
PGC1
ACSL
CPT1α
ATGL
B-actin
Milk +
Yogurt +
Cheese +
HFD +
F
SIRT1 protein abundance (relative to HFD group)
0.02
G
PPARα protein abundance (relative to HFD group)
0.01
H
PGC1 protein abundance (relative to HFD group)
0.04
I
ACSL protein abundance (relative to HFD group)
J
CPT1α protein abundance (relative to HFD group)
0.04
0.03
0.01
K
ATGL protein abundance (relative to HFD group)
HFD
Milk
Yogurt
Cheese

Figure 6. 3 Effects of milk, yogurt, and cheese on hepatic steatosis in HFD-fed C57BL/6 mice

(A) Representative microscopic images of liver sections stained with H&E from each group. (B) Frequency distribution of the liver lipid droplet size and box plot quantification of the liver lipid droplet size (in μm^2). Colorimetric quantification of (C) triglycerides and (D) total cholesterol concentrations in the liver. Western blot results indicating liver lipid metabolism pathway enzyme abundance in the fasting state for lipid oxidation, including (E) pAMPK/AMPK, (F) SIRT1, (G) PPARα, (H) PGC1α, (I) ACSL, (J) CPT1α, and (K) lipolysis enzyme ATGL. (L) Representative immunoblots (fasted state). Data represent the mean ± SE of n=6-8 mice. P-values are indicated for pairwise comparisons following ANOVA and Tukey's post-hoc test.

Figure S2

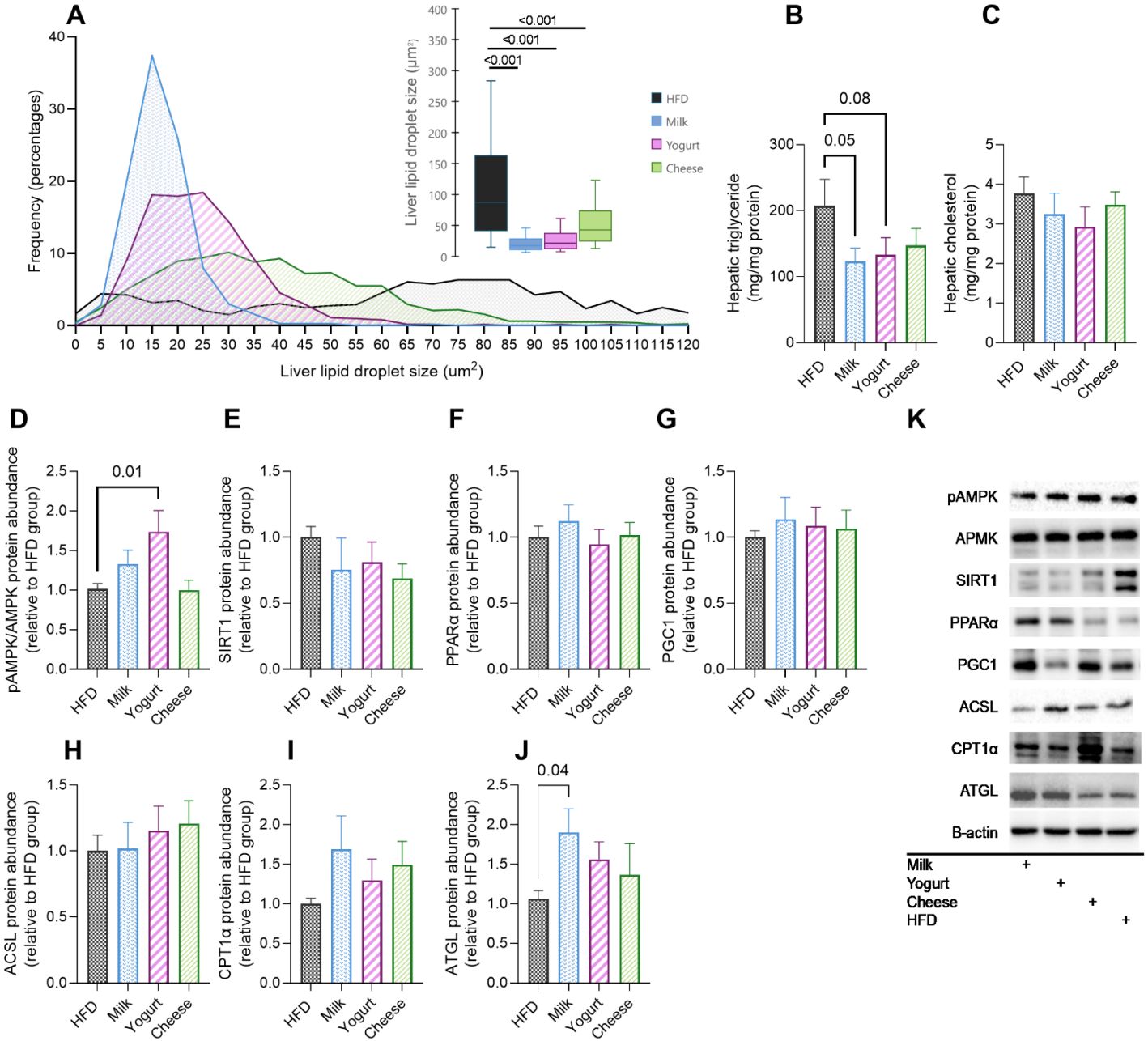
A
B
C
D
E
F
G
K
H
I
J
Frequency (percentages)
Liver lipid droplet size (um²)
Liver lipid droplet size (μm²)
<0.001
<0.001
<0.001
HFD
Milk
Yogurt
Cheese
Hepatic triglyceride (mg/mg protein)
0.08
0.05
Hepatic cholesterol (mg/mg protein)
pAMPK/AMPK protein abundance (relative to HFD group)
0.01
SIRT1 protein abundance (relative to HFD group)
PPARα protein abundance (relative to HFD group)
PGC1 protein abundance (relative to HFD group)
ACSL protein abundance (relative to HFD group)
CPT1α protein abundance (relative to HFD group)
ATGL protein abundance (relative to HFD group)
0.04
pAMPK
APMK
SIRT1
PPARα
PGC1
ACSL
CPT1α
ATGL
B-actin
Milk
Yogurt
Cheese
HFD

Figure 6. 4 Effects of milk, yogurt, and cheese on hepatic steatosis in HFD-fed C57BL/6 mice in the refed state.

(A) Representative microscopic images of liver sections stained with H&E from each. (B) Frequency distribution of the liver lipid droplet size and box plot quantification of the liver lipid droplet size (in μm^2). Colorimetric quantification of (C) triglycerides and (D) total cholesterol concentrations in the liver. Western blot results indicating liver lipid metabolism pathway enzyme abundance in the refed state for lipid oxidation, including (E) pAMPK/AMPK, (F) SIRT1, (G) PPARα, (H) PGC1α, (I) ACSL, (J) CPT1α, and (K) lipolysis enzyme ATGL. (L) Representative immunoblots (fasted state)). Data represent the mean ± SE of n=6-8 mice. P-values are indicated for pairwise comparisons following ANOVA and Tukey's post-hoc test.

Effect of dairy product consumption on liver lipogenesis, lipid import and export

Adipogenesis is controlled by PPAR-γ, which promotes fatty acid storage in lipid droplets in the liver. All groups had a similar abundance of PPAR-γ relative to the HFD group in both fasted (Fig. 6.5A) and refed (Fig. 6.6A) states. In the study of lipogenic enzymes, immunoblotting revealed a notable increase in the phosphorylation of acetyl-CoA carboxylase (pACC/ACC), a rate-limiting enzyme whose activation suppresses *de novo* lipogenesis, in the fasting state (Fig. 6.5B) of MILK, YOG, and CHE groups compared to HFD. No significant differences among the groups in the fasting state were observed in fatty acid synthase (FAS) (Fig. 6.5C), phosphorylated ATP-citrate lyase (pACL)/ACL (Fig. 6.5D), and acetyl-CoA synthetase (AceCS) (Fig. 6.5E). Similarly, refeeding did not result in significant differences in those proteins across the groups (Fig 6.6B-E). Furthermore, the abundance of microsomal triglyceride transfer protein (MTP; lipid export) and cluster of differentiation 36 (CD36; fatty acid uptake) did not change in response to dairy intervention in either fasted (Fig. 6.5F,G) or refed states (Fig. 6.6F,G). All groups had similar fibroblast growth factor 21 (FGF21) relative to the HFD group in both the fasted (Fig. 6.5H) and refed states (Fig 6.6H); likewise, no differences in OXPHOS complex was detected (Fig. 6.5I and Fig. 6.6I).

Figure 3

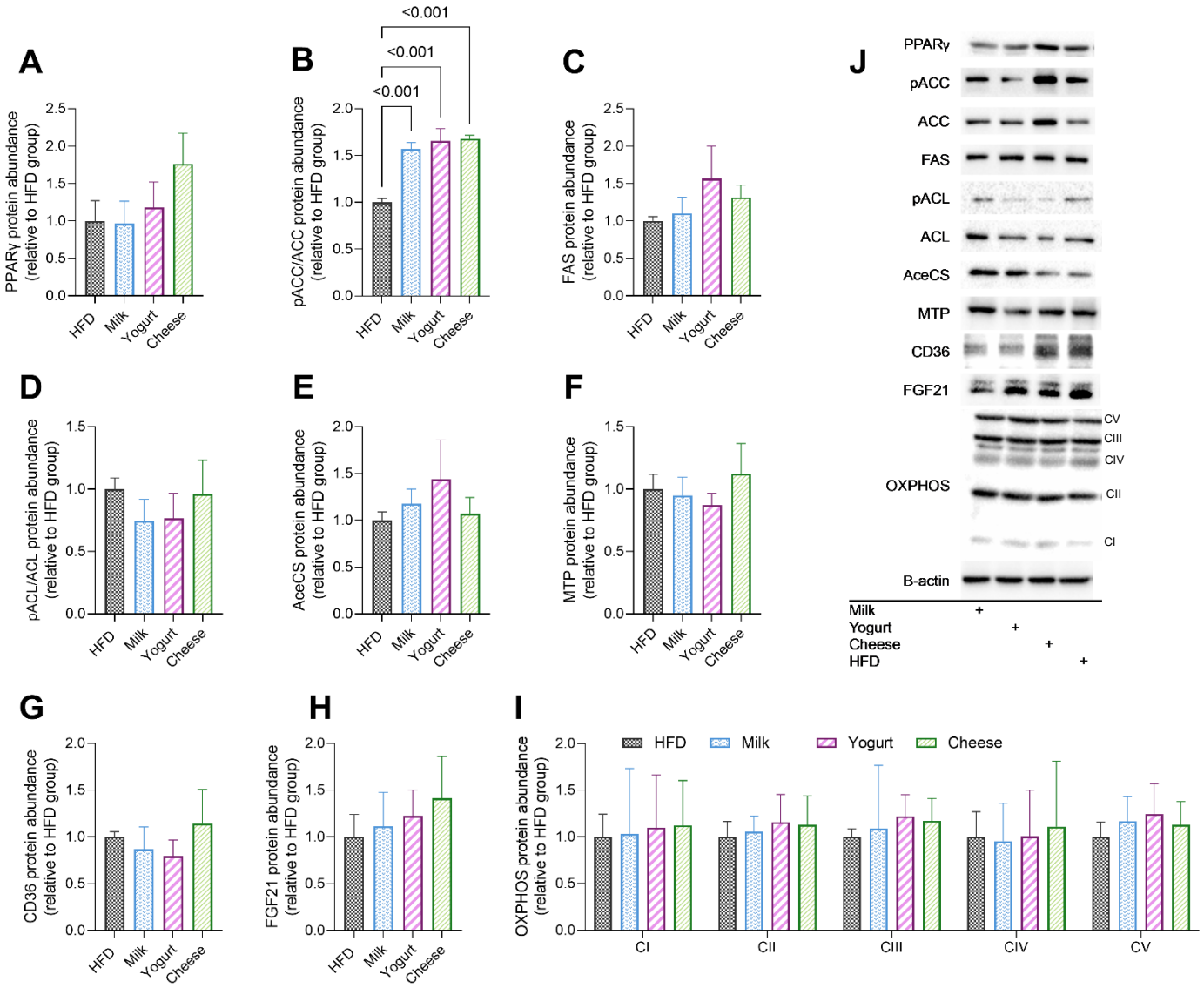
A
PPARγ protein abundance (relative to HFD group)
B
pACC/ACC protein abundance (relative to HFD group)
<0.001
<0.001
<0.001
C
FAS protein abundance (relative to HFD group)
J
PPARγ
pACC
ACC
FAS
pACL
ACL
AceCS
MTP
CD36
FGF21
OXPHOS
CV
CIII
CIV
CII
CI
B-actin
Milk
Yogurt
Cheese
HFD
D
pACL/ACL protein abundance (relative to HFD group)
E
AceCS protein abundance (relative to HFD group)
F
MTP protein abundance (relative to HFD group)
G
CD36 protein abundance (relative to HFD group)
H
FGF21 protein abundance (relative to HFD group)
I
OXPHOS protein abundance (relative to HFD group)
HFD
Milk
Yogurt
Cheese
CI
CII
CIII
CIV
CV

Figure 6. 5 Effects of milk, yogurt, and cheese on hepatic de novo lipogenesis and lipid uptake and export in HFD-fed C57BL/6 mice.

Western blot results indicate liver lipid metabolism pathway enzyme abundance in the fasting state involved in de novo lipogenesis, including (A) PPARγ, (B) pACC/ACC, (C) FAS, (D) pACL/ACL, and (E) AceCS. Relative abundance of proteins involved in lipid export (F) MTP and lipid uptake (G) CD36. Overall energy metabolism-related enzymes include (H) FGF21 and (I) OxPhos complex subunits. Representative immunoblots (fasted state, n = 6-8 mice). Data represent the mean ± SE of n=6-8 mice. P-values are indicated for pairwise comparisons following ANOVA and Tukey's post-hoc test.

Figure S3

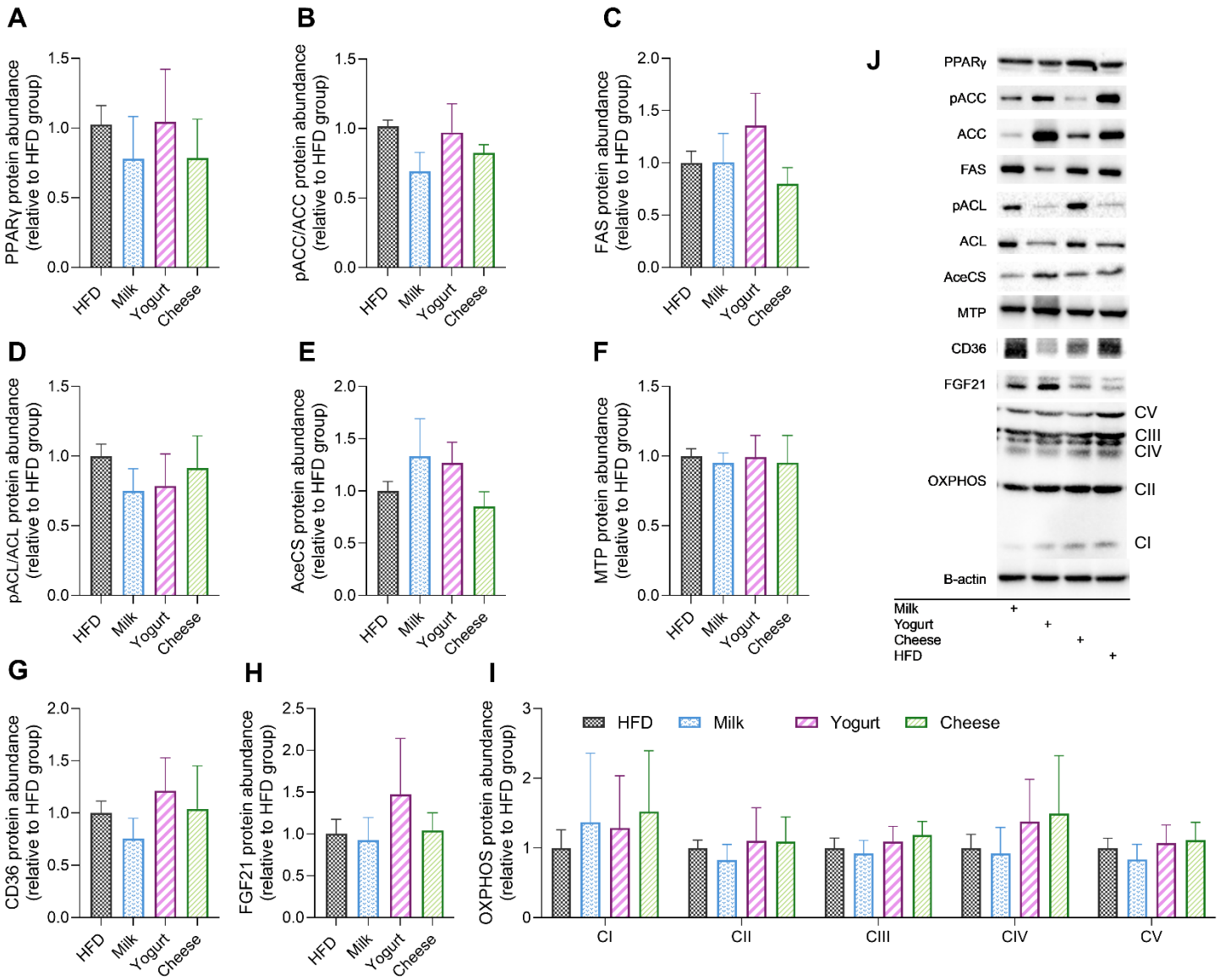
A
PPARγ protein abundance (relative to HFD group)
B
pACC/ACC protein abundance (relative to HFD group)
C
FAS protein abundance (relative to HFD group)
D
pACL/ACL protein abundance (relative to HFD group)
E
AceCS protein abundance (relative to HFD group)
F
MTP protein abundance (relative to HFD group)
G
CD36 protein abundance (relative to HFD group)
H
FGF21 protein abundance (relative to HFD group)
I
OXPHOS protein abundance (relative to HFD group)
HFD
Milk
Yogurt
Cheese
CI
CII
CIII
CIV
CV
J
PPARγ
pACC
ACC
FAS
pACL
ACL
AceCS
MTP
CD36
FGF21
OXPHOS
B-actin
Milk
Yogurt
Cheese
HFD

Figure 6. 6 Effects of milk, yogurt, and cheese on hepatic de novo lipogenesis and lipid uptake and export in HFD-fed C57BL/6 mice in the refed state.

Western blot results indicate liver lipid metabolism pathway enzyme abundance in the refeding state involved in de novo lipogenesis, including (A) PPARγ, (B) pACC/ACC, (C) FAS, (D) pACL/ACL, and (E) AceCS. Relative abundance of proteins involved in lipid export (F) MTP and lipid uptake (G) CD36. Overall energy metabolism-related enzymes include (H) FGF21 and (I) OxPhos complex subunits. Representative immunoblots (fasted state, n = 6-8 mice). Data represent the mean ± SE of n=6-8 mice. P-values are indicated for pairwise comparisons following ANOVA and Tukey's post-hoc test.

Effect of dairy product consumption on insulin-regulated liver enzymes

The phosphorylation of AKT, as a marker of hepatic insulin action, was significantly increased in the refed state and showed a trend of remaining increased in the fasted state (Fig. 6.5A) in the MILK group. Consistent with enhanced insulin sensitivity, further investigation of enzymes controlling hepatic glucose production revealed a decreased abundance of essential gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) among MILK, YOG, and CHE in the refed state (Fig 6.7B) and glucose 6-phosphatase (G6PC) in the YOG group compared with HFD (Fig. 6.7C). In the fasting state, the PEPCK was lower in MILK than HFD (Fig. 6.7B).

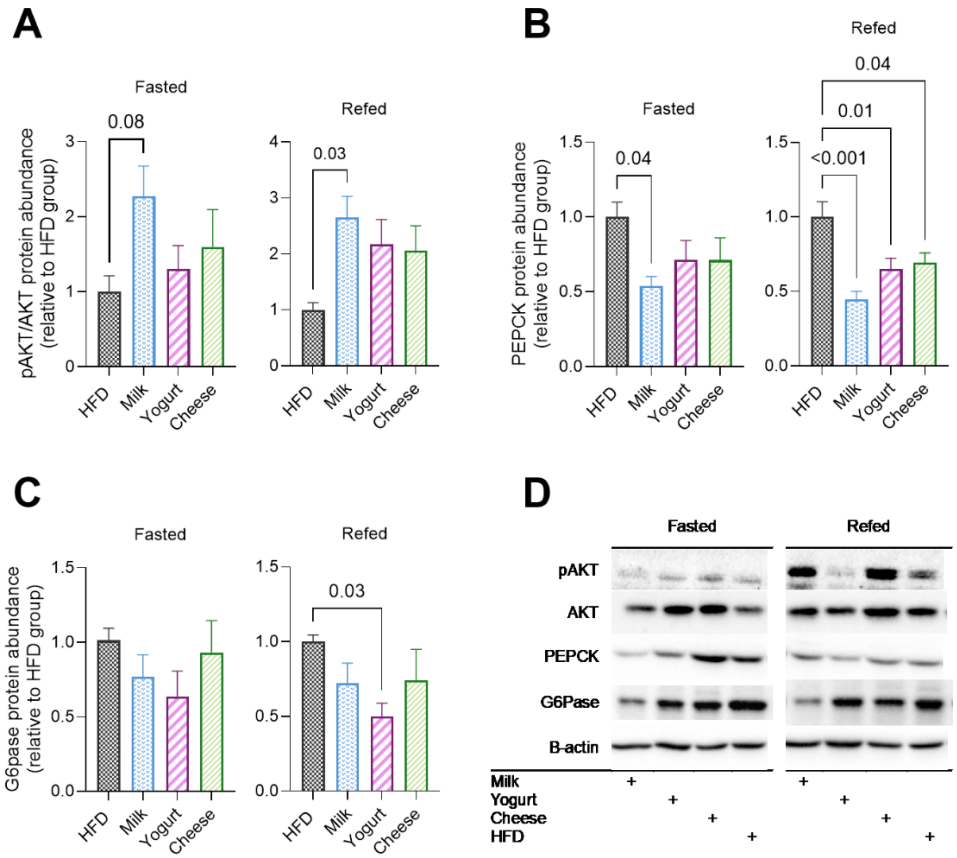

Figure 6. 7 Effects of milk, yogurt, and cheese on enzymes involved in hepatic insulin signaling and gluconeogenesis in HFD-fed C57BL/6 mice.

The western blot results indicate liver enzyme abundance in the fasting and refed states of a key regulator of insulin signaling, (A) pAKT/AKT, and rate-limiting gluconeogenesis enzymes, including (B) PEPCK and (C) G6Pase. Representative immunoblots (refed and fasted state). Data represent the mean ± SE for n = 6-8 mice. P-values are indicated for pairwise comparisons following ANOVA and Tukey's post-hoc test.

Effect of dairy product consumption on the liver lipidome

Quantitative analysis of liver lipid metabolites was performed to compare overall liver metabolic changes in mice fed with a HFD with and without dairy supplementation. Unsupervised PCA (Fig. 6.9A) and supervised PLS-DA (Fig. 6.8A) did not show distinct clusters of liver lipid species across groups. However, sPLS-DA models, which achieve better discrimination by considering 100 of the most discriminative features, showed clustering by dairy intervention with a clear separation of the MILK group from the HFD-control group and also from the YOG and CHE groups (Fig. 6.8B). Variable importance in projection (VIP) scores were used to identify the most critical lipid molecules for the clustering (Fig. 6.8C). Among the lipidomic features, phosphatidylserine (PS) 38:1, DG O-42:3, hexosylceramides (HexCer):40:2;O2, prenol (PR) 23:3;O2, sulfatides (SHexCer) 38:6;O, phosphatidylcholines (PC) -34:4, and TG 41:1'O were recognized as driving the separation on the basis of VIP scores. To contrast differences across the groups, a heatmap (Fig. 6.8D) was generated from group averages with clustering by Ward linkage of the top 25 highly significant differences in lipids identified, revealing their discrepancies across groups. The unique lipidomic profiles in response to each intervention were demonstrated through these analyses, with HFD illustrating a distinct pattern from MILK and YOG but not different from CHE.

Compared to the HFD-fed mice, 498 hepatic lipid species were significantly altered in the MILK group, including 252 up-regulated and 246 down-regulated lipids (Fig. 6.8E) while 664 lipids were changed significantly in YOG vs. HFD, of which 397 lipids were up-regulated and 267 lipids were down-regulated (Fig. 6.8F). In addition, 253 lipids were up-regulated, and 175 lipids were down-regulated in CHE group compared with HFD group (Fig 6.8G). When compared to HFD, 80 lipids were consistently up-regulated in all 3 dairy intervention groups (Fig. 6.8H), and 34 lipids were consistently down-regulated (Fig. 6.8I). The up-regulated lipids common between dairy products consisted of 13 TG, 8 DG, 10 Cer, 6 HexCer, 4 PI, 4 PS, 6 PE, 5 phosphatidylglycerols (PG) and various other lipids. The 34 down-regulated lipids comprised 6 PG, 4 HexCer, 3 DG, 5 TG, and others (Fig. 6.9B). In addition, several lipid species were identified that were consistently upregulated in the MILK and YOG but not in the CHE group (Fig 6.9C). Notably, DG such as DG O-39:0;O2 and DG O-61:5, Cer including Cer 44:6;O3 and Cer 38:5;O2, and TG like TG 74:3;O2 and TG 16:0_22:0_22:4 were significantly elevated in the MILK and YOG groups. In addition, distinct lipid signatures in both MILK and YOG groups were also found, with several differentially

upregulated lipid species that may be related to beneficial effects on liver fat accumulation (Fig. 6.9D,E).

Lipid ontology through LION enrichment analysis allowed the identification of potential pathways underlying the impact of consuming dairy products on hepatic lipid metabolism. Lipid species significantly downregulated in the MILK compared with the HFD group were linked to diradylglycerols (GL02), diacylglycerols (GL0201), glycerolipids (GL), and headgroup with neutral charge. The most enriched lipid ontology term in the YOG group was associated with a significant reduction of pathways involving fatty acids with three double bonds, sphingolipids (SP), C20:3, steryl esters (ST0102), diacylglycerols (GL0201), diacylglycerophosphocholines (GP1010), fatty acids with 20 carbons, hexosylceramides, ceramide phosphocholines (sphingomyelins) (SP0301) (Fig 6.8J). However, YOG points to up-regulated pathways linked to lipids headgroup with negative charge, C12:0, glycerophospholipids (GP), positive intrinsic curvature, triradylglycerols (GL03), glycerophosphates (GP10), and triacylglycerols (GL0301) (Fig. 6.8K). CHE group showed no significant upregulation or downregulation in specific lipid pathways compared to HFD (Fig 6.9F).

Figure 5

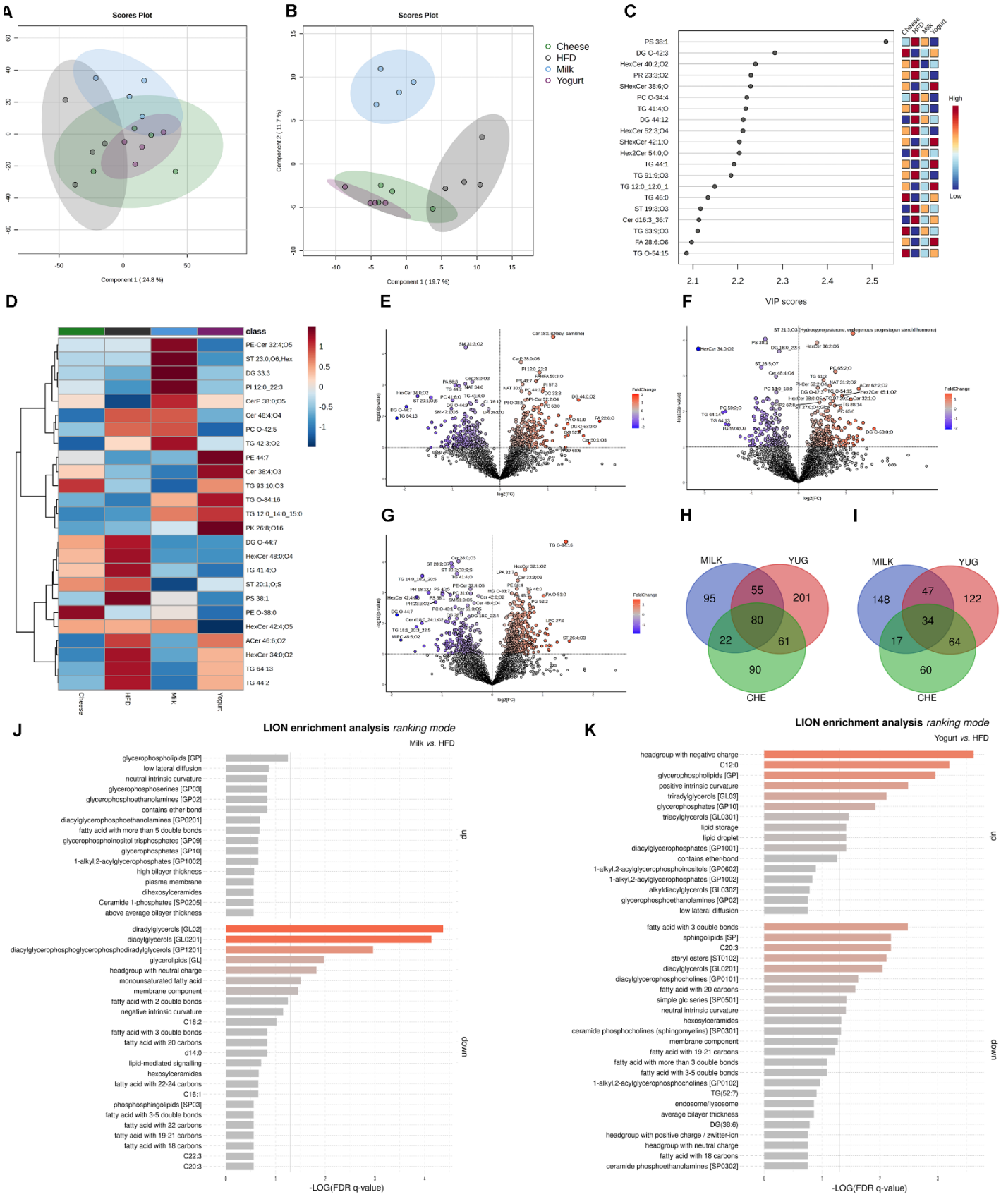
A
Scores Plot
Component 1 (24.8 %)
B
Scores Plot
Component 1 (19.7 %)
Component 2 (11.7 %)
Cheese
HFD
Milk
Yogurt
C
PS 38:1
DG O-42:3
HexCer 40:2;O2
PR 23:3;O2
SHexCer 38:6;O
PC O-34:4
TG 41:4;O
DG 44:12
HexCer 52:3;O4
SHexCer 42:1;O
Hex2Cer 54:0;O
TG 44:1
TG 91:9;O3
TG 12:0_12:0_1
TG 46:0
ST 19:3;O3
Cer d16:3_36:7
TG 63:9;O3
FA 28:6;O6
TG O-54:15
High
Low
VIP scores
D
class
PE-Cer 32:4;O5
ST 23:0;O6;Hex
DG 33:3
PI 12:0_22:3
CerP 38:0;O5
Cer 48:4;O4
PC O-42:5
TG 42:3;O2
PE 44:7
Cer 38:4;O3
TG 93:10;O3
TG O-84:16
TG 12:0_14:0_15:0
PK 26:8;O16
DG O-44:7
HexCer 48:0;O4
TG 41:4;O
ST 20:1;O;S
PS 38:1
PE O-38:0
HexCer 42:4;O5
ACer 46:6;O2
HexCer 34:0;O2
TG 64:13
TG 44:2
Cheese
HFD
Milk
Yogurt
E
F
G
H
MILK
YUG
CHE
95
55
201
80
22
61
90
I
MILK
YUG
CHE
148
47
122
34
17
64
60
J
LION enrichment analysis ranking mode
Milk vs. HFD
glycerophospholipids [GP]
low lateral diffusion
neutral intrinsic curvature
glycerophosphoserines [GP03]
glycerophosphoethanolamines [GP02]
contains ether-bond
diacylglycerophosphoethanolamines [GP0201]
fatty acid with more than 5 double bonds
glycerophosphoinositol trisphosphates [GP09]
glycerophosphates [GP10]
1-alkyl,2-acylglycerophosphates [GP1002]
high bilayer thickness
plasma membrane
dihexosylceramides
Ceramide 1-phosphates [SP0205]
above average bilayer thickness
diradylglycerols [GL02]
diacylglycerols [GL0201]
diacylglycerophosphoglycerophosphodiradylglycerols [GP1201]
glycerolipids [GL]
headgroup with neutral charge
monounsaturated fatty acid
membrane component
fatty acid with 2 double bonds
negative intrinsic curvature
C18:2
fatty acid with 3 double bonds
fatty acid with 20 carbons
d14:0
lipid-mediated signalling
hexosylceramides
fatty acid with 22-24 carbons
C16:1
phosphosphingolipids [SP03]
fatty acid with 3-5 double bonds
fatty acid with 22 carbons
fatty acid with 19-21 carbons
fatty acid with 18 carbons
C22:3
C20:3
up
down
-LOG(FDR q-value)
K
LION enrichment analysis ranking mode
Yogurt vs. HFD
headgroup with negative charge
C12:0
glycerophospholipids [GP]
positive intrinsic curvature
triradylglycerols [GL03]
glycerophosphates [GP10]
triacylglycerols [GL0301]
lipid storage
lipid droplet
diacylglycerophosphates [GP1001]
contains ether-bond
1-alkyl,2-acylglycerophosphoinositols [GP0602]
1-alkyl,2-acylglycerophosphates [GP1002]
alkyldiacylglycerols [GL0302]
glycerophosphoethanolamines [GP02]
low lateral diffusion
fatty acid with 3 double bonds
sphingolipids [SP]
C20:3
steryl esters [ST0102]
diacylglycerols [GL0201]
diacylglycerophosphocholines [GP0101]
fatty acid with 20 carbons
simple glc series [SP0501]
neutral intrinsic curvature
hexosylceramides
ceramide phosphocholines (sphingomyelins) [SP0301]
membrane component
fatty acid with 19-21 carbons
fatty acid with more than 3 double bonds
fatty acid with 3-5 double bonds
1-alkyl,2-acylglycerophosphocholines [GP0102]
TG(52:7)
endosome/lysosome
average bilayer thickness
DG(38:6)
headgroup with positive charge / zwitter-ion
headgroup with neutral charge
fatty acid with 18 carbons
ceramide phosphoethanolamines [SP0302]
up
down
-LOG(FDR q-value)

Figure 6. 8 Lipidomic and enrichment analysis of liver samples from HFD-fed C57BL/6 mice (n=4 per condition) treated with milk, yogurt, and cheese.

(A) PLS-DA scores plot showing separation between groups based on liver lipidomic profiles. (B) sPLS-DA scores plot depicting clustering of lipidomic data by intervention groups using the 100 most significant lipid species. (C) VIP scores from the PLS-DA model identify the top lipid species contributing to group separation. Heatmap of the top 25 lipid species with the most significant differences between intervention groups. (E-G) Volcano plots displaying differentially abundant lipid species in (E) Milk vs. HFD, (F) Yogurt vs. HFD, and (G) Cheese vs. HFD. (H-I) Venn diagrams illustrate the increased (H) and decreased (I) number of lipids species shared between intervention groups compared to HFD. (J-K) LION enrichment analysis shows significantly altered lipid pathways in (J) Milk vs. HFD and (K) Yogurt vs. HFD.

Figure S4

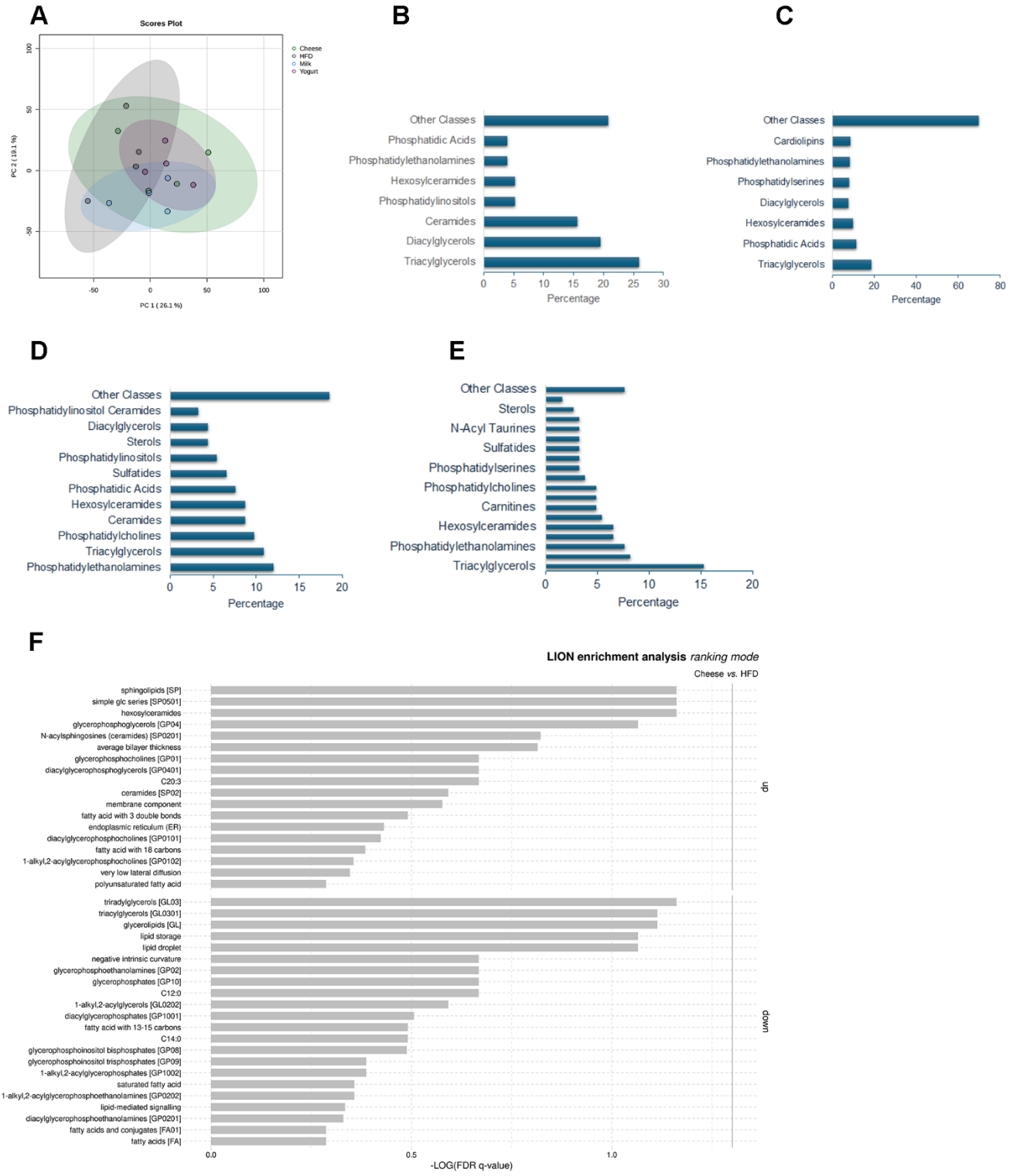
A
Scores Plot
Cheese
HFD
Milk
Yogurt
PC 2 (19.1 %)
PC 1 (26.1 %)
B
Other Classes
Phosphatidic Acids
Phosphatidylethanolamines
Hexosylceramides
Phosphatidylinositols
Ceramides
Diacylglycerols
Triacylglycerols
Percentage
C
Other Classes
Cardiolipins
Phosphatidylethanolamines
Phosphatidylserines
Diacylglycerols
Hexosylceramides
Phosphatidic Acids
Triacylglycerols
Percentage
D
Other Classes
Phosphatidylinositol Ceramides
Diacylglycerols
Sterols
Phosphatidylinositols
Sulfatides
Phosphatidic Acids
Hexosylceramides
Ceramides
Phosphatidylcholines
Triacylglycerols
Phosphatidylethanolamines
Percentage
E
Other Classes
Sterols
N-Acyl Taurines
Sulfatides
Phosphatidylserines
Phosphatidylcholines
Carnitines
Hexosylceramides
Phosphatidylethanolamines
Triacylglycerols
Percentage
F
LION enrichment analysis ranking mode
Cheese vs. HFD
sphingolipids [SP]
simple glc series [SP0501]
hexosylceramides
glycerophosphoglycerols [GP04]
N-acylsphingosines (ceramides) [SP0201]
average bilayer thickness
glycerophosphocholines [GP01]
diacylglycerophosphoglycerols [GP0401]
C20:3
ceramides [SP02]
membrane component
fatty acid with 3 double bonds
endoplasmic reticulum (ER)
diacylglycerophosphocholines [GP0101]
fatty acid with 18 carbons
1-alkyl,2-acylglycerophosphocholines [GP0102]
very low lateral diffusion
polyunsaturated fatty acid
triradylglycerols [GL03]
triacylglycerols [GL0301]
glycerolipids [GL]
lipid storage
lipid droplet
negative intrinsic curvature
glycerophosphoethanolamines [GP02]
glycerophosphates [GP10]
C12:0
1-alkyl,2-acylglycerols [GL0202]
diacylglycerophosphates [GP1001]
fatty acid with 13-15 carbons
C14:0
glycerophosphoinositol bisphosphates [GP08]
glycerophosphoinositol trisphosphates [GP09]
1-alkyl,2-acylglycerophosphates [GP1002]
saturated fatty acid
1-alkyl,2-acylglycerophosphoethanolamines [GP0202]
lipid-mediated signalling
diacylglycerophosphoethanolamines [GP0201]
fatty acids and conjugates [FA01]
fatty acids [FA]
up
down
-LOG(FDR q-value)

Figure 6. 9 Liver lipidomic profiles and LION enrichment analysis liver samples from HFD-fed C57BL/6 mice (n=4 per condition) treated with milk, yogurt, and cheese

(A) Principal component analysis (PCA) scores plot showing the separation of liver lipidomic profiles among the four groups. Lipid class distribution of significantly altered lipid classes in (B) Milk vs. HFD, (C) Yogurt vs. HFD, (D) Cheese vs. HFD, (E) Combined Milk and Yogurt vs. HFD. (F) LION enrichment analysis shows significantly altered lipid pathways in Cheese vs. HFD.

Effect of dairy product consumption on the gut microbiome

The gut microbiome composition was compared between the 4 groups. Alpha-diversity analysis was carried out to assess the within-group diversity using several metrics. The number of observed OTUs that evaluated the richness of microbiota was not different between the groups (Fig 6.10A); hence, a similar richness of microbial features exists between the dairy-supplemented and HFD groups. However, bacterial richness and evenness in YOG mice, as compared to HFD and CHE, as evidenced by increased Shannon (Fig 6.10B) and Simpson indices (Fig 6.10C), significantly increased. The beta-diversity index estimated the differences in microbiota composition across the 4 groups. Overall comparison of groups by Bray-Curtis dissimilarity analysis indicated a significant difference between groups ($p = 0.012$), and post-hoc testing illustrated pairwise differences between MILK-HFD diets and MILK-YOG diets (Fig 6.10D). However, there was no significant difference between HFD and either YOG or CHE. The same pattern was observed when beta diversity was calculated based on unweighted UniFrac distance (Fig 6.10E, $p = 0.001$) and weighted UniFrac distance (Fig 6.10F, $p=0.012$), indicating the bacterial microbiota of MILK clustered apart from that of HFD and YOG. At the genus level, DESeq log 2-fold changes analysis (Fig 6.10G) showed that the MILK supplementation versus HFD significantly enriched the abundance of *Anaerotignum_189125* and *oscillospiraceae_88309* while depleting the abundance of *Avispirillum*, *Longicatena*, and *Turicibacter*. The YOG treatment significantly increased the abundance of *Streptococcus* and *Clostridium* and decreased the *Evtepia*, and *Lachnospiraceae* abundance. Compared with HFD, CHE treatment led to an enrichment of *Sporofaciens* and *Streptococcus* and a depletion of *Limivicinus* abundance.

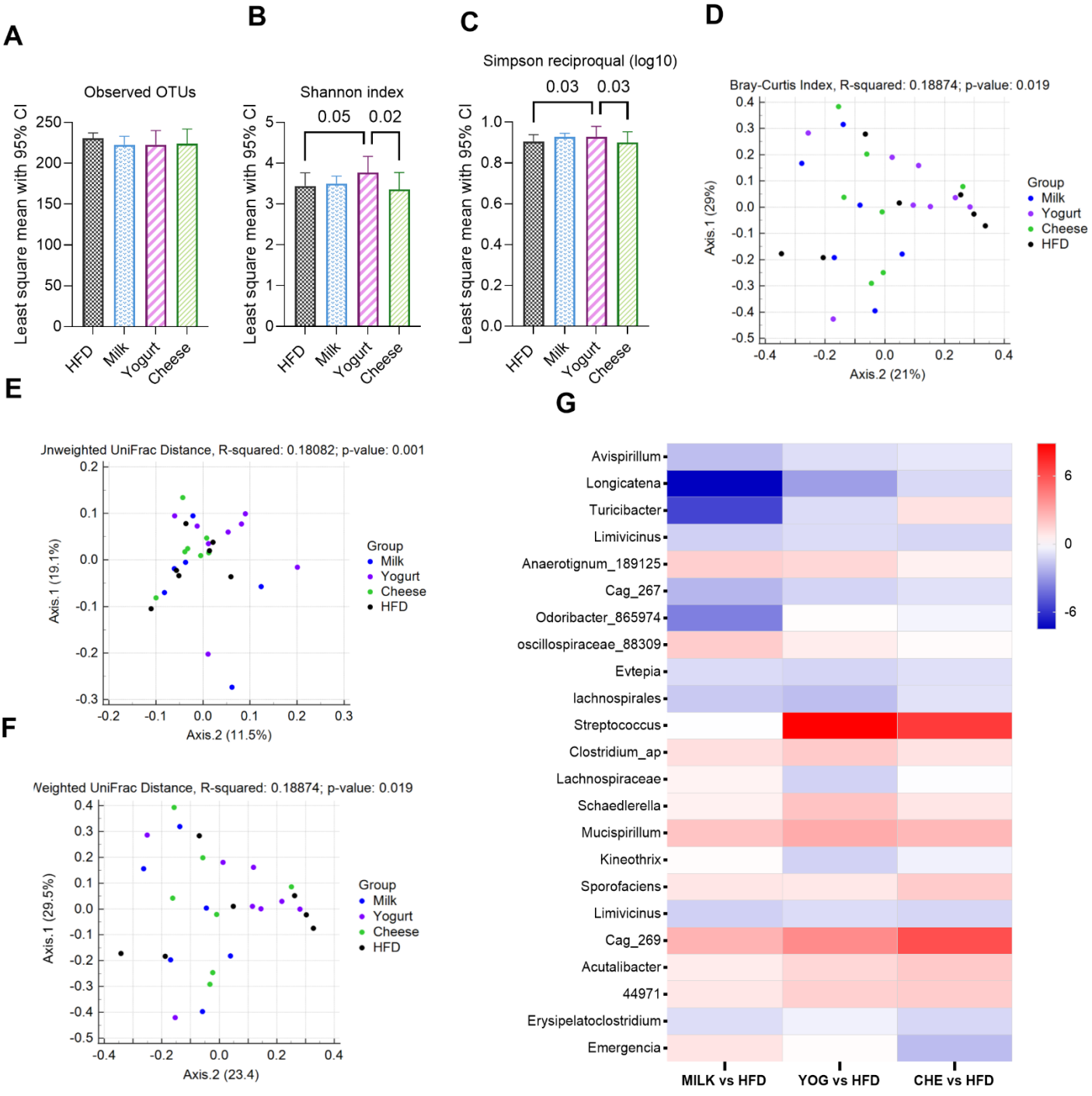

Figure 6. 10 Effects of milk, yogurt, and cheese on gut microbiota composition in HFD-fed C57BL/6 mice.

(A-C) Alpha-diversity indices: (A) Observed OTUs, (B) Shannon index, and (C) Simpson reciprocal (log10), showing the least square mean with 95% confidence intervals (95%CI).

Significant p-values are indicated for pairwise comparisons. (D-F) Beta-diversity analysis: (D) Bray-Curtis index, (E) Unweighted UniFrac distance, and (F) Weighted UniFrac distance. (G) Heatmap displaying the relative abundance of bacterial genera significantly changed by the dietary interventions compared with the HFD group.

6.5 DISCUSSION

The present study aimed to directly compare the effect of consuming MILK, YOG, and CHE in amounts similar to 2 servings per day by humans (about 10% of total energy intake) on obesity phenotype markers, glucose homeostasis, and hepatic steatosis in HFD-induced obese, male mice. To determine the effects of dairy product consumption on lipid and glucose metabolism under physiological conditions, we examined the outcomes during fasting and 4 hours after food reintroduction. We demonstrate that, despite similar energy intake across groups, an 8-week feeding trial with MILK, YOG, and CHE reduces weight gain, increases energy expenditure, and lowers FBG, while YOG and MILK increase the ratio of lean-to-fat mass. Interestingly, only MILK significantly reduces fasting serum insulin and HOMA-IR, indicating enhanced insulin sensitivity, which is consistent with greater activation of liver AKT and suppression of PEPCK during refeeding. Additionally, we report that MILK and YOG lead to lower serum TG in the refed state and lower liver TG content during fasting, with a similar trend in the refed state, which is accompanied by increased fatty acid oxidation-related proteins and decreased DNL enzymes in the liver. Thus, we confirm the hypothesis that MILK, YOG, and CHE all mitigate the development of obesity, independent of changes in caloric intake. Contrary to the belief that fermented dairy products have superior effects, MILK yields a greater impact on IR indicators and hepatic steatosis than YOG or CHE. MILK also exhibits a different serum (Chapter 5) and liver lipid profile than CHE and distinct effects on the gut microbiome. In the following paragraphs, MILK and YOG results will be discussed together based on several similarities in outcomes, whereas CHE diverged in its actions and will be discussed separately.

Recent RCTs show that milk (264, 312) and plain yogurt (483) as part of a habitual diet can improve IR by decreasing fasting insulin, c-peptide, and HOMA-IR. However, the mechanism of action and the liver's contribution to this improvement remain to be elucidated. The liver is a major hub for coordinating fasting to feeding transitions, given its functions in maintaining blood glucose and regulating whole-body energy metabolism (484). Alterations in hepatic gene expression occur during the transition from fasting to fed states (485). During fasting, when blood insulin is minimal, hepatic glucose production is increased through activating PEPCK and G6PC, regulators of the rate of gluconeogenesis (486, 487, 488). In the postprandial state, insulin inhibits hepatic gluconeogenesis by suppressing gluconeogenic enzymes (488). However, in HFD-induced obesity and IR, the insulin regulatory effect through the activation of AKT pathways on the gluconeogenic

control is impaired, and insulin fails to decrease *Pepck* or *G6Pase* mRNA expression during the refeeding period, leading to increased glucose release to the blood, which can cause hyperglycemia (485, 489). We observe that MILK and YOG consumption may attenuate hepatic gluconeogenesis in HFD-fed mice by reducing PEPCK. The MILK and YOG-mediated repression of hepatic gluconeogenesis in our IR model appears to be due to improved hepatic insulin signaling in fed conditions, evidenced by increased phosphorylation of AKT (activation) in the liver. The abundance of the activated form, pAKT, tends to stay high even during a prolonged fasting period, along with a reduced abundance of PEPCK in the MILK group compared to the HFD group, further highlighting the impact of milk in modulating glucose production pathways, both in the fed and fasted states and a probable mechanism to improve IR by reducing FBG and fasting insulin levels, which leads to improved calculation of HOMA-IR in MILK mice. Although there are limited studies investigating the tissue-specific mechanism of milk consumption in animal models of IR, a few studies have shown the beneficial effect of milk components such as whey protein (490, 491) and polar lipids (492) in mitigating the detrimental effect of HFD on glucose homeostasis. In a previous study, Li et al. demonstrated that supplementing the diet with milk-derived polar lipids resulted in enhanced insulin sensitivity and reduced the relative abundance of PEPCK and G6PC in the livers of HFD-fed mice. This was concomitant with elevated p-AKT levels. However, it was not specified whether the tissue was collected during the fasted or refed state (492). Whey protein supplementation effectively decreased *Pepck* and *G6Pase* in the livers of mice fed a high-fat diet to similar levels as observed mice on a low-fat diet, the healthy control group (493). Regarding the effect of yogurt consumption on impaired glucose metabolism, in HFD-induced IR mice, yogurt consumption reduced the HOMA-IR (266, 271) and improved insulin sensitivity in the liver as evidenced by augmented PI3K and p-AKT with suppressed abundance of PEPCK (266). It should be noted that the reduced PEPCK and G6PC in the refed state observed in the YOG group did not reflect on HOMA-IR, which is a fasting indicator of IR; however, utilizing techniques such as glucose tolerance tests or hyperinsulinemic-euglycemic clamps, which assess insulin sensitivity more dynamically, could reveal more subtle beneficial effects of YOG.

In humans, prospective cohort studies (468, 494) and meta-analyses (466, 495) suggest the potential of dairy products to reduce the risk of MASLD. There is growing evidence that dairy products provide health benefits beyond their individual components, likely because of their complex matrices, and should be evaluated separately in terms of outcome assessment. Both MILK

and YOG ameliorate the MASLD phenotype as evidenced by the reduction of hepatic lipid droplet size and TG content, suggesting a considerable reduction in hepatic steatosis and consistent with previous studies in humans that report a negative association between the frequency of newly diagnosed MASLD in the general population and high milk and yogurt intake (391, 468). In our previous meta-analysis, higher milk and yogurt consumption intakes were associated with a lower risk of MASLD compared with those with lower intake (466). Fat accumulation in the liver is a hallmark of MASLD development due to excess fatty acid substrate that surpasses the hepatocytes' ability to oxidize lipids (87). At the molecular level, YOG and MILK both prevent the reduction in AMPK phosphorylation induced by HFD. This would inactivate its downstream target ACC, a key regulatory enzyme of fatty acid synthesis, thereby suppressing the formation of malonyl-CoA, which is used to synthesize fatty acids catalyzed by FAS. Thus, both MILK and YOG may reduce DNL. Similar to our finding, others report a preventive effect of yogurt on HFD-induced MASLD in golden hamsters through the suppression of liver fatty acid production, with activation of AMPK leading to reduced gene expression of *Srebp1*, *Acaca*, and *Fas* (325).

MILK and YOG may also alleviate hepatic steatosis by activating fatty acid β-oxidation by distinct mechanisms (Figure 6.11). Regarding YOG effects, PGC-1α is upregulated downstream of pAMPK, which would be expected to reverse HFD-decreased mitochondrial metabolic imbalance by inducing genes related to mitochondrial biogenesis, OXPHOS, and fatty acid β-oxidation (496). Although the stimulatory impact of the AMPK-PGC-1α pathway did not lead to an increase in OXPHOS subunits, we observed an increase in CPT-1α in the YOG group. When malonyl CoA is low, as during fasting (see above paragraph), CPT1-α enhances the transport of long-chain fatty acids into mitochondria. However, in the MILK group, higher pAMPK occurred with an increased abundance of SIRT1 and PPAR-α, which are involved in enhancing lipid oxidation and lipolysis, as well as lower DNL enzymes. Indeed, several studies show a link between the activation of AMPK and increased abundance of SIRT1 (497, 498), both of which are stimulated by various milk-derived bioactive molecules (499, 500, 501, 502), and their activation protects against HFD-induced hepatic lipotoxicity (503). AMPK-SIRT1 signaling pathway upregulates PPAR-α, thereby promoting fatty acid oxidation via increasing CPT-1α and suppressing lipogenesis via deactivating ACC (497, 504). In addition to effects enzymes involved in DNL and β-oxidation, MILK increases liver ATGL, which promotes lipolysis, leading to smaller lipid droplets and reduced liver TG, as we observe. Our results are consistent with a human tissue experiment demonstrating enhanced

SIRT1 activity and expression in muscle and adipose tissue after incubating adipocytes and myocytes with serum from patients on a high-dairy diet (447).

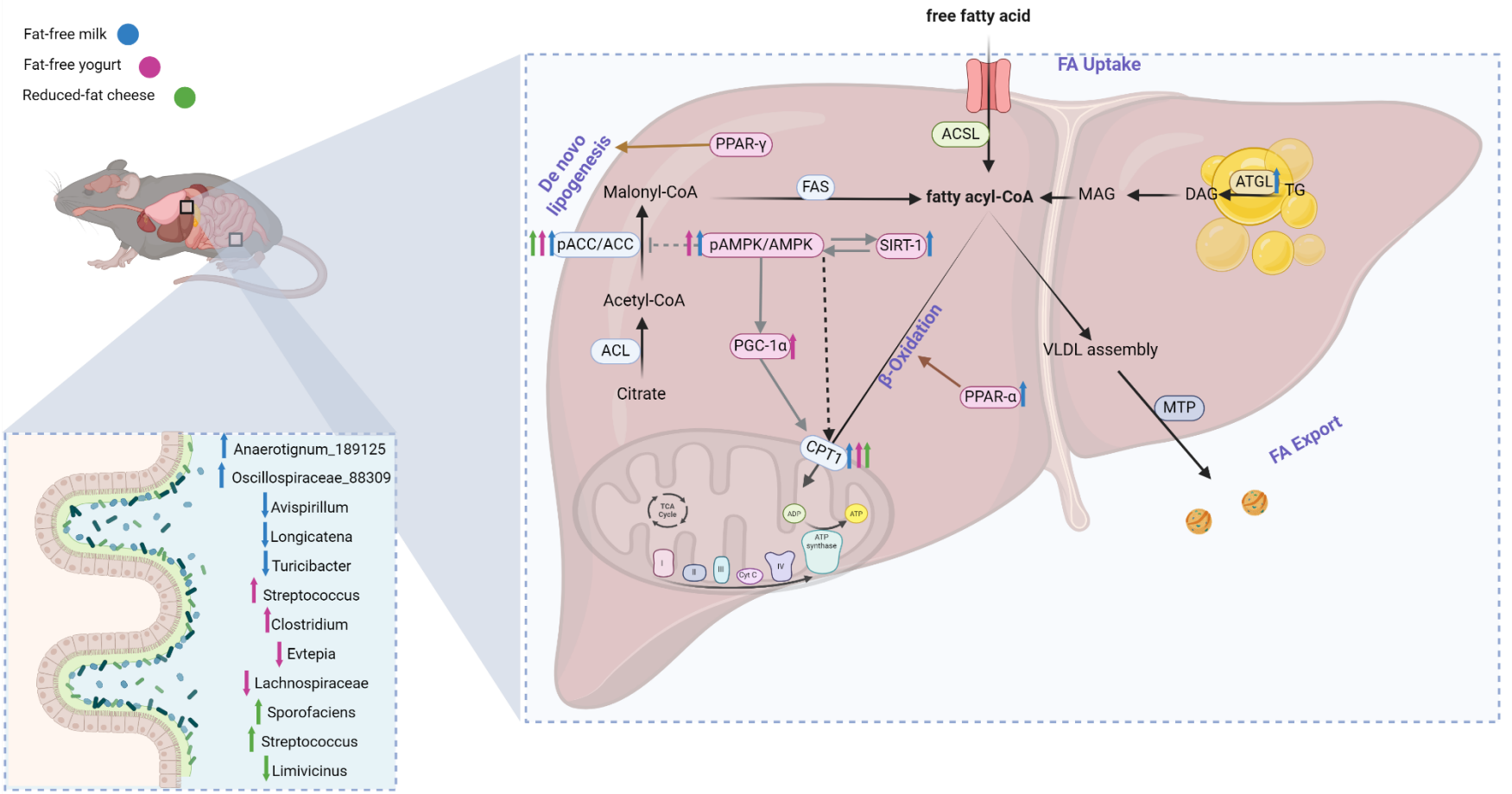

Figure 6. 11 Proposed mechanisms by which fat-free milk, fat-free yogurt, and reduced-fat cheese influence hepatic lipid metabolism and gut microbiota composition in high-fat diet (HFD)-induced obese mice.

There are several critical metabolic pathways in the liver. Phosphorylation of AMP-activated protein kinase (AMPK) is increased, promoting fatty acid oxidation and inhibiting de novo lipogenesis through acetyl-CoA carboxylase (ACC) phosphorylation. Upregulation of Sirtuin 1 (SIRT1) activates AMPK and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α),

enhancing fatty acid oxidation and mitochondrial biogenesis. Increased expression of peroxisome proliferator-activated receptor -α (PPAR-α) stimulates fatty acid oxidation and decreases lipid accumulation, while PPAR-γ modulates lipid storage. Carnitine palmitoyltransferase 1 (CPT1) facilitates the transport of fatty acids into mitochondria for β-oxidation. Adipose triglyceride lipase (ATGL) promotes the breakdown of triglycerides (TG) into free fatty acids (FFAs). Acyl-CoA synthetase long-chain family member 1 (ACSL1) converts FFAs into fatty acyl-CoA, a substrate for β-oxidation and lipid synthesis. Fatty acid-binding protein (FABP) transports FFAs across the cell membrane. Microsomal triglyceride transfer protein (MTP) is involved in very-low-density lipoprotein (VLDL) assembly and lipid export from the liver. Created with BioRender.com by EY.

To understand how milk and yogurt consumption is associated with health benefits, we conducted an extensive computational lipidomic study to assess lipid-mediated signaling and membrane remodeling induced by dairy feeding. Our data suggests that after 8 weeks of intervention with MILK, YOG, and CHE, the patterns of liver lipid species show a clear separation between the dairy intervention groups and show that the liver lipid profiles of the HFD-control group are markedly distinct pattern from those of the MILK group, less different from YOG, and with more similarities with CHE group. Interestingly, both MILK and YOG significantly reduced DGs, which are identified as biomarkers of lipid-induced IR, formation of lipid droplets, and increased lipotoxicity (505). This aligns with the reduced hepatic steatosis and improved lipid metabolism after MILK and YOG intervention. However, the analysis indicates that YOG and MILK also have distinct effects on hepatic lipid profile. Diradylglycerols, glycerolipids, and headgroups with neutral charge are frequently elevated in metabolic disorders (506). Thus, their downregulation of the MILK group is an indication that these molecules are disposed of more efficiently and not accumulated in the liver, reflecting improved lipid metabolism and reduction in lipotoxicity (506). In contrast, YOG supplementation decreases hepatic sphingolipids, long-chain fatty acids, and ceramides associated with pro-inflammatory actions and hepatic fat accumulation; therefore, their decrease signifies reduced inflammation and healthier metabolism (507, 508). YOG also increases glycerophospholipids, important structural components of cellular membranes that influence membrane fluidity and are involved in signaling (509). The upregulation of glycerophospholipids and headgroups of negative charge and positive intrinsic curvature could indicate an enhancement of membrane dynamics and improvement in cellular functions (510, 511). The concomitant increase in the metabolization of medium-chain fatty acid C12:0 and decrease in the metabolism of long-chain fatty acids in YOG may also lead to better energy utilization and less fat accumulation since C12:0 is rapidly metabolized compared to long-chain fatty acids (512).

The current understanding of how TG and intermediate-disease markers are linked is that there is a distinct advantage of measuring postprandial and non-fasting TG concentration over fasting TG concentration in the prediction of early metabolic dysfunction (513, 514, 515, 516). Plasma TG rises after a meal, and higher peak concentrations or delayed clearance of postprandial TG may indicate an aberrant response to food intake, a condition linked to numerous metabolic abnormalities, including IR (515) and MASLD (516). Lower TG after feeding indicates that YOG and MILK specifically improve lipid handling, reducing the surge of TG that typically follows

consuming a HFD and appearing similar to TG after intake of the LFD. While some studies investigating the matrix effects of dairy products on fasting TG show a neutral effect (264, 517, 518, 519), data on non-fasting TG is scarce in human and rodent studies. Also, the postprandial TG-lowering ability of yogurt and milk tested in human intervention trials (520, 521) supports its potential benefit for attenuating harmful lipid spikes arising from the intake of high-fat diets. Several mechanisms that uniquely contribute to high TG in the fed state have been proposed, many of which are tied to IR (513). Given the poor suppression of VLDL secretion by insulin in an IR liver, MILK and YOG may improve the non-fasting TG by enhancing hepatic AKT activation, leading to better suppression of VLDL postprandially. This improved AKT activation in the liver likely contributes to the observed reductions in non-fasting TG, indicating a beneficial impact of MILK and YOG on lipid metabolism by promoting insulin signaling.

Most of the beneficial effects of yogurt on IR and hepatic steatosis are attributed to its probiotics, which may mitigate dysbiosis. Our data shows *Streptococcu*s enrichment in the gut microbiome of the YOG group, consistent with the results of others. These bacteria enhance gut barrier function and generate beneficial metabolites, such as lactic acid, that suppress the growth of pathogenic bacteria (522). Furthermore, during milk fermentation, yogurt starter culture organisms like *Streptococcus thermophilus* generate metabolites, including branched-chain hydroxy acids (BCHA), which are concentrated in yogurt (523). Yogurt consumption increases plasma and liver BCHA levels, improving insulin action on glucose metabolism in the liver and suppressing hepatic glucose production (273). In addition, we report that the *Clostridium* genus, known as short-chain fatty acid (SCFA)-producing bacteria, is increased in the feces of mice in YOG. In the host, SCFAs enhance insulin sensitivity and reduce inflammation, preventing excessive fat accumulation in the liver (524, 525). YOG group also shows the depletion of *Lachnospiraceae* and Evtepia, which are associated with gut dysbiosis and predispose individuals to develop metabolic disorders. They increase after consuming a Western-style diet and in HFD-induced obesity (526, 527). Colonization of *Lachnospiraceae* in the gut of germ-free mice exacerbates the development of obesity and impairs glucose tolerance, leading to T2D (527). Interestingly, Clarke et al. demonstrate a relative increase in the abundance of bacteria from *Lachnospiraceae* in HFD-induced obese versus lean mice (528). By modulating the gut microbiota, yogurt can help mitigate the adverse effects of an HFD, lead to a healthier gut environment, improve insulin sensitivity, and reduce the risk of hepatic steatosis.

MILK, which contains carbohydrate molecules with prebiotic properties (223), increased beneficial gut bacteria such as Anaerotignum_189125 and oscillospiraceae_88309 and reduced potentially harmful bacteria *Avispirillum*, *Longicatena*, and *Turicibacter*, which is an indication of a healthier gut environment (529). *Anaerotignum* and *Oscillospiraceae* are anaerobic bacteria that contribute to preserving the integrity of the gut barrier. Reduced abundance of these genera allows an increase in gut permeability, leading to translocation of endotoxins like lipopolysaccharides into the circulation, hence enhancing inflammation and IR (530). These genera produce SCFAs such as butyrate, which modulates hepatic glucose and lipid metabolism by an AMPK-dependent mechanism (531, 532), consistent with our liver metabolism findings. While the individual contributions of *Avispirillum* and *Longicatena*to metabolic health are not well defined, the decrease found in response to MILK treatment indicates a gut microbiome profile that promotes better metabolic functions (533), and might be part of a broader beneficial shift in the gut microbiota induced by MILK.

Compared with MILK and YOG interventions, the pattern of response to CHE is quite different, consistent with its distinct nutrient profile and matrix. CHE attenuates body weight gain and improves the lean-to-fat ratio and FBG compared with HFD, to a similar extent as MILK and YOG as discussed elsewhere (Chapter 5), but the effect on insulin sensitivity is less consistent, both systemically as measured by HOMA-IR being intermediate between MILK and HFD, and at the molecular level, which demonstrates less PEPCK abundance in the refed state although pAKT was not enhanced. Similarly, plasma and liver TG are not statistically different between CHE and HFD groups, although reducing trends are observed. Regarding hepatic lipid metabolism, increased phosphorylation of ACC in the fasting condition may suggest suppression of DNL, which indicates a shift to reduced hepatic lipid synthesis, consistent with the smaller but still distinctly visible lipid droplets. Moreover, no significant changes are observed in the lipidomic analysis for CHE compared with the HFD. This would indicate that, although cheese supplementation had overall effects on improving glucose and lipid metabolism, the connection with changes in lipid species is not as pronounced as observed with other dairy interventions. Cheese intake has demonstrated inconsistent results on its effect on IR and MASLD. Hanning et al. indicate that both low- and regular-fat cheeses enhanced insulin sensitivity and reduced hepatic glucose production by suppressing hepatic PEPCK (similar to our finding) with no profound effect on fat accumulation in the liver (283). However, In humans, studies show mixed results; some findings indicate neutral

or adverse effects on metabolic health (225, 315, 323, 534). As such, the relationship between cheese consumption and metabolic health is very complex, and it may be context-dependent; therefore, this requires more research.

Similarly, there are fewer changes in the gut microbiome composition. The increase in *Sporofaciens* and *Streptococcus* is representative of enhanced gut health and a lower propensity to develop systemic inflammation, a prime factor for improving insulin sensitivity and reducing hepatic steatosis. Enrichment of *Streptococcus* is expected because it is used in the cheese-making process and is beneficial, as discussed previously. Although the CHE group shows the depletion of *Limivicinus* in the gut of HFD-fed mice, the health-related characteristics of this genus are not well determined. These microbiome changes likely account for the key link to observed metabolic improvements.

Conclusion

Our study demonstrates that an 8-week feeding trial with milk, yogurt, or cheese ameliorated the development of obesity and improved metabolic health markers in HFD-induced obese mice. Specifically, it was revealed that MILK and YOG reduced weight gain, increased energy expenditure, and improved insulin sensitivity concurrent with enhanced hepatic AKT activation and reduced gluconeogenic enzymes, which suggests improved insulin signaling in the liver. Additionally, both MILK and YOG decreased the content of TG in the liver, possibly by increasing fatty acid oxidation and suppressing de novo lipogenesis. Liver lipidomic analysis revealed that MILK and YOG have a unique lipid profile, indicating improved lipid metabolism and reduced lipotoxicity. Both interventions significantly ameliorated gut microbiota dysbiosis, supported by an increased abundance of beneficial bacteria such as Streptococcus in YOG and Anaerotignum in MILK. Conversely, CHE weakly impacted insulin sensitivity and lipid metabolism. The findings would, therefore, suggest that dairy products, particularly milk and yogurt, mitigate obesity and related metabolic dysfunctions, including non-alcoholic steatohepatitis, via multiple mechanisms involving both glucose and lipid metabolism, possibly influenced by the gut microbiome.

7 A SCOPING REVIEW ON THE IMPACT OF CONSUMPTION OF DAIRY PRODUCTS ON PHOSPHATIDYLCHOLINE AND LYSOPHOSPHATIDYLCHOLINE IN CIRCULATION AND THE LIVER IN HUMAN STUDIES AND ANIMAL MODELS

A scoping review on the impact of consumption of dairy products on phosphatidylcholine and lysophosphatidylcholine in circulation and the liver in human studies and animal models

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7.1 ABSTRACT

Dairy consumption is inversely related to the risk of developing type 2 diabetes in epidemiological research. One proposed hypothesis is that phospholipid (PL) species associated with dairy consumption mediate this relationship. This scoping review aimed to identify the existing literature in animal and human trials investigating the impact of dairy products, including milk, yogurt, and cheese as well as dairy-derived PL supplementation on PL and its species in the circulation, summarizing the characteristics of these studies and identifying research gaps. A systematic search was conducted across three databases (PubMed, Scopus, and Web of Science) in March 2021. Of 2427 identified references, 15 studies (7 humans and 8 animal studies) met the eligibility criteria and were included in the final narrative synthesis. The evidence base was heterogeneous, involving a variety of clinical and pre-clinical studies, metabolically healthy or obese/diabetic participants or animal models, and displayed mixed findings. Circulating postprandial concentrations of total PL were elevated acutely but unchanged after longer intervention with dairy products. PL concentration remained stable even after a high dosage of milk supplemented with dairy-derived PL, which may be related to increased fecal excretion; however, certain phosphatidylcholine (PC)

or lysophosphatidylcholine (LPC) species were increased in circulation by interventions. These include several PC species with 32-38 total carbons in addition to the dairy biomarkers C15:0 and C17:0. The results of this scoping review demonstrate a small body of literature indicating that dairy products can influence blood concentrations of PC and LPC species in both rodents and humans without alteration of total PL and PC. There is a lack of well-designed trials in humans and animals that explore the potential differences between individual dairy foods on PL species. In addition, trials to understand the bioactive properties of PC and LPC species on cardiometabolic risk are needed.

Keywords: Dietary intake, yogurt, diabetes, lipid metabolism

7.2 INTRODUCTION

Metabolic disorders such as insulin resistance, type 2 diabetes, metabolic dysfunction-associated fatty liver disease (also called non-alcoholic fatty liver disease), and dyslipidemia are related pathophysiological conditions in which normal metabolic processes of the body are disrupted (535). Investigations into identifying plasma metabolite signatures of obesity and metabolic dysfunction have uncovered crucial information on the molecular processes that cause cardiometabolic diseases. Strong evidence from mass spectrometry-based-lipidomic studies indicates that the pattern of circulating or tissue phospholipid (PL) is a unique biochemical signature associated with diagnosed metabolic disorders (536, 537). Phospholipids, which have hydrophilic phosphate heads and hydrophobic lipid tails, are important molecules for signal transduction pathways and play important roles in membrane structure and cell division. Phosphatidylcholine (PC) is the most prevalent PL in mammalian cells. Lysophosphatidylcholine (LPC) is derived by hydrolytic cleavage of PC catalyzed by the phospholipase A2 enzymes (538). PL panels captured using high-throughput technology have been widely used in epidemiological studies to improve prediction and better biological understanding of diseases. Disturbances in the proportion of PL subclasses and individual species may alter glucose and lipid homeostatic pathways leading to the development of glucose intolerance and to the progression of insulin resistance and type 2 diabetes (539). PLs species can modulate the activity of the peroxisome proliferator-activated receptors and the production of eicosanoids and other lipid mediators that exacerbate obesity and low-grade inflammation (540). Thus, some PL species have been identified

as biomarkers of type 2 diabetes (541, 542). For instance, reduced levels of LPC 18:2 are consistently associated with obesity, dysglycemia, insulin resistance, and type 2 diabetes and have been proposed as a predictive biomarker for metabolic dysfunction (543, 544, 545) while PC 38:3 has a positive association with obesity (537). However, whether normalizing PL profiles is associated with improvements in outcomes is unclear.

The liver plays a crucial role in lipid metabolism. Hepatic tissue is the primary site of PL production and metabolism (546). Accumulating evidence supports the hypothesis that hepatic lipid deposition as triacylglycerides may not be inherently harmful in people with obesity and obesity-related diseases (547, 548). However, as metabolic abnormalities progress, a more unhealthy hepatic lipid composition profile may lead to poorer metabolic health and a more aggressive fatty liver disease trajectory (549). In this context bioactive lipid intermediates such as PCs and LPCs and their species proposed to be linked to the development of hepatic lipotoxicity and/or insulin resistance (550, 551). Therefore, rather than an absolute quantity, hepatic lipid composition may underpin hepatic and systemic metabolic dysfunction.

Diet is a key modifiable risk factor for cardiometabolic diseases and altering the dietary composition is an effective option to achieve and maintain healthy metabolic function (552). From a food group point of view, dairy products have beneficial effects on metabolic health (302, 553, 554) and are well recognized as nutrient-rich foods providing high-quality protein and calcium, among others (555). However, dairy foods have a complex influence on metabolism because of differences in their industrial processing as well as the complex matrix of nutrients of each food (332, 556). Several mechanisms have been evaluated to explain how dairy foods can influence metabolism and ameliorate metabolic risk factors (557, 558, 559), but a full understanding remains incomplete.

Milk and some by-products of dairy products are a considerable natural source of PLs (553, 558, 560). Milk-based foods consist of many products with clear differences in nutritional profile and matrix. In particular, processing alters dairy lipid composition and distribution, especially PL, in the final matrix (561). Compared with cows' raw milk, cream, butter and buttermilk contain 2-5-fold more PL by weight but even skimmed milk retains PL comparable to whole milk, similar to hard cheeses and yogurt (561). However, cream and butter are naturally high in saturated fats and therefore have been limited by dietary guidelines to support a healthy diet (562). On the other

hand, milk, yogurt, and cheese have been recommended by MyPlate (562) as dairy products and account for 75-95% of total daily milk-based product intake in various populations (346, 563, 564). Thus, milk, cheese, and yogurt contribute the majority of dairy-derived PLs in most diets. A hypothesis is proposed that the beneficial effects of dairy products on metabolic health may pertain to normalizing PL metabolism, mainly compensating circulating species of PC and LPC. Observational studies investigating modifications of the plasma metabolome associated with dairy consumption in individuals with metabolic disorders support this hypothesis (216, 560, 565).

However, the effect of various kinds of dairy products or supplementation with dairy-derived PL on circulating PL subclasses and their potential relation with cardiometabolic health are still generally unknown. A more explicit description of the evidence base on human and animal studies is required to understand how dairy products or dairy-derived PL may alter the total or individual PL species in the circulation and their consentation in the liver. Therefore, we conducted a scoping review of the existing literature of both animal and human trials in order to synthesize the available data on the influence of dairy products and dairy-derived PL on circulating PL, its subgroups, and species to better understand their metabolic effects and, more specifically, their impact on cardiometabolic health. The specific research questions were: (1) In adults, what is the impact of increasing dairy PL on circulating PL, PC or LPC (2) In rodent models, what is the impact of increasing dairy PL on circulating or hepatic PL, PC or LPC? We also provide an overview of available data on the cardiometabolic risk factors in the included studies. In this context, human intervention trials providing a diet modifying the amount of dairy PL (using dairy products with or without PL enrichment) were reviewed to provide evidence of the impact of dairy PL on circulating PL subclasses and individual species concentrations. Animal experiments were included to provide insights into the mechanisms of action of dairy on circulating PL. The effects of interventions on liver PL observed in animal studies were also analyzed as a secondary outcome of interest. The knowledge gap and research needs were also identified, and a theoretical basis for future research and practical applications were provided.

7.3 METHODS

Search strategy

Three online databases, including PubMed, Scopus, and Web of Science, were searched in March 2021 to find relevant studies published between January 2000 to January 2021. Search terms were determined using the MeSH database and relevant reviews. In addition to terms identified in the MeSH database, the following search terms were used to define specific search syntax (Appendix 1) for each database: “dairy, milk, yogurt, and cheese (562)” combined with “phospholipid, phosphatidylcholine, and lysophosphatidylcholine.” After a careful manual search of all included studies' reference lists, additional studies were added. We selected studies that were conducted on either humans or animals. A summary of the review and the reasons for excluding studies are presented in the PRISMA flow chart (Fig. 1).

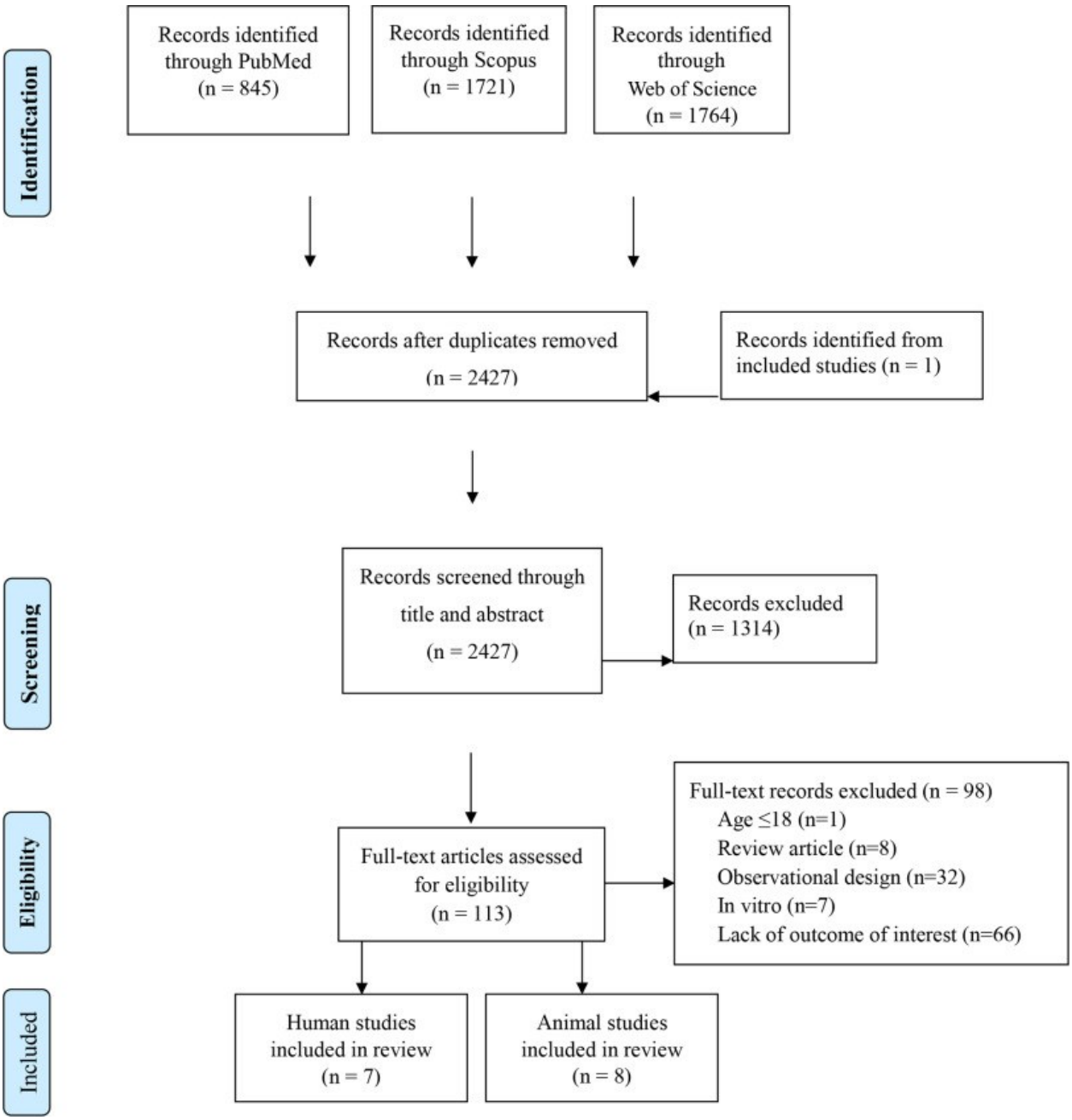

Figure 7. 1 Flowchart of the study selection process in systematic literature review, according to PRISMA guidelines

Eligibility criteria

Studies included were interventional trials (animal and human) that investigated the effect of total or individual dairy products on the circulating or liver content of PL, PC, and LPC. Studies assessing dairy products based on MyPlate definition, including milk, skim, low-fat, or full-fat milk, yogurt, and cheese custard, were included, but studies evaluating ice confections, ice creams, cream, sour cream, or butter were excluded. We also included studies that administered dairy-derived PL, for example, milk or other types of dairy food enriched with dairy-derived PL. Studies that supplemented participants with PL from other sources were excluded. The outcome measures of interest were serum/plasma concentration and liver content of PL, PC, LPC, and their species. We excluded *in vitro* studies and narrative, non-systematic reviews, and conference abstracts and those not published in English.

Selection process

Relevant studies were stored in EndNote, and the title/abstract screening process initiated after removing duplicate articles. The full texts of the remaining studies were read to check inclusion and exclusion criteria, detailed above.

Data charting

Charting forms were developed separately for human and animal studies, and one reviewer (EY) charted information. The second reviewer (SM) checked all of the records. From each human study, the following data were charted: authors' names, publication year, aim, sample size, age, study design, study duration, participants' health conditions, results related to PL and its subclasses, and the main metabolic outcome of the study. The charted data for animal studies were: authors' names, publication year, aim, animal species, sex, age, number of animals in intervention groups and control groups, intervention characteristics, and main results including metabolic outcomes. In the case of missing data or unclear pieces of information, it was considered that the authors did not report such variables.

Risk of bias

The risk of bias (RoB) in human randomized trials was assessed using the revised Cochrane's Risk of Bias (RoB2) assessment tools for randomized trials (566). For animal studies, SYRCLE's Risk of Bias tool was used to assess the RoB (567). Both RoB results had been visualized by robvis

(568). Both tools assess the studies' methodological quality by evaluating selection, performance, detection, attrition, reporting, or other bias. Evaluation of potential bias for each included study was done by one reviewer (EY) and checked by a second (CBC).

Summarization of data

The data on the response of PL, PC, LPC, and their species to the dairy consumption were summarized as significantly increased, decreased or not changed by the intervention(s). We grouped the included studies by health conditions of the participants/animal models and duration of the intervention. The results were synthesized as a narrative summary of human and animal studies separately.

7.4 RESULTS

Overview of publications

In this review, 2427 articles were identified and screened after removing duplicates. After evaluation of the titles and abstracts, 133 papers were selected for full-text assessment, of which 15 studies were identified that met the inclusion criteria. The citation lists of all included references were then reviewed to identify any additional relevant articles. Studies were classified according to their type, resulting in 7 human trials (569, 570, 571, 572, 573, 574, 575) and 8 animal studies (283, 576, 577, 578, 579, 580, 581, 582).

All human studies were randomized trials, either parallel (571, 574, 575, 583) or cross-over design (569, 570, 572, 573) with duration ranging from 1-4 hours to 12 weeks. Participants mean ages ranged from 25 to 63 years. The participants of 3 studies were apparently healthy, or there was no restriction on the inclusion criteria (570, 572, 573), while others selected individuals with a high risk for cardiovascular disease (CVD) (569), overweight, or obesity (571, 574, 575, 583). Of 7 human studies, only 4 considered plasma concentration of PL as the primary outcome (570, 571, 572, 573). Interventions varied from milk supplemented with 2 or 3, or 6 g milk-derived PL (571, 572, 573) and manipulation of dairy product consumption in the regular daily diet of participants (569, 570, 574, 575). A summary of the included studies' details and findings are presented in Table 7.1, sorted by year of publication.

Table 7. 1 Summary of the human interventional studies with evaluated the effect of dairy on phospholipid and its subclasses concentration

Author, year	Participants (age/ n/ risk factors / gender)	Design	Intervention	Duration (week)	Main results	Outcome of interest
Markey (569), 2017	25-70 y/ 54 / High risk for CVD/ both	Cross-over RCT	Isocaloric HF diet (38% total energy) with SFA-reduced, MUFA-enriched dairy products (modified) or regular dairy (control)	12	*Modified vs. control:* ↓ PC species including 14:0, 15:0, 17:0, 20:3, total SFA ↑ PC species including 16:0, 18:1, total MUFA	There was no significant treatment effect for anthropometric measurements.
Weiland (571), 2016						
Trial 1	50-76 y/ 62 / overweight or obese/ men	Double-blind, parallel-group RCT	Participants consumed 200 mL/d LF milk enriched with 2 g milk-PL (PL group) or 200 mL/d milk enriched with 2 g milk-fat	8	Plasma total PL not affected by the intervention (1.97±0.32 *vs.* 1.93±0.37)	GGT activity decreased.
Trial 2	50-76 y/ 57 / overweight or obese/ men	Double-blind, parallel-group RCT	Participants consumed 250 mL/d LF milk enriched with 3 gr milk-PL (PL group) or 250 mL/d milk enriched with 2.8 g soy-PL	7	Plasma total PL not affected by the intervention (2.27±0.41 *vs.* 2.22±0.35)	GGT activity slightly decreased.

Meikle (570), 2015	40-46 y/ 16 / healthy/ men	Cross-over RCT	Participant consumed a breakfast containing HF dairy products or a breakfast containing soy oil-based foods	Postprandial (1-4 h after eating)	*Dairy meal vs. baseline:* No change in total LPC ↑ plasma total PC ↑ PC species including 28:0, 29:0, 30:0, 32:0, 32:1, 32:2, 32:3, 33:2, 33:3, 34:2, 34:3, 35:2, 35:3, 36:1, 36:3, 36:5, 36:6, 38:3, and LPC species including 18:3, 24:0 ↓LPC species including 20:1, 22:5 *Dairy meal vs. soy meal:* ↑ PC and PC species with odd-chain FAs (15:0 and 17:0) and PC species including 29:0, 28:0, 30:0, 31:0, 32:0, 32:1, 35:1, 36:3, 36:4, 36:5, 38:3, 38:5, 38:6, 40:5, and 40:6	There were no metabolic parameters measured
Keller (572), 2014	Adults/ 39/ atopic dermatitis-metabolically health/ both	Double-blind, cross-over RCT	Participants consumed 250 mL/d LF milk enriched with 3 g milk-PL (PL group) or 250 mL/d whole milk (control)	6	Plasma total PL (2.13 ±0.36 *vs.* 2.09 ± 0.29 in PL group and 2.12 ±0.33 *vs.* 2.09 ±0.35 in control), PC, and LPC not affected by the intervention	There was no treatment effect on lipid profile and inflammation parameters.
Keller (573), 2013	Adults/ 14/ healthy/ women	Open-label, cross-over RCT	Participants consumed LF milk enriched with 3 g milk-PL (low-PL group) or LF milk enriched with 6 g milk-PL (high-PL group) or LF milk enriched with 2 g plant sterol (PS-PL group)	10 days	Plasma total PL (2.27 ± 0.44 *vs.* 2.35 ± 0.36 *vs*. 2.29 ± 0.41) and PC not affected by the interventions PS-PL group *vs.* baseline and low-PL group: ↑ LPC	TC in plasma was lower after low-PL in comparison with baseline. TC and LDL cholesterol rose significantly compared with low-PL PS-PL, resulted in a lower LDL

						cholesterol compared with high-PL.
Tardy (575), 2009	18-50 y/ 63/ abdominal obesity/ women	Double-blind, parallel-group RCT	Participants consumed industrial source TFA lipids contain hydrogenated vegetable oil (IP-TFA diet) or ruminant TFA–vaccenic acid-rich milk fat (R-TFA diet) or low-TFA diet: palm oil with hydrogenated sunflower oil (Low-TFA)	4	Plasma total PL not affected by the intervention *R-TFA vs. Baseline:* ↑ 18:1trans-11 and PL content in total n-3 PUFA ↓ PL content in total SFA and n-6 PUFA	There was no treatment effect for fasting glycemia, insulinemia, lipid profile, and markers of liver function and inflammation.
Hlavaty (574), 2008	Adults/ 40/ obese/ women	Parallel-group RCT	Participants receive one serving regular fat yogurt (LCD); prescribed caloric intake was 60% of energy expenditure	3	*LCD vs. baseline:* ↑ PC species including 16:0, 18:1n-7, ↓ PC species including 14:0, 18:0, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, total SFA	BMI, LDL-cholesterol, and CRP were lower after a yogurt in comparison with baseline.

CVD, cardiovascular disease; RCT, randomized controlled trial; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PL, phospholipid; PC, Phosphatidylcholine; LPC, lysophosphatidylcholine; PS, plant sterols; HF, high-fat; LF, low-fat; TFA, trans fatty acids; LCD, low calorie diet; PUFA, polyunsaturated fatty acid; GGT, γ-glutamyl transferase; TC, Total cholesterol.

Most animal studies (n=7) were conducted in mice (576, 577, 578, 579, 580, 582) except for 2 studies, which selected rats (283, 581) (Table 7.2). Interventions included milk supplemented with milk-derived PL (577, 578, 579, 580, 582), cheese (283), MUFA-enriched dairy foods (576), and one that used milk fat (581). Only 2 out of 7 studies considered PL or its subclasses as a primary outcome (283, 577).

Table 7. 2 Summary of the animal studies which evaluated the effect of dairy on phospholipid and its subclasses concentration

Author, year	Model	Intervention	Duration (week)	Main results
Millar (580), 2020	mice LDLr$^{-/-}$ C57BL/6J	HFPL1: HF diet + milk-PL 1% by wt HFPL2: HF diet + milk-PL 2% by wt Control: HF diet	14	No change in PC and PL of liver
Zhou (579), 2019	mice C57BL/6J ob/ob	GG: Standard diet + milk gangliosides at 0.2 g/kg of diet PL: Standard diet + with milk-PL at 10 g/kg of diet Control: standard diet	2	↑ total serum PL in the PL group compared with the GG and control groups. No change in PC and PL of liver
Hanning (283), 2019	rat Sprague Dawley	REG: HF diet + regular-fat cheese diet LOW: HF diet + LF cheese diet HF diet: HF diet Control: LF diet	8	*LOW vs. HF diet:* ↑ LPC species including 14:0, 16:0, 16:1, 17:0, 18:1, 20:3, and 20:4 and PC species including 30:0, 32:2, 34:1, 34:3, 34:4, 36:3, 36:5, 38:3, 38:0 *REG vs. HF diet* ↑ LPC species including 14:0, 16:0, 16:1, 17:0, 18:1, 20:3, and 24:0 and PC species including 30:0, 32:1, 32:2, 34:1, 34:3, 34:4, 36:3, 36:5, 38:3, 38:0, 42:0 *REG vs. LOW* ↑ PC specie including 30:0 and LPC specie including 14:0 ↓ PC species including 32:1, 34:3, 30:0, 36:5, 42:0, LPC specie including 16:0 Plasma and liver total PL, PC, LPC were not affected by intervention
Milard (582), 2019	mice C57Bl/6	HFPL1: HF diet + milk-PL 1.9% (w/w) HFPL2: HF diet + milk-PL 3.8% (w/w) HF: HF diet LF: LF diet	8	Liver content in PL, total SFA, MUFA, and PUFA did not differ among groups. Liver: *HFPL1 vs. HF:*

				↑ PL species including 20:2, 23:0, 24:0 *HFPL2 vs. HF:* ↑ PL species including 20:2, 23:0, and 24:0 ↓ PL species including 18:1(n-7) *HFPL2 vs. HFPL1:* ↑ PL species including 20:2, 23:0 ↓ PL species including 18:1(n-7) *HFPL1 vs LF:* ↑PL species including 18:1(n-9), 22:0, 20:3(n-3), 20:4(n-6), 23:0, 24:0 ↓ PL species including 14:0, 15:0, 16:1, 18:1(n-7), 18:2(n-6), 20:1, 20:5, 24:1 *HFPL2 vs LF:* ↑PL species including 18:1(n-9), 22:0, 20:4, 23:0, 24:0 ↓PL species including 14:0, 15:0, 16:1, 18:1(n-7), 18:2 (n-6), 20:1, 20:5, 24:1
Kamili (577), 2010	mice C57BL/6	HFPL: HF diet + milk PL Control: HF diet	8	Plasma PL was not affected by the intervention. *HFPL vs. control:* Feces: ↑ all PL species and PC Plasma: ↑ PC species including 32:1, 34:1, 32:0, 34:0 Liver: total PL was not affected by the intervention. ↓ total PC, PC species including 34:1, PC 34:2, PC 38:6; PC 36:3
Wat (578), 2009	mice C57BL/6	HFPL: HF diet + milk-PL 2.5% (w/w) HF diet: HF diet NPL: Standard diet + milk-PL 2.5% (w/w) N: Normal diet	8	*HFPL vs. HF diet* Liver: ↓ PL Serum: ↓ PL
Higuchi (576), 2008	mice Crlj:CD-1 (ICR)	10% yogurt 30% yogurt Control	12	Plasma: 10% yogurt and 30% yogurt had lower PL concentrations than control group No change in PC and PL of liver

Ramaprasad (581), 2003	rats wistar	Standard diet + milk fat GNO: Standard diet + groundnut oil High-cholesterol diet + milk fat GNO: High-cholesterol diet + groundnut oil	8	*Standard diet* Plasma: ↑ PL in milk fat Liver: No difference between groups *Cholesterol-enriched diet* Plasma: No difference between groups Liver: No difference between groups

HFLP, high-fat diet plus phospholipid; HF, high-fat; LF, low-fat; PL, phospholipid; PC, Phosphatidylcholine; LPC, lysophosphatidylcholine; GG, gangliosides; REG, regular; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; NPL, standard diet with phospholipid

Study quality and risk of bias

The results of the RoB evaluation for human and animal studies are shown in Figures 7.2 and 7.3, respectively. Three human studies were assigned high RoB (569, 573, 574) with the main reason being a lack of information on randomization, concealment methods and blinding of participants and investigators, which is a limitation of most nutrition interventions. The results for RoB were assessed as low for 2 studies (572, 575) and as some concerns in 2 RCT (570, 571) because of the randomization method.

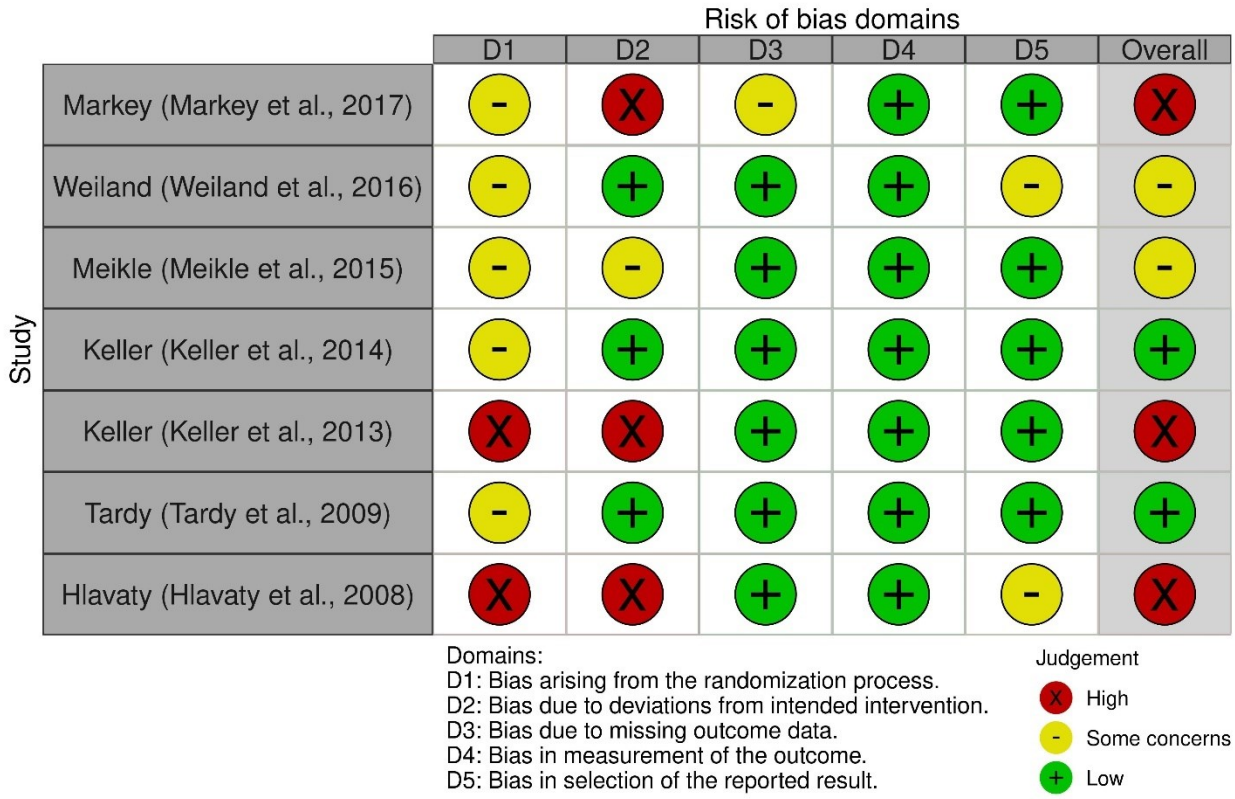

Figure 7. 2 Risk of bias in included human studies: review authors' judgements about each risk of bias item for each included study

The domains of risk are shown for each study. Colour coding is used to depict a three-point scale for bias. Green (+) reflects a low risk of bias, red (X) a high risk of bias and yellow (-) an unclear risk of bias.

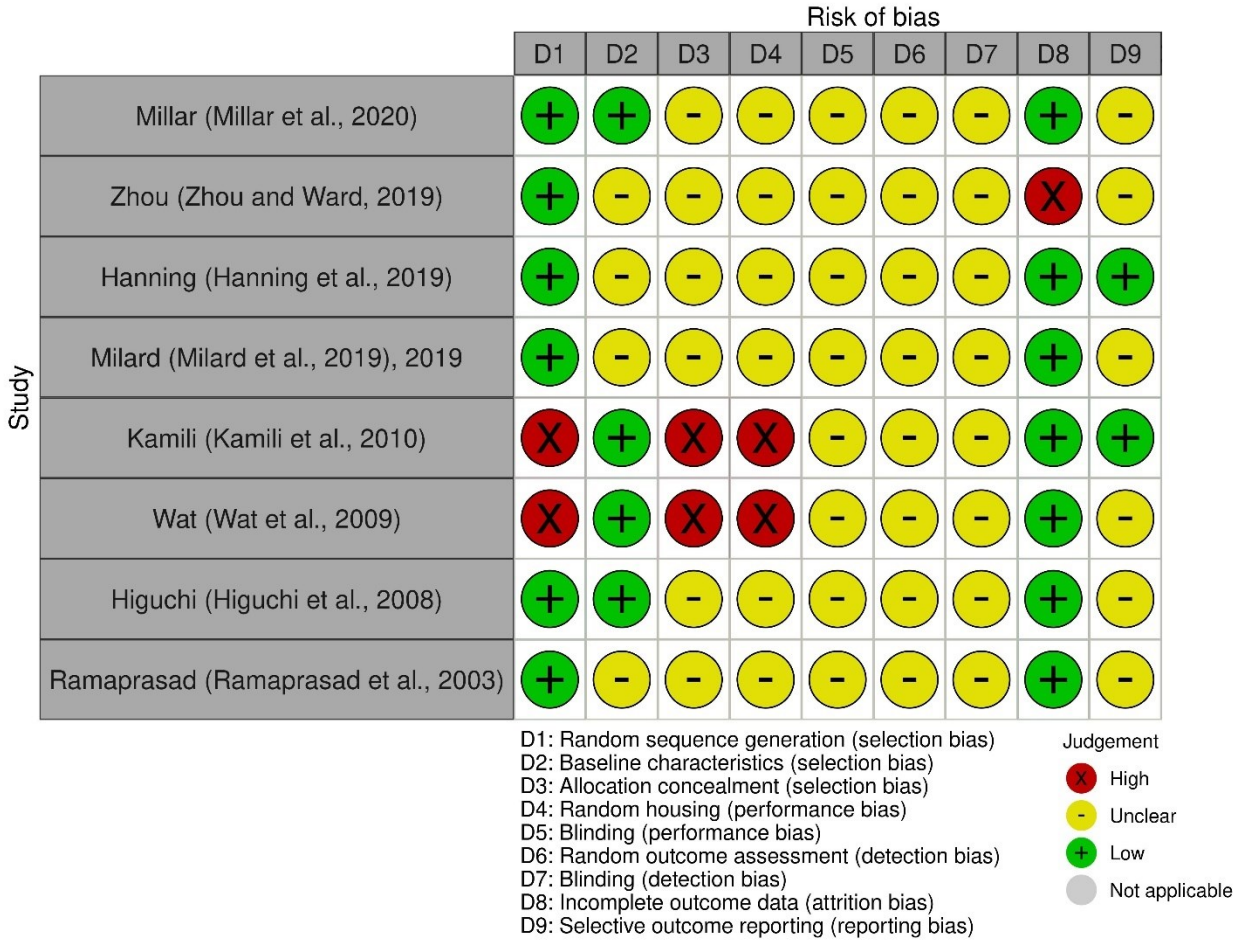

Figure 7. 3 Risk of bias in included animal studies: review authors' judgements about each risk of bias item for each included study.

The domains of risk are shown for each study. Colour coding is used to depict a three-point scale for bias. Green (+) reflects a low risk of bias, red (X) a high risk of bias and yellow (-) an unclear risk of bias.

The ARRIVE guidelines for reporting animal studies (https://arriveguidelines.org) have not been as widely adopted as the CONSORT guidelines for reporting RCT. Thus, assessing their RoB based on methodological quality is more difficult. Two studies (577, 578) did not report the randomized allocation of animals, leading to high RoB, while other authors (283, 576, 579, 580, 581, 582) stated that the allocation was randomized, but procedural details were not reported. Baseline characteristics between groups were poorly reported; therefore, we selected the initially reported body weight to evaluate RoB. Only 4 studies received low RoB (576, 577, 578, 580) based on no body weight differences between groups at baseline, while the rest were unclear. None of the studies reported data on blinding of caregivers or investigators or on the random selection of animals for outcome assessment, leading to the assignment of unclear RoB. No blinding of the outcome assessor was reported; however, the outcome of interest (PL) was an objective measurement less subject to bias than subjective measures. Regarding attrition bias, only one study reported that a considerable number of animals died during the study (579); for other studies, information about the animal loss was adequate, and a similar number of analysed animals in each group of the studies was reported. On this basis, all studies were assigned a low RoB for attrition. The assessment of PL and its subclasses was the primary outcome of 2 included studies, so the RoB for this domain was judged as low (283, 577).

Human Studies

In Table 1, the main study characteristics and results of the effect of dairy consumption on PL concentration are summarized. Overall, circulating PL ranged from 1.93 to 2.23 mmol/L in all human subjects, regardless of their health. In the following, the studies are described in detail based on the initial health of participants.

Healthy participants: Only 2 studies focused on healthy participants. One trial evaluated the acute impact of dairy products on circulating postprandial PL. Compared with soy-based foods, a breakfast meal comprised of high-fat (HF) dairy foods (consisting of cheddar cheese, butter, and extra creamy whole milk) increased postprandial total plasma PC and PC containing dairy-derived fatty acids C15:0 and C17:0 but not LPC or circulating TG (570). After the dairy meal, post-prandial plasma PCs species including 29:0, 33:2, 33:3, 35:2, 35:3, 28:0, 30:0, 32:0, 32:1, 32:2, 32:3, 34:2, 34:3, 36:1, 36:3, 36:5, 36:6, 38:3, and LPC 18:3 and 24:0 increased significantly. However, plasma LPC species including 20:1, 22:5, and 18:1 decreased after an HF dairy meal

(570). In healthy women, a short-term (10-day) intervention with milk enriched with milk-derived PL of 3 or 6 g dose had no effect on plasma PL, PC, and LPC (573). Decreased HDL-C compared with baseline after 3g milk-PL intervention was noted, but no correlations with plasma PL were presented. Interestingly, measurements in feces indicated a slight milk-PL dose-dependent increase in PL excretion (573). Similarly, a randomized cross-over trial with 6-week intervention arms found no effect of consuming LF milk supplemented with 3 g milk-PL compared with whole milk on either plasma total PL, PC or LPC or on lipid profile or inflammatory markers (572). However individual PC and LPC species were not reported in the latter 2 studies (572, 573).

Participants with cardiometabolic risk factors: No acute studies were identified. In women with obesity, a 3-week intervention with regular yogurt showed that, compared with baseline, PC 16:0 and 18:1n-7 increased whereas PC species including 14:0, 18:0, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3 and total SFA decreased compared to the baseline valuses without alterations in total PL (574). Participants' BMI, LDL-cholesterol, and CRP were lower after yogurt provision in comparison with baseline; however, this result was confounded by prescribed 30% reduction in energy intake as part of the intervention.

The impact of dairy products on circulating PL was consistent in 4 studies with interventions ≥4 weeks. Three studies (2 trials) were conducted on people with obesity (571, 575), with one considering additional cardiovascular risk factors (569). one of the parallel-arm RCT (using milk-PL enriched LF milk) found no effect on total plasma PL (571). This study also found no intervention effect on most metabolic outcomes. However, Weiland et al. found either increased gamma-glutamyl transferase (GGT) in the milk-fat control group (trial 1) or reduced GGT in the PL group (compared with a soy-PL control, trial 2), which was interpreted as a benefit of milk-PL on fatty liver (571).

Dairy-derived biomarkers PL 15:0 and 17:0 in the blood can indicate intervention compliance (584, 585). Two trials examined the incorporation of specific fatty acids into the plasma PL fraction. Tardy et al. (575) found that provision of ruminant *trans* fats increased their abundance in circulating PL while also increasing total MUFA and reducing total SFA and PUFA in PL. Markey et al. (569) likewise found that feeding dairy enriched in MUFA and depleted in SFA (dcreased amount of PC 15:0 and 17:0) led to a similar pattern in fatty acids incorporated into plasma PL. Thus, although most studies show null effects on total PL, differences can be observed

when PL species are quantified. In addition to being a measure of compliance when C15:0 and C17:0 are measured, it is possible that certain species may exert biological activities. However, Tardy (575) did not find differences in body weight or metabolic outcomes in women with abdominal obesity, and Markey et al. (569) performed no metabolic measurements.

Animal Studies

Eight studies assessed the effect of dairy intake on PL metabolism in animals, with 6 employing obese rodent models or HF background diet to induce obesity. Regarding total plasma PL in obese mice and rats, Hanning et al. (283) and Kamili et al. (577) found no difference, whereas Zhou (579) reported an increase and Wat et al. (578) reported a decrease after dairy consumption. Hanning et al. (283) was the only study to use cheese, whereas the other groups employed milk-PL or milk gangliosides; all studies intervened for 8 weeks. Total hepatic PL was likewise unchanged by the intervention in five studies (283, 577, 579, 580, 582), with Wat et al. (578) noting a decrease. Interestingly, fecal PL excretion increased by 2.8-fold in the latter study (578) and Kamili et al. also noted increased excretion of PL (577). On a standard LF diet background, enrichment with milk-PL had no effect on plasma or hepatic PL in ob/ob mice (579).

In non-obese rodents, PL increased in plasma but not liver in a rat study using standard LF diet plus milk fat compared with LF control diet (581). However, PL concentration and liver content were stable after adding a certain amount of PL in their standard diet (578). Conversely, on a high cholesterol diet background, milk fat had no effect on PL in plasma or liver (576). Even in the absence of effects on total PL, multiple 8-week trials in obese rodent models that measured PL species reported alterations (283, 577, 582). A combination of HF diet with 1.9% or 3.8% (w/w) milk-PL increased hepatic PL 20:2, 23:0, and 24:0 compared with the HF diet group (582), but plasma concentrations were not reported. Plasma PC species 32:1, 34:1, 32:0, 34:0 increased but liver total PC and species 34:1, 34:2, 38:6; 36:3 decreased (577). In a study in rats, several PC and LPC species decreased in the HF diet group compared with the LF diet. However, intervention with regular- or reduced-fat cheese increased their concentration towards normal. For example, HF diet-induced reductions in LPC species such as 14:0, 16:0, 16:1, 17:0, 18:1, and 20:3 and PC species including 30:0, 34:1, 34:3, 34:4, 36:3, 36:5, and 38:3 were increased to the LF diet values by the cheese interventions (283).

7.5 DISCUSSION

This scoping review synthesized the research on the effects of dairy intake or milk-PL on circulating PL and its subclasses, including LPC and PC. Although the design and quality of included intervention studies were heterogeneous, and some were not specifically designed to investigate the effects of dairy intake, the findings were relatively consistent. The main finding is that, in contrast to acute intervention, chronic human studies 10 days to 12 weeks long generally demonstrate that total circulating PL, PC, and LPC are not affected by intervention with dairy products, remaining stable even after a high dosage of milk-PL. However, the serum concentration of PC and LPC species can be altered by dairy products or dairy-derived PL. This finding was consistent with the results of rodent studies. Effects on total PL may be limited by increased fecal excretion. To date, associations with cardiometabolic outcomes are weak and poorly supported by mechanistic studies.

Associations of PC and LPC species with metabolic dysfunctions have been reported in several studies (536, 537, 541, 543, 544, 586, 587). In a recent cross-sectional study using a targeted metabolomics approach, some PC species (32:0, 32:1, 32:2, 34:1, 34:2, 34:3, 36:2, 36:3, 40:5, 40:6, 42:3, 42:4, and 42:5) were associated with a lower risk of insulin resistance (586). In addition, LPC 18:0, 18:1, and 17:0 have been identified as negative predictors of type 2 diabetes (587). In a human study, circulating PC and LPC species rapidly change after even one meal that contains a high amount of specific dairy-derived PL, and these extensive alterations remain up to 4 h (570). The PL content in dairy can possibly compensate for those PC or LPC species that change in the context of metabolic dysfunction. Although mice exhibit significant differences in lipid metabolism versus humans (588, 589), some animal trials provide data regarding PC and LPC in serum that associate with benefits on insulin sensitivity (283) or liver associated with reduced weight gain (582). The circulating PC species including 32:0, 32:1, 32:2, 34:1, 34:3 and 36:3 were considerably elevated after a course of dairy intervention in rodents (283, 577). Furthermore, LPC species including 18:0, 18:1, and 17:0 also tended to increase (283). Other studies found that milk consumption was associated with lower adiposity, increased insulin sensitivity, and improved glucose homeostasis among diabetic and prediabetic rats (590, 591). In addition to milk intake, yogurt consumption enhanced insulin sensitivity in rodents (274, 534, 592). However, these studies did not report PC and LPC, and hence the degree to which long-term dairy food intervention increase food-specific PC and LPC species is only reported by Hanning et al (283).

Furthermore, plasma PL species are highly sensitive markers of the fatty acid composition of acute interventions (570), persisting up to 3 weeks (574). However, the circulating PC and LPC species that were increased following the intervention were not all specifically dairy-derived (569, 570). Untargeted metabolomic analyses also indicated a great many more PL species were altered that might not be directly attributable to intake of dairy products or milk-PL (570, 593). The capacity of meal-derived lipids to alter plasma PL pools is determined by absorption and subsequent metabolic processes, as well as the quantity of lipid in the meal and the size of the plasma PL pools (594). Extensive processing or differences in the food matrix likely affect the PL content of dairy foods and their absorption; thus it is tempting to speculate that individual dairy products have a specific impact on the serum concentration of PL species. Therefore, short- and long-term human studies are needed to elucidate how PL and its species are regulated in response to different types of dairy products. In addition, animal studies are also required to determine the mechanism and improve our understanding of how dairy foods change PL species, particularly those not attributable to dairy sources (283, 570).

Human trials of healthy participants using dairy enriched in PL (572, 573) found alterations in circulating cholesterol but since total circulating PL and PC were not changed, the mechanism is unclear. Possibly, measuring incorporation of dairy-derived PL into membranes could shed additional information. Studies of people with CVD risk yielded variable cardiometabolic outcomes, but these could not be attributed to dairy interventions in most cases (571). In contrast, in trials where the intervention increased dairy-derived fatty acids in the PL fraction, no metabolic improvements including insulin sensitivity or plasma lipids were observed (30) and one study did not report metabolic outcomes (569). A current meta-analysis of 30 human RCTs of dairy interventions showed a beneficial effect on homeostatic model assessment for insulin resistance (HOMA-IR) (307); however, the impact of individual dairy foods was not presented nor were PL measured. Furthermore, based on findings from clinical studies (595, 596, 597), an RCT in humans with impaired PL subclasses is required to measure the efficacy of dairy PL from different dairy foods on PL subclasses and species and any associated benefits on glucose and lipid metabolism or body composition.

In the current scoping review, we focus on the most highly consumed dairy products, milk, yogurt, and cheese, as the greatest source of dairy-derived PL in the diet. For example, in a nationally representative sample of adult Canadians, these 3 products plus frozen dairy products contribute

>95% of total dairy intake (206). There are also various milk-based products with high content of PL that are consumed in lower amounts, such as buttermilk, cream and butter that were excluded from this review. In this regard, an 8-week single-blind, parallel-group RCT with 1 dL per day whipping cream (139 to 190 mg PL per 100 g) did not affect plasma total PL and PL species. However, increases in LDL cholesterol, non-HDL cholesterol, ApoB, and total cholesterol were observed compared with a control group consuming milk protein isolate (598), which might be attributable, at least in part, to the saturated fat content. Another double-blinded randomized crossover placebo-controlled study indicated that 4 weeks of buttermilk (93.7 mg PL per day) consumption significantly reduced serum total cholesterol and TG concentrations compared with the placebo (17.3 mg PL) group but effects on circulating PL were not reported (599). Results of these 2 studies do not alter our conclusion that consumption of dairy PL does not alter total circulating PL. However, further studies comparing individual dairy foods' effects, including those with naturally high PL concentrations, on specific species of PL and potential biological outcomes are warranted.

This review has identified other limitations and gaps in the current literature. Heterogeneity in designs, methodology, and reporting in the included studies, makes conclusions challenging. Lipid metabolism differs between species limiting the transposition of findings from rodents to humans; however, some general similarities between humans and rodents support using rodents as a model for many aspects of human lipid metabolism (600, 601). Questions requiring further consideration are the extent to which PC or LPC species are raised after intervention by dairy products and the involvement of metabolic pathways that regulate the balance of PL species. In the current scoping review, we included studies that intervened with milk, yogurt, and cheese, all classified according to dairy products on MyPlate, the United States Department of Agriculture's dietary guidelines. However, other milk-based foods high in fat but still rich in PLs, such as cream and buttermilk, were excluded. It is a possible limitation of the current work, and the effect of PL concentration might also be interesting. Another limitation was the potential confounding effect of participants' habitual dietary intake before and during the intervention in human trials. Dairy foods may be consumed in processed or other foods, leading to underestimating the actual intake of dairy products, especially in the control groups. Since PL or its subclasses was not the primary outcome in most studies, results should be interpreted with caution because non-significant changes might result from lack of power rather than actual effect. The physiological basis for benefits of dairy

PC and LPC on metabolism remains unclear, with evidence to the contrary also reported (602). Future research is needed to fill up the gaps and limitations to explore the impact of dairy foods on PL metabolism. Nonetheless, the current scoping review covered research using a variety of methodologies, allowing us to compile data from human and animal studies evaluating dairy-derived PL under various settings and, as a result, identify features that could be improved in future studies. As a strength of this study, the application of multiple databases provides a broader range of published papers and contribution of 2 researchers to conduct the literature search and study assessments for quality control.

Conclusion

In conclusion, experimental and clinical studies provide evidence that total circulating PL is tightly regulated, even in interventions with a high milk-PL dosage. Data regarding the impact of dairy on total PC and LPC remain inconclusive. However, their species in the blood or liver have been repeatedly altered in response to dairy products without significant changes in total circulating PC. PC 17:0 and 15:0, identified as dairy biomarkers, were most commonly increased after dairy interventions but other species with 32-38 carbons were also commonly reported. Our scoping review identified the need for additional human studies with a large number of participants and with a specific focus on individual dairy products, detailed dietary data, and strict intake control to provide stronger evidence and overcome the limitations previously discussed. Rodents appear to be suitable models for studying dairy effects on PL metabolism. We highlight areas in which additional animal trials are warranted to describe better the PL-related metabolic pathways linked to the individual dairy products. A deeper understanding of the metabolic pathways that regulate PL species in response to dairy consumption would provide new ideas about the relationship to cardiometabolic risk that have consistently been reported in meta-analyses of cohort studies.

Acknowledgments

E.Y. and C.B.C. conceptualized, designed the study; E.Y. and S.M. conducted the search, selection, and evaluation of studies; E.Y. prepared the first draft of the manuscript, which was subsequently finalized in close collaboration with C.B.C. and S.M.; C.B.C. supervised the project and revised and approved the final version of the manuscript; and all authors read and approved the final manuscript.

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8 DISCUSSION AND FUTURE DIRECTIONS

8.1 OVERALL DISCUSSION

The WHO defines an individual having a BMI of 25 kg/m² or more as overweight and an individual whose BMI exceeds 30 kg/m² as obese (603). Presently, almost 2.5 billion adults around the world are now in the category of being overweight and obese, with younger populations' prevalence of this health-threatening condition dramatically rising (604). Being overweight and obese predisposes a person to the development of IR, which leads to chronic diseases, including T2D and MASLD (605). Poor energy balance and a suboptimal diet are linked with obesity and its related metabolic disorders in many epidemiological studies (125). Nutritional interventions are now known to manage such conditions effectively (606) but compliance can be poor. Therefore, there is a need to determine how familiar food groups that shape daily eating patterns and are widely consumed affect the progress and treatment of obesity and related metabolic disorders. It should be noted that foods are not simply single nutrients or components; they are a mixture of molecules forming sophisticated matrices in which molecules interact in ways that have complicated effects on health and disease. In this context, because dairy products are highly heterogeneous in terms of the contents of components as well as their physical structures, it is plausible that intakes of these foods have different effects on health (205). The contribution of milk and dairy products to modern metabolic disorders is an active and still developing research area. There is a debate over the right types and quantities of dairy intake and how they are effectively related to metabolic health. Thus, in my dissertation, I aimed to advance our understanding of the impact of consuming different types of dairy products on obesity-related metabolic outcomes by integrating various approaches, including nutritional epidemiology, synthesizing available data, and examining the mechanisms behind physiological changes in mouse models.

In this project, we consistently show that each dairy product (or several products in combination) can distinctly contribute to the mitigation of obesity-related metabolic outcomes. A pattern of dairy products with mixed low- and whole-fat milk consumption was significantly associated with a lower risk of T2D in males, and it was more pronounced in high-risk individuals. Our observation in the prospective cohort study is parallel with our findings of the mouse model of obesity, which

shows the most beneficial improvement in insulin sensitivity for milk consumption in male mice fed with HFD. It should be noted that the male mice were fed with fat-free milk, not a mix of low- and high-fat milk. Furthermore, our results on 11 pooled observational studies indicate that dairy products, particularly milk, followed by yogurt, are associated with preventing MASLD risk, which is supported by lower hepatic steatosis seen in HFD-fed mice when supplemented with non-fat milk or yogurt. Noteworthy in both observational human studies and our preclinical trial is that milk has the most significant health improvements, followed by yogurt, with cheese having a neutral effect. By integrating the findings of the observational study with mechanistic testing in the mouse model of obesity, we comprehend that milk consumption could mitigate the adverse effect of the Western diet on metabolic health by increasing energy expenditure, improving insulin sensitivity, reducing hepatic steatosis, and enhancing overall metabolic function, likely mediated by modulation in lipid metabolism and presence of beneficial bacteria in the gut. The association of yogurt consumption in reducing the risk of MASLD appears moderate relative to milk's impact, which is also seen in the preclinical study indicating some beneficial effects for yogurt on energy expenditure and glucose and lipid metabolism in the liver, particularly related to modulation of the gut microbiome. This finding is interesting because it supports my hypothesis that dairy products are most beneficial for metabolic health in people at high risk of developing T2D, such as overweight and obese individuals. These findings can be relevant to any Western nation, including Canada, whose diet is characterized by Westernized food items high in fats and sugars. Keeping or adding low-fat dairy products, especially fat-free milk, to a diet could help consumers gain immense health benefits due to reduced obesity-associated metabolic diseases. The results could also be applied to updates of nutritional recommendations on dairy types for the improvement of metabolic health. This study, therefore, provides a rationale for targeted dietary interventions with milk as a priority food to reduce prevalence not just in T2D but also in MASLD, consequently enhancing the population health in Western countries.

In this dissertation, I first aimed to investigate the association of dairy product consumption with T2D using a novel approach by considering clustering or patterns of participants' dairy food choices. There are a number of studies investigating the effect of dairy products and their subtypes on the prevalence and incidence of T2D. The findings of these observational studies have been synthesized in the current and previous meta-analyses, indicating the preventive association of total dairy consumption, in addition to milk and yogurt, with T2D (234, 301, 303, 307). However,

so far, dairy products have been categorized into two main groups: high-fat and low-fat but these *priori* groupings based on the arbitrary fat content of dairy products incapacitates distinguishing between dairy product matrixes and do not mirror people's behavior regarding dairy product consumption. We know that people select a variety of dairy food products in their daily basket, be based on factors such as quality, perceptions of healthiness, price, availability, and personal preferences and not necessarily based on their fat content (607, 608). Thus, for the first time, I evaluated the association of peoples' patterns of dairy consumption based on linear combinations of dairy food items and the risk of T2D incidence (418). In agreement with my primary hypothesis, I observed that people followed different patterns with respect to dairy product consumption in their daily lives, which led to a distinct association with T2D. Among males, following a dairy consumption pattern characterized by high consumption of whole milk, regular cheese, and non-fat milk and low consumption of 2% milk was associated with a 40% lower risk of incident T2D. Interestingly, in the subgroup analysis, the prevention of T2D by this pattern was more pronounced in overweight and obese individuals and also in elderly populations. This finding suggests that males who are more at risk of T2D, including older people with overweight and obesity, may have more benefit from dairy product consumption.

The second aim of this dissertation was to summarize the current evidence on the association between dairy product consumption and the risk of MASLD, combining information from separate primary observational studies to describe the entire body of work better. When I started my Ph.D., no systematic review or meta-analysis on this topic had been conducted. In my meta-analysis (updated to September 2022), I synthesized data from 11 observational studies with a total sample of 43,649 participants. I calculated that consuming more total dairy products lowers the incidence of MASLD by 10%. Dairy products, however, differ in their nutritional composition and physical characteristics based on the processing techniques used (outlined in 2.2.2); thus, the effects of dairy products on liver structure and function may vary. According to my study, consuming milk is linked to a 14% decreased risk of MASLD, the benefits of yogurt consumption are slightly lower at a 12% lower risk of MASLD development, and there is no discernible advantage to cheese consumption (466). Another meta-analysis published more recently indicated that total dairy consumption was associated with a 3% lower risk of MASLD (495). However, these researchers did not analyze further to provide data for dairy subtypes. Three cohort studies have been published after my meta-analysis. A large prospective cohort study conducted on 190,145 participants from

the UK Biobank Study with a mean of 11 years of follow-up showed that the risk of MASLD and cirrhosis among people in the highest quartile of total dairy consumption is 14% and 25% lower compared with those with the lowest intake, respectively. Furthermore, a higher intake of low-fat dairy products was associated with a 22% and 33% lower incidence of MASLD and cirrhosis, respectively. However, the types of dairy products were not analyzed separately in this article (467). Another cohort study conducted on 12,204 Chinese people free of MASLD at baseline found that 1 serving/d increase in total dairy consumption was associated with a 6% lower risk of MASLD. Subgroup analysis found that increasing yogurt consumption by 1 serving/d decreased the risk of incident MASLD by 8%, but milk had no association. This study does not include cheese consumption because of minimal intake due to cultural preferences (494). A cohort study was conducted on 7,540 Iranian adults. Keshavarz et al. evaluated the relationship between an array of milk products, including milk, yogurt, and cheese, among others, and the incidence of MASLD and found that only milk consumption was associated with a 4% lower risk of NAFLD (468).

Through the third aim, I address the need to define precise mechanisms behind the relationship between dairy consumption and obesity-related metabolic disorders mentioned in recent meta-analyses and systematic reviews (234), which is not clear at present. Therefore, I investigated how two fat-free dairy products, milk, and plain yogurt, and one reduced-fat dairy product, cheddar cheese (19% fat), when added to the HFD, contribute to the regulation of metabolism using a mouse model of obesity. As expected, HFD mice gained more body weight than LFD mice, whereas treatment with milk, yogurt, and cheese mitigated weight gain. My data suggest that milk consumption, followed by yogurt, is the most effective in reducing liver lipid accumulation, enhancing thermogenic activity, and improving metabolic health. At the same time, cheese also reduces body weight but by undiscovered mechanisms not related as strongly to thermogenesis (Figure 8.1).

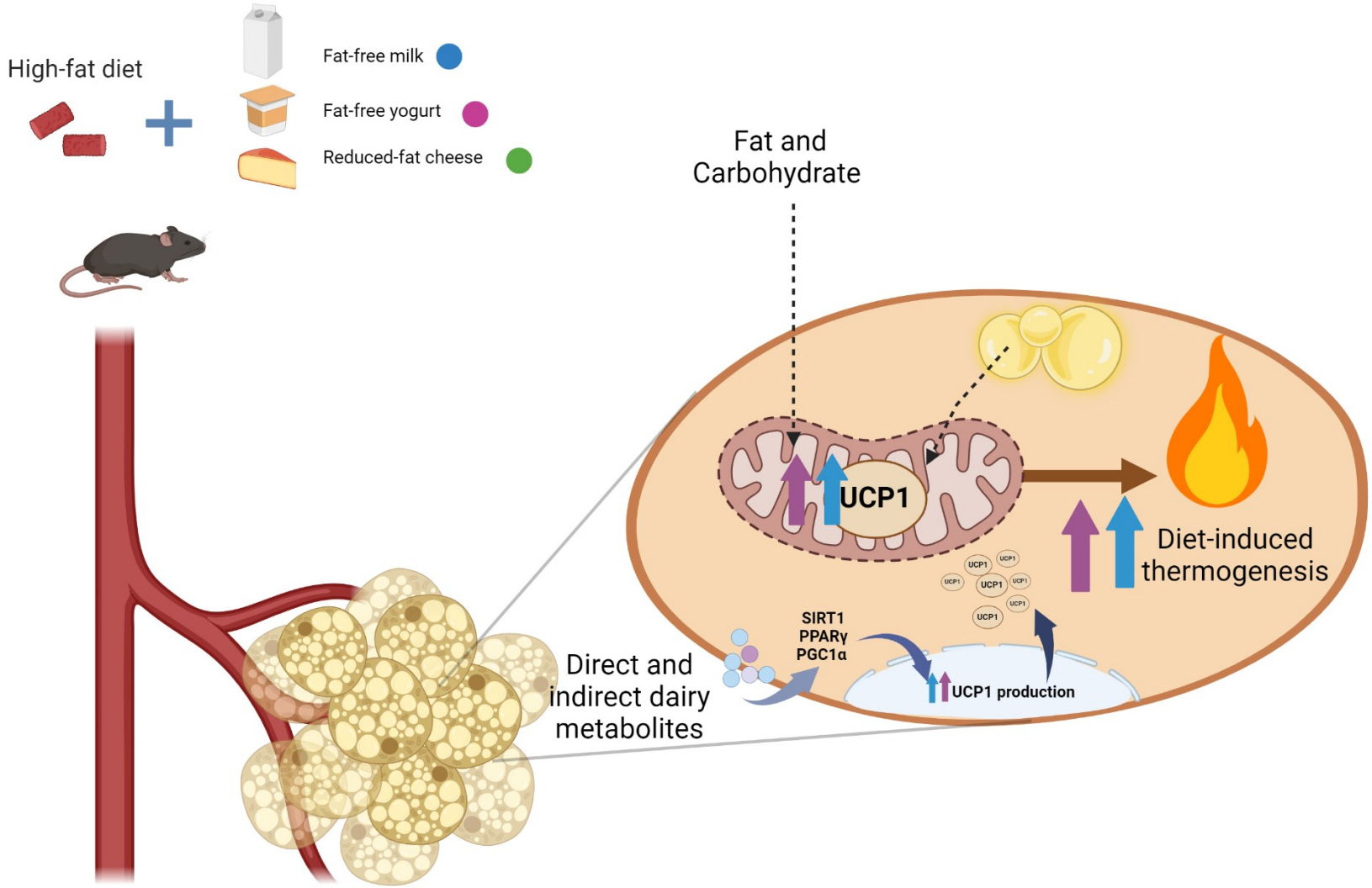
High-fat diet
Fat-free milk
Fat-free yogurt
Reduced-fat cheese
Fat and
Carbohydrate
UCP1
Diet-induced
thermogenesis
SIRT1
PPARγ
PGC1α
UCP1 production
Direct and
indirect dairy
metabolites

Figure 8. 1 The graphical abstract summarizes the effects of fat-free milk, fat-free yogurt, and reduced-fat cheese on brown adipose tissue (BAT) thermogenesis in high-fat diet (HFD)- induced obese mice.

The figure illustrates how dairy consumption influences diet-induced thermogenesis through the upregulation of uncoupling protein 1 (UCP1). Direct and indirect dairy metabolites activate key regulatory pathways, including SIRT1, PPARγ, and PGC1α. These pathways enhance UCP1 production, leading to increased mitochondrial activity and heat production, contributing to improved energy expenditure and metabolic health.

In this study, a histological alteration in the liver was observed in milk and yogurt groups, which contained considerably smaller lipid droplets with lower TG content than the HFD-fed mice; however, in the milk group, this reduction of TG content was greatest and accompanied by the improved glucose homeostasis and insulin sensitivity. These improvements in mice supplemented with milk can possibly be attributed to a potential for increased BAT thermogenesis by activating UCP1 through SIRT1-PPARγ-PGC-1α axis pathways. Yogurt consumption increased UCP1, AMPK, and PGC1α, but cheese was less effective in changing thermogenic proteins in BAT (Figure 8.2). BAT is specialized for thermogenesis, which expends considerable energy dissipated in producing heat (609). The primary energy source for BAT thermogenesis is lipid. Activation of BAT by stimuli rapidly induces the breakdown of intracellular TGs from lipid droplets into FAs and leads to thermogenesis and FA oxidation. TG stores in BAT are replenished mainly by the uptake of NEFAs from the bloodstream. As a result, BAT thermogenesis is an important part of total energy expenditure, which favors lipid combustion in particular (610). My results suggest that milk also increased liver lipolysis, as evidenced by increased ATGL; the liberated FFA can be subsequently taken up by BAT and utilized as an energy substrate for non-shivering thermogenesis. Adipose tissue dysfunction, which occurs through the expansion of fat as a result of the excess energy intake in both BAT and WAT (68), contributes to the systemic metabolic dysregulation in diet-induced MASLD and affects the pathophysiology of MASLD through a range of mechanisms mediating liver-adipose tissue crosstalk (611). However, the activation of BAT may exert beneficial metabolic effects in preventing/reversing NAFLD by promoting negative energy balance, reducing the availability of NEFAs to be accumulated within the liver, and secreting endocrine signals (611). These results appear to be translatable to humans. A recent study of participants with MASLD indicated that those with lower BAT activity had elevated hepatic fat content (612). Furthermore, BAT-based strategies have been introduced to activate energy dissipation and improve metabolic dysfunction in rodents and humans (613, 614, 615). Recently, the enhancement of energy expenditure by food ingredients and natural products has gained significant interest in combating MASLD via promoting adaptive thermogenesis and stimulating BAT (616). Milk and dairy products contain bioactive molecules that have been shown to induce BAT activation in isolation (253, 435, 440).

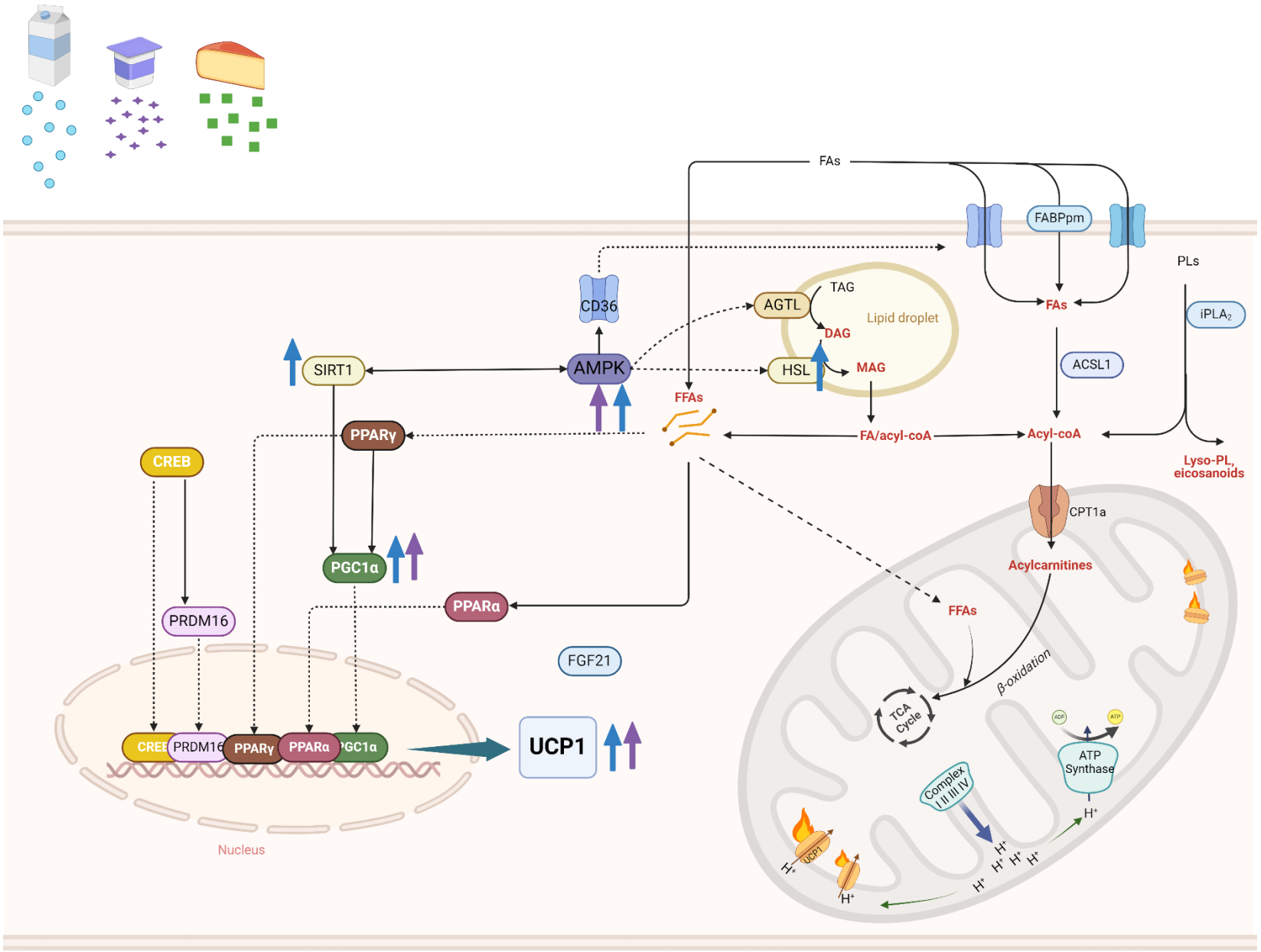
FAs
FABPpm
PLs
CD36
AGTL
TAG
Lipid droplet
DAG
FAs
iPLA2
SIRT1
AMPK
HSL
MAG
ACSL1
FFAs
PPARγ
FA/acyl-coA
Acyl-coA
Lyso-PL, eicosanoids
CREB
CPT1a
PGC1α
Acylcarnitines
PPARα
FFAs
PRDM16
FGF21
β-oxidation
TCA Cycle
CREB
PRDM16
PPARγ
PPARα
PGC1α
UCP1
ATP Synthase
Complex I III IV
Nucleus

Figure 8. 2 Proposed mechanism of action for milk, yogurt, and cheese on brown adipose tissue thermogenesis in high-fat diet (HFD)-induced obese mice.

The figure illustrates the molecular pathways dairy consumption affects, leading to thermogenesis. The arrows indicate the direction of abundance of specific proteins and pathways due to dairy consumption. When AMP-activated protein kinase (AMPK) is promoted, it enhances the Sirtuin 1 (SIRT1) and vice versa. This cascade leads to the activation of peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1α). PGC-1α upregulates uncoupling protein 1 (UCP1) and facilitates thermogenesis by uncoupling ATP synthesis from mitochondrial respiration, leading to heat production and increased energy expenditure. Furthermore, the presence of fatty acids in the cell can activate peroxisome proliferator-activated receptor -α (PPAR-α) and PPAR-γ as ligands, further enhancing their regulatory roles in lipid metabolism.

In other respects, an HFD resulted in hyperglycemia as well as IR. Observed decreases in fasting glucose, fasting insulin, and HOMA-IR suggest improved glucose homeostasis and insulin sensitivity in milk-fed HFD mice. An insulin tolerance test (ITT) and pyruvate tolerance test (PTT) were also conducted at the end of the study; however, I found the results for ITT and PTT tests to have high variability between animals. This variability can be attributed to so many factors that are independent of the dietary interventions, which suggests that our data coming from these tests might not be an accurate reflection of the impact caused by these interventions on metabolic phenotypes. Several potential factors may have contributed to this variability. With these possible sources of variation, the accuracy of my data raises a big question; thus, I decided not to include the data for ITT and PTT in my paper. Of note, despite variability observed in ITT and PTT results, we found elevated pAKT, which suggests that dairy ameliorates the hepatic insulin signaling pathway. A reduction in the levels of PEPCK suggests reduced gluconeogenic potential of the liver. Overall, the *in vivo* tests were not entirely reliable, but these findings support that dairy supplementation presents beneficial effects on glucose homeostasis and insulin sensitivity.

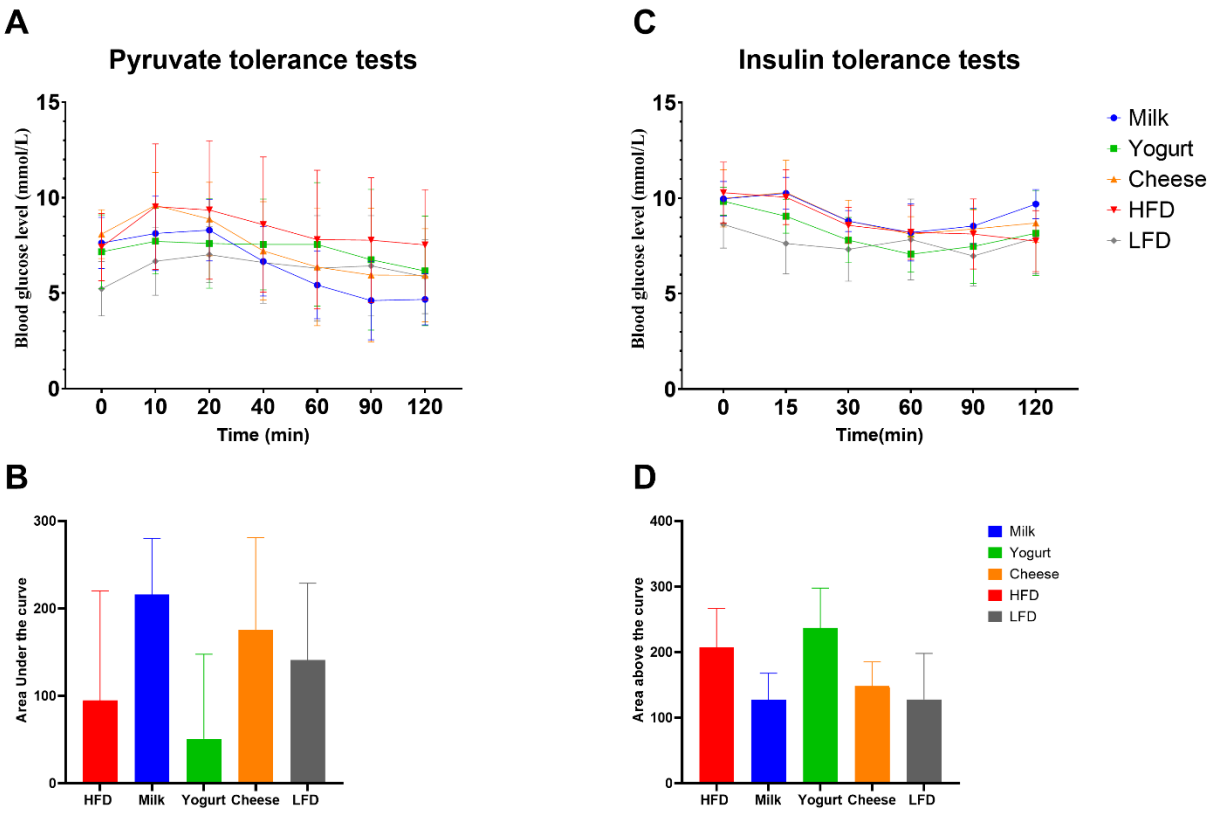

Figure – (A) In week 8, The pyruvate tolerance test (PTT) was performed in 12-h fasted mice after an intraperitoneal injection of 2 mg/g of body weight of sodium pyruvate (Sigma-Aldrich, St Louis, MO, USA) saline solution. Blood glucose was measured using a glucometer (Contour Next, Bayer, Leverkusen, Germany) at the baseline before pyruvate injection, and then blood glucose was measured 10, 20, 40, 60, 90, and 120 min after the challenge. (B) The area under the curve for PTT. (C) In week 8, an insulin tolerance test (ITT) was performed in 4-h fasted mice who received an intraperitoneal injection of insulin (2.6 mg/g), and blood glucose was measured at 0, 15, 30, 60, and 120 min later. (D) the area under the curve was calculated.

Through these results, my study has provided further insight into the importance of different dairy matrices impacting metabolism through a thorough analysis of the liver and serum lipid molecule profiles. An interesting observation I made in the mouse trial was of the significant divergence in lipidomic profiles across the different dairy interventions, which highlights the unique metabolic pathways influenced by each dairy product. After the integration of liver and serum lipidomic data findings, I found that consumption of milk and yogurt significantly alters lipid metabolism, both hepatically and systemically. These alterations include the regulation of key lipid molecules and pathways, suggesting beneficial effects on liver fat accumulation, activation of BAT, and overall metabolic health. In contrast, cheese exhibited a less pronounced impact on lipid metabolism. For yogurt and milk, the integrated analysis reveals complex interactions between dairy interventions and lipid metabolism. For instance, the downregulation of sphingolipids and phosphosphingolipids in the serum and liver after feeding milk indicates a systemic reduction of these classes of lipids, which modulate pro-inflammatory processes, possibly insinuating some beneficial effect on lipid metabolism (617, 618). The upregulation of glycerolipids and triacylglycerols in both the serum and liver of mice supplemented with yogurt likely reflects increased fatty acid mobilization, possibly in a compensatory response to the HFD-induced disruption (619, 620). The cheese group, characterized by less induction of these specific lipid pathways, is consistent with reduced-fat cheese having a more neutral effect on lipid metabolism compared to milk and yogurt.

My results align with mouse studies finding that fecal bacteria may affect BAT function (621, 622, 623). Transferring the microbiota of mice exposed to the cold to germ-free mice increases the expression of UCP1 in the host BAT, demonstrating the profound influence of microbes in the gut on energy homeostasis (624). At the genus level, milk supplementation enriched beneficial genera like *Anaerotignum, Oscillospiraceae, and Bariatricus.* In addition, yogurt consumption increased the abundance of beneficial genera like *Streptococcus* and *Clostridium*. Various members of these genera are known to produce SCFAs, which are correlated with decreased obesity and improved metabolic health, suggesting that the alterations in gut microbiota composition induced by milk and yogurt consumption are mainly responsible for metabolic changes. SCFA-producing bacteria, such as those enriched by milk and yogurt, can increase the production of compounds like butyrate, which enhances BAT function (625, 626, 627). *Streptococcus* genus is used as a probiotic within the yogurt matrix can be increased in the gut of humans and rodents to influence the gut microbiota positively and may help prevent or treat obesity-induced metabolic dysfunctions (628, 629, 630,

631). Besides, *Oscillospira* and *Clostridium* genera were also present in metabolically healthy obese individuals compared to the metabolically unhealthy obese individuals (625), which further supports the role of these genera in promoting metabolic health. These findings show the potential for dairy products, including milk and yogurt, to modulate gut microbiota that may promote metabolic health through improved BAT function, consequently reducing obesity and improving energy homeostasis.

A growing body of research indicates that the various dairy products have complicated effects on the body's metabolism due to possible interactions between various nutrients and other properties, including homogenization, fermentation, and probiotics (205). In order to understand how dairy product consumption can induce metabolic changes and bring health benefits, study methodology is becoming more sophisticated, and instead of only examining the relationship between dairy consumption (as determined by dietary records or biomarkers) and metabolic health outcomes, they started to apply integrated strategies, including untargeted metabolomic analyses of serum, urine, and feces samples (632). Metabolomic techniques have revealed that metabolic disorders significantly alter the plasma metabolome, particularly affecting the metabolism of phospholipids, acylcarnitines, biogenic amines, and amino acids, as compiled in recent reviews (633, 634). Dr. Chan's previous study hypothesized that the beneficial effect of regular and low-fat cheese on improving lipid and glucose homeostasis in HFD rats might be attributed to improvements in specific phospholipid species that are disrupted by HFD-induced obesity (283). This hypothesis is further elaborated in a scoping review that I undertook to compile the existing evidence on dairy products or their derivatives, like phospholipids, to assess where the current literature stands and what areas need further exploration. By summarizing the literature (by Feb 2021), I observe that the disruption of the phospholipid profile in metabolic diseases can be partially compensated by dairy products or their high phospholipid derivatives. Perhaps predictably, after considering changes in the phospholipids in response to dairy products across studies, it can be learned that liver PC and LPC species, including those containing C15:0 and C17:0 fatty acids, which are known as dairy consumption biomarkers, are more affected by dairy products. However, some other altered PC and LPC species are not inherently present in milk or other dairy products, indicating a profound impact on lipid metabolism regulation. This study provided the rationale for me to examine the lipidomics of mice that received fat-free milk, yogurt, or reduced-fat cheese over 8 weeks, as discussed above. Our findings partially support the hypothesis that fat-free milk,

followed by yogurt, increased certain serum lipid molecules, including PC and LPC with C15:0. However, the liver lipid profile showed more stability than serum in response to dairy consumption, and milk and yogurt reduced some lipid species of diacylglycerol and sphingosine, thereby improving hepatic lipotoxicity. Findings from recent RCTs show that serum PC with C15:0 is not affected by fat-free milk or whole-fat milk consumption through the 12 weeks of intervention (635). Supplementation of a standard diet of mice with milk polar lipid change serum PC 14:0/20:2 and 14:0/18:2 without any impact on C15:0 and C17:0 (636).

Nowadays, people's concern for their diets is increasing as they become more aware of their health and the importance of nutrition; consumers are interested in functional foods, typically defined as foods that provide health advantages to the host other than basic nutrition (637). Humans have consumed milk and dairy products for thousands of years as part of their diet. Dairy products contribute ~10% of all calories in the US diet (638). Milk alone provides 7.5% of the total calorie intake in the Canadian diet (204). Yet, for such a significant share of the food supply, a relatively high debate exists to question the direct health impact of consuming dairy foods. Milk is a complex mixture of proteins, fats, and carbohydrates. It also contains various bioactive ingredients such as casein hydrolysates, whey protein hydrolysate, α-lactalbumin, galactooligosaccharides, conjugated linoleic acid (CLA), and glycomacropeptide. My colleagues and I have thoroughly reviewed their impact and underlying mechanisms on glucose homeostasis (223). Consequently, milk qualifies as a naturally occurring functional food (223).

In critically assessing the relative efficacy of dairy products compared with other food and bioactive ingredients previously reported to increase insulin sensitivity and lower liver lipids (639), it is apparent that one must consider both physiological mechanisms and practical translational relevance for people, especially those at high risk for IR and hepatic steatosis. A wide range of food ingredients, such as IRW (Isoleucine–Arginine–Tryptophan, a bioactive peptide from egg white), quercetin, mung bean protein, Kefir peptide, astaxanthin, pectin, and Ginkgo polysaccharides are just a few of thousands of discovered molecules which have shown evidence of anti-obesity and anti-diabetes properties (639). Mechanistically, these foods often work through specific pathways like the enhancement of lipid metabolism, reduction in inflammation, or modulation of gut microbiota. In fact, dairy products, namely low-fat milk and yogurt, have shown similar benefits, indicating improvement in insulin sensitivity, increased energy expenditure, and alterations in lipid profiles through mechanisms such as the modulation of gut microbiota and

enhancement of lipid metabolism. Another advantage that dairy brings to overweight or obese people in the prevention of IR and hepatic steatosis is its high availability and being easily consumed in a diet; moreover, it offers important nutrients like calcium and vitamin D, providing high-quality protein that is good for health. Last but not least, dairy products are relatively safe and well-tolerated foods, except for people who suffer from lactose intolerance (640).

By recommending low-fat dairy products, especially milk and yogurt, as part of an overall eating pattern, individuals may achieve significant health benefits related to metabolic health and liver lipid prevention or reduction. This integrated approach not only addresses the physiological mechanisms but also offers a practical, affordable, and nutritionally comprehensive option for people at risk of obesity-related metabolic diseases. Such considerations allow some synthesis to be made from the findings within the six chapters and help to focus some on the somewhat unique position of dairy products within the broader context of dietary interventions for metabolic health.

8.2 FUTURE DIRECTIONS

From the epidemiological point of view, extracting the pattern of dairy product consumption using a data reduction technique and examining its association with T2D incidence allows for assessing the contributions of various dairy consumption aspects and provides a better understanding of the association of T2D with dairy pattern consumption. It is the first study to undertake this approach and was made possible by the detailed nutritional assessment conducted in Alberta's Tomorrow Project. However, it should be noted that a more frequent assessment of both diet and T2D indicators is required for appropriately determining temporal relationships between them. The main limitation of my study is the lack of serial dietary information throughout the study follow-up period. Dietary intakes can change over time, and a greater number of repeated diet measures can theoretically be better able to capture this variation (assuming one can avoid recording fatigue and other forms of reporting bias) (641). In particular, the relaying of a one-time dietary assessment when the participants are followed longitudinally in relation to the presence of a health outcome may be inadequately captured by only a baseline measure, as in Alberta's Tomorrow Project. Thus, future observational studies need to be conducted to assess the relationship between dairy pattern consumption and T2D by considering the dietary changes through the follow-up time by frequent diet measures.

Furthermore, investigating and improving dietary patterns in a multi-ethnic and multi-cultural population like Canada is one of the most challenging tasks. Analyzing the association of dairy consumption patterns with obesity and its metabolic disorders among diverse populations adds another layer of complexity. My study explored the connection between overall dairy consumption patterns and T2D via principal component analysis (PCA), which will likely yield valuable data for hypothesis generation. I, however, have not fully addressed the variation in dairy item choices among subpopulations, including ethnic minorities. It is clear that further studies need to be conducted among Canadian subpopulations, considering ethnic and cultural backgrounds to evaluate the reproducibility of the findings. Only after developing a clear set of definitions for the dairy consumption patterns among the Canadian population can researchers determine correlations between dairy consumption patterns and disease outcomes in the future. The importance of these dairy-pattern-based findings is significant for policy-based health promotion because they will provide support for a healthy overall dietary pattern in disease prevention. This approach is more effective than promoting individual dairy food items or conventional categorization based on fat content, which is the most influential in health practice recommendations for preventing metabolic disorders such as T2D and MASLD in Western populations.

To the best of our knowledge, for the first time, a meta-analysis with observational studies has been performed to pool data on the association between dairy product consumption and the risk of MASLD. Yet, there is still no meta-analysis summarizing data from RCTs on the impact of dairy product consumption on parameters of liver function. It will be very important for the few RCTs (312, 324, 483, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652) carried out in various settings with small sample sizes to be pooled and analyzed in the framework of a meta-analysis to produce robust and high-level evidence supporting clinical practice guidelines. A meta-analysis would clarify dairy's role in liver health; it will guide more precise conclusions, and dietary recommendations and interventions will be made in the future.

The first and major limitation of my preclinical study is that it was conducted exclusively in male mice, without including female mice. This sex bias overwhelms the generalizability of the findings because male and female mice can have different responses to dietary interventions on a metabolic condition due to differences in sex hormones, body composition, and metabolic rates (653, 654, 655, 656). Besides, the presence of different metabolic profiles between males and females, along

with different risk factors for diseases such as T2D and MASLD, could modulate responsiveness toward dietary interventions. (657, 658, 659). Future studies should include both males and females in equal proportions to understand how dairy intake impacts metabolic health in both sexes. The inclusion of female mice would also allow the identification of sex-specific mechanisms or responses that will help ensure dietary recommendations for both males and females. In the research on how dairy products may affect metabolic health in both sexes, sex-specific benefits and risks may be identified for more tailored nutrition advice. This approach is expected to strengthen the effectiveness of dietary interventions and contribute to better health outcomes with respect to the incidence of metabolic disorders between both sexes, therefore averting the burden of metabolic disorders in the population.

Following this preclinical study, future research should focus on a detailed analysis of molecular mechanisms explaining how different dairy products exert their differential effect on energy homeostasis and obesity phenotypes. Accordingly, more research that would help establish the specific dairy bioactive components responsible for such effects on thermogenesis and lipid metabolism in BAT would be highly beneficial in combating obesity. This would then make it possible to discover the ingredients responsible for the same and thus enhance energy expenditure, fat oxidation, and reduced hepatic lipid accumulation. This information can potentially reveal the pathways through which dairy foods may elevate energy expenditure and suppress lipid accumulation. Identifying such components would also unravel, formulating extraction of potent compounds that may be utilized as supplements, or bioactive components may be concentrated in milk or fortified to increase their health benefit.

We show that the impact of dairy products on metabolic health can be through the modulation of the gut microbiome. Future studies need to address how this relates to changes in the microbiome induced by dairy consumption and whether these changes can be transferable to recapitulate the metabolic effects of dairy consumption. Such studies could be most effectively conducted with fecal microbiota transplantation experiments, in which the microbiota from dairy-supplemented animals would be transferred to germ-free hosts. The experiments would be carried out under controlled conditions in which one group of germ-free mice receives microbiota from milk-supplemented donors, and another group receives microbiota from yogurt-supplemented donors. A control group of germ-free mice would receive microbiota from non-dairy-supplemented

donors, which will then provide information on how far gut microbiota in itself can mediate the beneficial effects of dairy products for improved energy homeostasis, BAT function, and metabolic health. The findings of such studies might open a great window into the role of gut microbiota in health effects related to dairy products, possibly leading to the development of promising probiotics or prebiotics that would exploit these benefits. It could also guide better dietary guidelines and interventions for improving metabolic health and lowering obesity, in which modulation of gut microbiota presents a prime mechanism of action for these beneficial effects of dairy products.

It would also be interesting to find out whether these dairy products affect other tissues, such as the muscle, WAT, and brain, in order to give a more comprehensive view of the systemic changes in metabolism. Interventional studies in humans should now be carried out to validate the findings in rodent models and should focus on changes in energy homeostasis following the intervention. The modulating effect of dairy product consumption on DIT must be considered as an acute and chronic effect of dairy consumption. Such studies would provide a long-term perspective on the effects of low-and reduced-fat dairy foods on weight management, metabolic health, and energy homeostasis. Another point that needs to be highlighted is the role of the gut microbiota in mediating dairy's effect on energy homeostasis and metabolic health as a potential modifiable risk factor for obesity and its related metabolic disorders. Particular attention should be paid to the possibility that dairy products promote browning in WAT because that would suggest molecular triggers behind such a transformation and aspects of the environment that support it. Finally, a study using a multi-omics approach, including transcriptomics, proteomics, and metabolomics, integrated with microbiome analysis, is required to take a holistic look at the biological changes induced by dairy consumption. These will lead to a deep understanding of the interaction between dietary components and metabolic pathways and open ways for personalized nutrition strategies to prevent obesity and metabolic disorders.

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