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Obesity and type 2 diabetes mellitus: novel and alternative functional bioactive nutritional interventions

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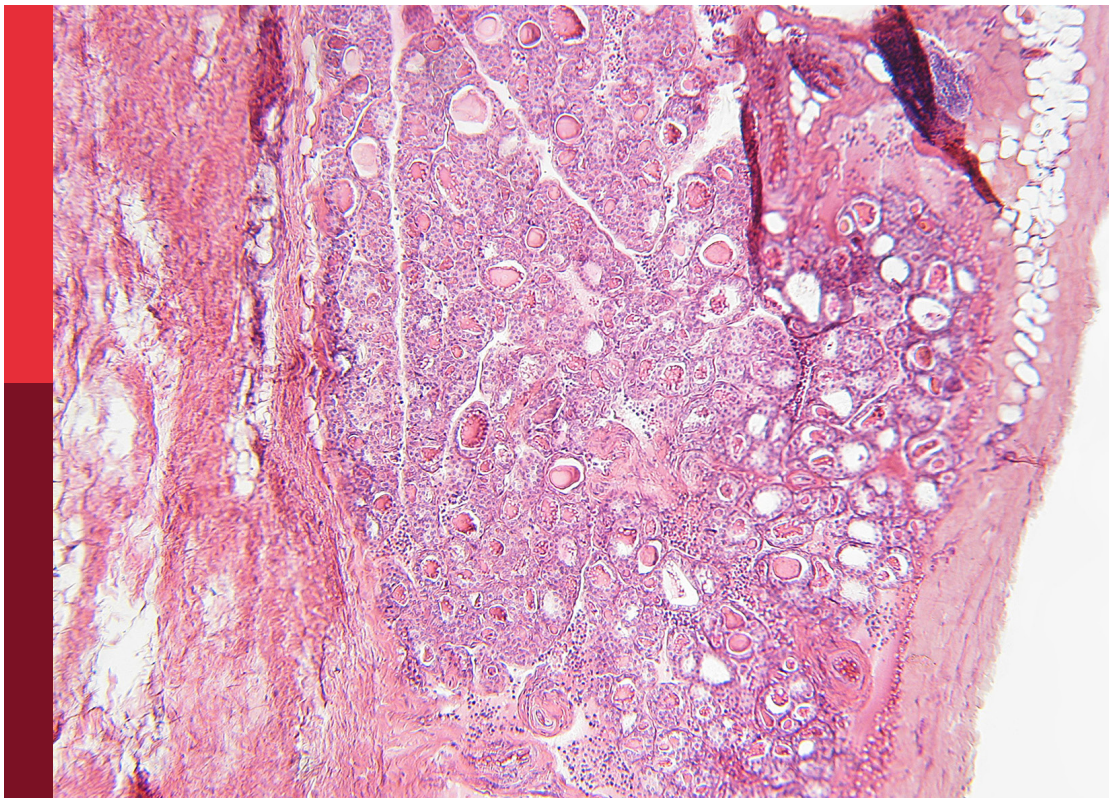

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Obesity and type 2 diabetes mellitus: novel and alternative functional bioactive nutritional interventions

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Editorial: Obesity and type 2 diabetes mellitus: novel and alternative functional bioactive nutritional interventions

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Editorial on the Research Topic
Obesity and type 2 diabetes mellitus: novel and alternative functional bioactive nutritional interventions

Obesity is a metabolic disorder that increases the risk of developing type 2 diabetes mellitus (T2DM), cardiovascular disease and cancer and requires effective, durable interventions (1). Whilst dietary and physical activity approaches are foundational, long-term adherence is challenging. Pharmaceutical and surgical therapies are proven options but often unaffordable. Therefore, innovative and economically viable nutritional interventions, focusing on bioactive compounds, nutrients, or molecules are urgently required to address these disparities in adherence and access to effective therapies. This collection of articles focuses on novel dietary supplements and bioactive molecules extracted from functional food as strategies to mitigate or fight obesity and T2DM.

Bioactive nutritional components show promise in addressing obesity and associated complications (2). Chen et al. discusses the potential of traditional Chinese medicine, including acupuncture and herbal therapy, for improving metabolic health by regulating various metabolic processes such as energy homeostasis, glucose metabolism, inflammation and enhancing insulin sensitivity. Similarly, Zhou et al. demonstrated that mulberry leaf extract and *Hippophae* protein peptides (MHP) have beneficial effects on blood glucose, lipid profile, and weight loss. The administration of MHP in obese rats, suggests its potential for reducing adiposity through pathways like PPARγ and FGFR1 signaling, although further research is needed for a complete understanding of its mechanisms. The authors demonstrated MHP induced weight loss and reduced adiposity through blocking adipocyte enlargement and fat depots, despite similar energy intake in rats on high-fat high-fructose diet. Combined data supports the findings that the protective effect of MHP on adiposity is at least partially associated with PPARγ and FGFR1 signaling pathways.

As showed by Yang et al., *Faecalibacterium prausnitzii* strains, a potential bioactive-compound producing species, displays beneficial metabolic effects contributing to the amelioration of obesity and associated metabolic disorders in an obesity-mice model induced by high-fat diet consumption. In their study, the authors showed that *F. prausnitzii* acts by modulating the gut-brain axis, inducing gut and neural hormone secretion that inhibited appetite in rats. Similarly, a randomized double-blind controlled trial by Savytska et al., assessed live multi-strain probiotics combined with absorbent smectite supplement effectiveness in participants with T2DM. It showed improvement in glucose homeostasis and in the pancreatic β-cell function.

Metabolic dysregulation in obesity and T2DM is associated with overall body metabolic dysfunction, which involves the action of different hormones including incretins. Glucagon-like peptide 1 (GLP-1) receptor agonists are applicable for use in combination with metformin in selected clinical settings: atherosclerotic cardiovascular disease, T2DM, and the presence of chronic kidney disease (3, 4). Xie et al. aimed to perform a network meta-analysis to evaluate the efficacy and safety of GLP-1RAs in combination with metformin. This review provides evidence-based support and reference for the selection of clinical treatment. More specifically, the data showed that GLP-1RAs are highly effective in lowering HbA1c and reducing body weight and did not cause hypoglycemic reactions. Finally, the results presented here may provide guidance and orientation for the selection of clinical therapeutic agents.

Early intervention and well established anti-diabetic therapies are accepted and shown to be pivotal in the fight against obesity and its complications. However, sex-specific effects on endocrine/metabolism responsiveness are often observed and must be taken into consideration. Herein, Ivic et al. studied the effects of long-term treatment with metformin and liraglutide in elderly male and female rats fed a high-fat high-sugar. Even though, metformin treatment appears to be better than liraglutide, by improving central-leptin and peripheral-insulin sensitivity in female rats; while liraglutide therapy display a positive response, as short but not as long-term effect in male-rats, on the satiety signaling and on the hyperinsulinemia.

Author contributions

RB: Writing – review & editing, Writing – original draft. LB: Writing – review & editing, Writing – original draft. II: Writing – review & editing, Writing – original draft. JO: Writing – review & editing, Writing – original draft.

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We extend our gratitude to the reviewers for their thorough evaluation and to the authors for their valuable contributions to this Research Topic. It is our hope that this Research Topic will offer comprehensive insights into the role of novel bioactive compounds and approaches in addressing T2DM and its complications. By exploring alternative and more affordable strategies, we aim to enhance our understanding of how these interventions can positively impact individuals living with obesity and T2DM.

Conflict of interest

Author LB was employed by Kallyope Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Exploring the underlying mechanisms of obesity and diabetes and the potential of Traditional Chinese Medicine: an overview of the literature

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Obesity and diabetes are closely related metabolic disorders that have become major public health concerns worldwide. Over the past few decades, numerous studies have explored the underlying mechanisms of these disorders and identified various risk factors, including genetics, lifestyle, and dietary habits. Traditional Chinese Medicine (TCM) has been increasingly recognized for its potential to manage obesity and diabetes. Weight loss is difficult to sustain, and several diabetic therapies, such as sulfonylureas, thiazolidinediones, and insulin, might make it harder to lose weight. While lifestyle changes should be the primary approach for people interested in lowering weight, drugs are also worth investigating. Since some of the newer glucose-lowering medications that cause weight loss, such as glucagon-like peptide-1 receptor agonists (GLP-1 RAs) and sodium-glucose cotransporter 2 inhibitors (SGLT2i), are additionally utilized or are under consideration for use as anti-obesity drugs, the frontier between glucose-lowering medication and weight loss drugs appears to be shifting. This review provides an overview of the literature on the underlying mechanisms of obesity and diabetes and the prospect of TCM in their management. We discuss the various TCM interventions, including acupuncture, herbal medicine, and dietary therapy, and their effects on metabolic health. We also highlight the potential of TCM in regulating gut microbiota, reducing inflammation, and improving insulin sensitivity. The findings suggest that TCM may provide a promising approach to preventing and managing obesity and diabetes. However, further well-designed studies are needed to confirm the efficacy and safety of TCM interventions and to elucidate their underlying mechanisms of action.

KEYWORDS

Traditional Chinese Medicine, diabetes, gut microbiota, obesity, literature review

1 Introduction

Obesity is the accumulation of excess fat tissue in the body, which can occur at any age, and it is characterized by increased body and fat mass, hormone imbalances, eating patterns, and genetic factors. This condition has significantly contributed to the global burden of chronic diseases, including type 2 diabetes (T2D), cardiovascular disease, and asthma. It is considered a worldwide pandemic, and approximately 2.8 million people die from its complications annually (1). Directly measuring fat throughout the body is impossible, so the body mass index (BMI) is commonly used to evaluate the relationship between weight and height. Other methods, such as waist circumference, waist-to-hip ratio, skinfold thickness, and bio-impedance, are also utilized to assess overweight and obesity (2). If individuals fall into the heavy range of BMI, they are more likely to develop other diseases such as T2D, hypertension, cardiovascular diseases, and gallstones. The risk is moderate for those in obesity class 1, severe for those in obesity class 2, and very high for those with extreme obesity, especially if they already have other obesity-related diseases (3). Figure 1 illustrates the BMI classification recommended by the World Health Organization and the National Institute of Health in the United States.

Visceral and subcutaneous are the two types of fats in the human body. Visceral fat is the one that gets deposited around organs such as the liver, pancreas, and kidneys, among others. This type of fat is also known as active fat because it significantly impacts hormonal activity. According to research, visceral fat accumulation can result in metabolic syndrome and insulin resistance, affecting appetite and body fat distribution (4). Subcutaneous fat, on the other hand, is the fat located beneath the skin and can be felt in areas like the underarms and legs. Fat distribution in the body can result in two types of shapes, apple, and pear. People with an apple shape tend to accumulate fat in the upper region of their waist, abdomen, neck, arms, and shoulders (5). This physique is predominantly linked with visceral fat, heightening the possibility of developing T2D.

Conversely, individuals with a pear-shaped body have fat stored in their hips and thighs, resulting in lower visceral fat levels and a diminished risk of weight-related ailments (6). Obesity can give rise to numerous complications, such as reproductive problems for both genders, respiratory and cardiovascular illnesses, and issues with the gastrointestinal system and pancreas, as illustrated in (Figure 2). The United States ranked first in obesity, followed by China and India, according to the Organization for Economic Co-operation and Development (OECD) in 2017 (7). There has been a significant surge in obesity rates from 1999-2000 to 2015-2016 (8). In 2016, the World Health Organization (WHO) stated that of 1.9 billion overweight adults aged 18 and above, 650 million had obesity (2). An estimated 25 million individuals perish yearly due to obesity or being overweight (9, 10).

Moreover, a study by WHO in 2019 found that approximately 38.2 million children under 5 were either overweight or suffering from obesity (11). The individual's lifestyle and dietary choices are crucial in contributing to the development of obesity (12). Foods that are high in fat and sugar tend to have low micronutrient content, which can lead to weight gain. Excessive intake of processed grains, unhealthy snacks, and sugary drinks may lead to a more excellent waist-to-hip ratio and heightened accumulation of body fat (13–16).

Diabetes is a chronic condition characterized by elevated blood sugar levels due to insufficient insulin production or the body's inability to use insulin effectively. Insulin is a hormone produced by

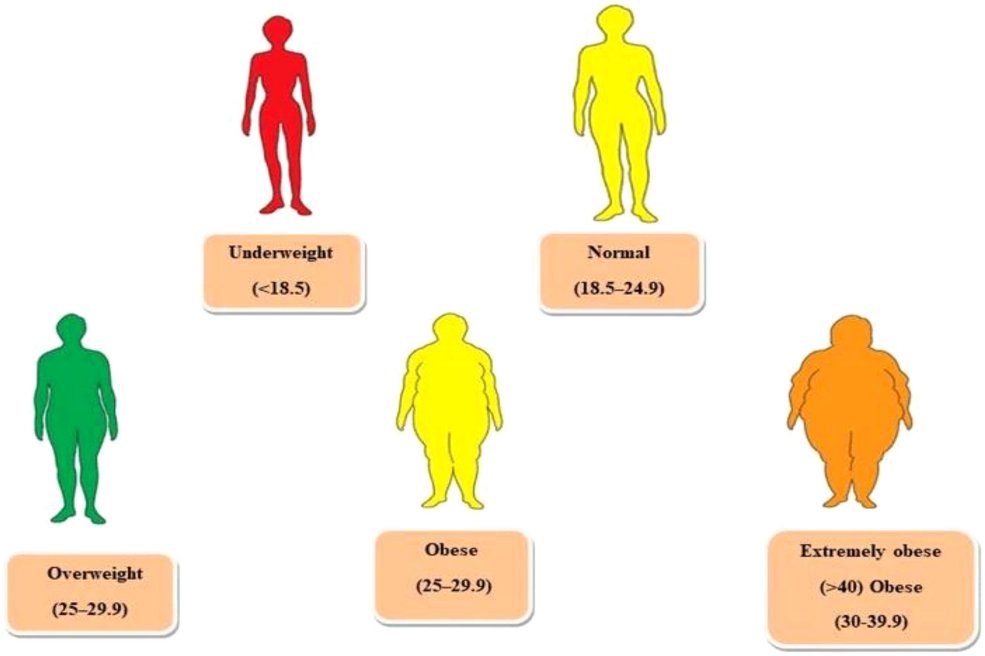

FIGURE 1
WHO recommended categorizing weight based on the body mass index (BMI).

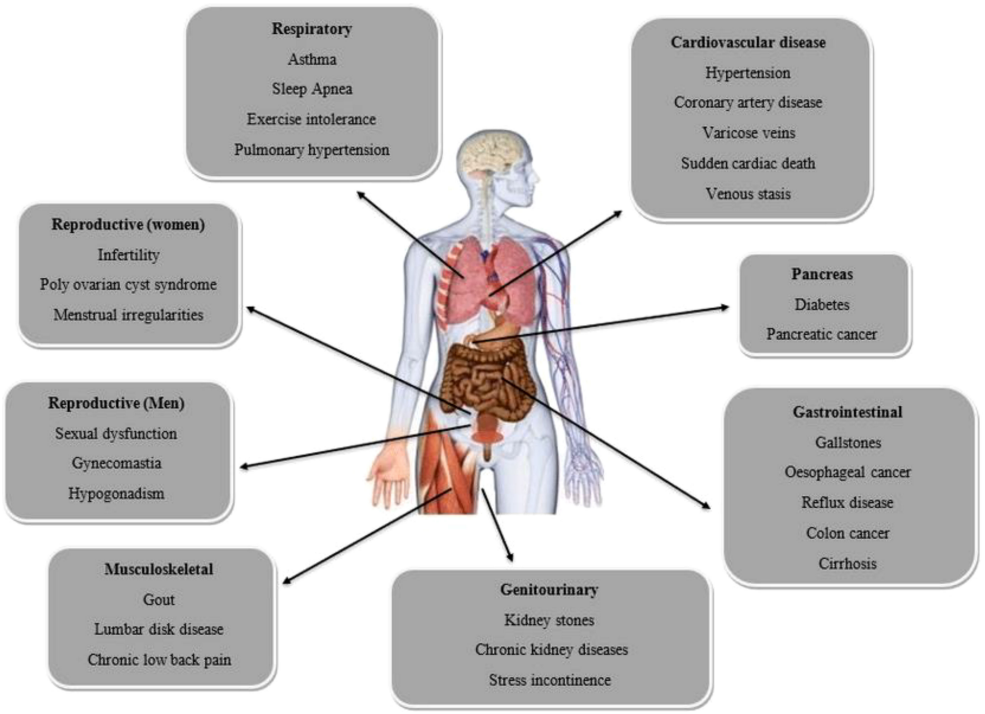

FIGURE 2
Obesity-related complications.

the pancreas that helps the body absorb and use glucose from food as energy (17). There are three primary types of diabetes: type 1, type 2, and gestational diabetes. Type 1 diabetes is an autoimmune disease that occurs when the immune system attacks and destroys the cells in the pancreas responsible for insulin production (18). This type of diabetes is typically diagnosed during childhood or adolescence and requires lifelong insulin therapy (19). T2D is the most common form, accounting for roughly 90% of cases, and occurs when the body becomes resistant to insulin's effects. The pancreas can no longer produce enough insulin to meet demand (20). T2D is often linked to lifestyle factors such as obesity and physical inactivity but can be managed through diet, exercise, medication, or insulin therapy (21). Gestational diabetes develops during pregnancy and is caused by hormones that make it harder for the body to use insulin effectively. While this type of diabetes typically resolves after giving birth, women who develop gestational diabetes have an increased risk of developing T2D later in life, as do their children (22).

If diabetes is not managed correctly, it can result in various complications, such as cardiovascular disease, nerve damage, kidney disease, blindness, and amputations. Nevertheless, individuals with diabetes can maintain a long and healthy life with proper treatment and management, which may involve a blend of medication, lifestyle alterations, and frequent monitoring of blood glucose levels. This can aid in preventing the development of complications associated with diabetes (23, 24). The focus of this review is to explore the underlying mechanisms of obesity and diabetes and to evaluate the potential of Traditional Chinese Medicine (TCM) in managing these conditions. The study examined the current understanding of the pathophysiology of obesity and diabetes and investigated how TCM may help address these conditions. The aim is to provide a comprehensive and critical analysis of the existing research in this field and to assess the potential of TCM as a complementary or alternative treatment option for obesity and diabetes. The present study used a comprehensive search in major scientific databases, including PubMed, Scopus, and Web of Science, to identify relevant studies. The search used keywords related to obesity, diabetes, Traditional Chinese Medicine, underlying mechanisms, and their various synonyms. The search was limited to articles published between 2019 and 2023, with no language restrictions. Inclusion criteria encompassed original research articles, review articles, and meta-analyses investigating the relationship between obesity, diabetes, and TCM at the molecular, cellular, and clinical levels. Studies focusing on the mechanisms of action of TCM interventions, such as herbal remedies, acupuncture, and dietary modifications, were also included. Exclusion criteria consisted of studies that primarily focused on non-TCM interventions or those unrelated to the specific topic of interest.

2 The underlying mechanisms of obesity and diabetes

2.1 Insulin resistance

Insulin resistance refers to the decreased sensitivity of cells to insulin, which increases insulin levels in the bloodstream. It is

considered a significant risk factor for obesity and is believed to be critical in its onset and advancement. The mechanisms by which insulin resistance mediates obesity are complex, but several key pathways have been identified (25). One of the primary mechanisms insulin resistance promotes obesity is its effects on regulating glucose metabolism. Insulin is an essential hormone for maintaining blood glucose levels within a healthy range. When insulin levels are high, glucose is taken up by cells and used for energy. However, in insulin-resistant individuals, cells become less responsive to insulin, leading to elevated glucose levels in the bloodstream (25, 26). The body produces more insulin to compensate, increasing insulin levels. These high insulin levels can promote the storage of excess glucose as fat, leading to weight gain and obesity. Another important mechanism by which insulin resistance promotes obesity is through its effects on the regulation of lipids. Insulin also regulates lipid metabolism; insulin-resistant individuals often have abnormal lipid profiles (27). For example, they may have elevated triglyceride levels, a type of fat in the blood. High triglyceride levels can promote fat storage in adipose tissue, leading to weight gain and obesity.

Insulin resistance can also promote obesity through its effects on regulating appetite and energy expenditure. Insulin regulates several hormones that control appetite and metabolism, including leptin, ghrelin, and adiponectin (28). Insulin-resistant individuals may have abnormal levels of these hormones, which can lead to increased appetite and reduced energy expenditure. This can lead to a positive energy balance, which promotes weight gain and obesity. Finally, insulin resistance can promote obesity through its effects on inflammation (29). Chronic low-grade inflammation is linked to insulin resistance, which may encourage the emergence of obesity and other metabolic disorders. Inflammatory cytokines produced by adipose tissue can impair insulin signaling and promote insulin resistance, further exacerbating the cycle of weight gain and metabolic dysfunction (30).

2.2 Inflammation

Obesity and diabetes are two closely related chronic diseases that are major health concerns worldwide. Research has shown that inflammation plays a crucial role in developing both conditions. When the immune system responds to infection or injury, inflammation occurs naturally. However, if it persists over a long period, chronic inflammation can result in various health issues, such as obesity and diabetes (31). One mechanism by which inflammation contributes to obesity and diabetes is releasing cytokines. Cytokines are signaling molecules secreted by immune cells and play a critical role in inflammation. In obese individuals, there is an increase in the production of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by adipose tissue (31, 32). The cytokines found to hinder the function of insulin, which is crucial in regulating blood sugar levels, have been demonstrated to promote insulin resistance, a defining characteristic of T2D. Persistent inflammation associated with obesity plays a role in developing this disease. Furthermore, inflammation also triggers the activation of the nuclear factor kappa B (NF-κB) pathway, which is a transcription factor that regulates gene expression during inflammation. Studies indicate that activation of the NF-κB pathway occurs in the adipose tissue of obese individuals and contributes to insulin resistance (33, 34). Additionally, NF-κB pathway activation has been linked to the onset of T2D. Inflammation can also contribute to obesity and diabetes by altering the gut microbiome. The gut microbiome refers to the trillions of microorganisms that live in the human gut and play a critical role in maintaining health (35). Studies have shown that obesity and diabetes are associated with alterations in the gut microbiome, and inflammation may be a contributing factor. Inflammation can lead to changes in the composition of the gut microbiome, which can, in turn, contribute to metabolic dysfunction (36).

2.3 Hormonal imbalances

Obesity and diabetes are two of the most prevalent chronic diseases worldwide. Hormonal imbalances can contribute to the development and progression of both conditions, as they can affect the regulation of appetite, energy metabolism, and glucose homeostasis. The two main hormones involved in these processes are insulin and leptin. In this essay, we will examine how imbalances in insulin and leptin can lead to obesity and diabetes (37). Insulin is a hormone the pancreas produces that is critical in regulating glucose metabolism. The process of glucose uptake into cells and its subsequent storage as glycogen in the liver and muscles is enhanced by insulin (38). Obesity and T2D commonly display insulin resistance, characterized by a reduced responsiveness of the body's cells to insulin. Consequently, the pancreas compensates by producing more insulin to maintain normal blood glucose levels. This can result in hyperinsulinemia, characterized by elevated insulin levels in the bloodstream (39). Hyperinsulinemia has been linked to obesity, as it facilitates fat storage in adipose tissue and impedes the breakdown of stored fat. It can also contribute to the development of diabetes by inhibiting glucose uptake into cells and promoting glucose production by the liver. Leptin is a hormone adipose tissue produces critical in regulating energy balance. It acts on the hypothalamus, a brain region controlling appetite and energy expenditure (40). Leptin signals the brain when energy stores are sufficient, reducing appetite and increasing energy expenditure. Leptin resistance is another common feature of obesity. It occurs when the brain becomes less responsive to leptin, and the body produces more leptin to compensate. This condition can lead to hyperleptinemia, characterized by high levels of leptin in the blood (40, 41). Hyperleptinemia can contribute to obesity by promoting fat storage in adipose tissue and inhibiting the breakdown of stored fat. Impaired glucose uptake into cells and increased glucose production by the liver, both of which can be caused by it, may also play a role in the development of diabetes (42).

2.4 Genetic predisposition

Obesity and diabetes are complex disorders resulting from genetic and environmental factors interplay. The genetic predisposition to these disorders has been extensively studied,

and several mechanisms have been proposed to explain their mediation by genetics. One tool of obesity mediation by genetics is regulating appetite and energy expenditure. Several gene roles in appetite regulation have been identified, such as the leptin and melanocortin-4 receptor genes (43). Leptin is a hormone adipose tissue produces that regulates appetite and energy expenditure by acting on the hypothalamus. Mutations in the leptin gene or its receptor can lead to leptin resistance, resulting in increased appetite and reduced energy expenditure, leading to obesity (44). Similarly, mutations in the melanocortin-4 receptor gene can also increase hunger and obesity. Another mechanism of obesity mediation by genetics is regulating adipose tissue distribution. The distribution of fatty tissue in the body, particularly visceral adipose tissue, strongly predicts metabolic disorders such as diabetes and cardiovascular disease (45). Several genes that play a role in adipose tissue distribution have been identified, such as the FTO and PPARG genes. Variants in the FTO gene have been associated with increased body mass index and obesity. This effect is believed to be mediated by regulating adipose tissue distribution (45, 46). Similarly, variants in the PPARG gene have been associated with increased visceral adipose tissue, insulin resistance, and T2D. The genetics behind the development of diabetes are intricate and involve multiple factors. One way in which genetics contributes to diabetes is through the regulation of insulin secretion and sensitivity. Specific genes, such as the TCF7L2 gene and the insulin receptor gene, are involved in this process. Research has shown that variants in the TCF7L2 gene are strongly linked to a higher risk of T2D, which is believed to occur due to reduced insulin secretion (47). Similarly, mutations in the insulin receptor gene can lead to insulin resistance, leading to T2D. Genetics can also mediate diabetes by controlling the maintenance of glucose balance, which is another disease mechanism (48). Glucose homeostasis is tightly regulated by a complex interplay between several hormones, including insulin, glucagon, and amylin. Several gene roles in glucose homeostasis have been identified, such as the KCNJ11 gene and the HNF1A gene. Variants in the KCNJ11 gene have been associated with impaired insulin secretion and an increased risk of T2D. In contrast, mutations in the HNF1A gene can lead to impaired glucose homeostasis and maturity-onset diabetes of the young (49).

2.5 Lifestyle factors

Obesity and diabetes are two of the most prevalent chronic diseases worldwide, and their incidence is rising due to lifestyle factors. Obesity, characterized by excessive body fat accumulation, is a significant risk factor for T2D due to insulin resistance and impaired insulin secretion. Lifestyle factors such as sedentary behavior, unhealthy diet, and inadequate sleep are known to mediate the mechanisms of obesity and diabetes (50). Sedentary behavior, such as prolonged sitting or inactivity, has been linked to increased obesity and diabetes risk. Physical inactivity leads to decreased energy expenditure, reduced muscle mass, and impaired glucose metabolism, contributing to insulin resistance and diabetes development (51). Regular exercise can improve insulin sensitivity, glucose uptake, and body weight, reducing the risk of diabetes and obesity. An unhealthy diet, characterized by a high intake of refined carbohydrates, saturated and trans fats, and a low fiber intake of fruits and vegetables, is another crucial factor in developing obesity and diabetes (52). The high glycemic load of refined carbohydrates leads to rapid glucose absorption, causing insulin spikes and subsequent insulin resistance. The saturated and trans fats in unhealthy diets contribute to weight gain, insulin resistance, and inflammation, leading to obesity and diabetes. In contrast, a healthy diet that includes whole grains, fruits, vegetables, and moderate amounts of healthy fats, can help prevent and manage obesity and diabetes (53). Inadequate sleep, characterized by insufficient duration and poor quality, has been linked to increased obesity and diabetes risk. Sleep deprivation disrupts the regulation of hormones that control appetite and energy metabolism, leading to increased food intake, decreased physical activity, and altered glucose metabolism, contributing to obesity and diabetes (54). Adequate sleep, on the other hand, can improve insulin sensitivity, reduce appetite, and promote weight loss, reducing the risk of diabetes and obesity (55).

2.6 Gut microbiota

Obesity and diabetes are chronic metabolic disorders that affect a large portion of the global population. Recent research has highlighted the potential role of gut microbiota in developing and progressing these diseases. Gut microbiota refers to the trillions of microorganisms that inhabit the human gut, including bacteria, viruses, and fungi (56). These microorganisms regulate various metabolic processes, including energy homeostasis, glucose metabolism, and inflammation. One of the critical mechanisms by which gut microbiota mediates obesity is regulating energy balance. Gut bacteria have been shown to influence the amount of energy extracted from the diet by breaking down complex carbohydrates and other nutrients that are resistant to digestion by human enzymes (56, 57). This produces short-chain fatty acids (SCFAs), which the host can use as an energy source. However, excessive production of SCFAs can lead to increased fat storage and obesity. Another mechanism by which gut microbiota contributes to the development of obesity is through the regulation of appetite and satiety (58).

Studies have shown that gut bacteria, such as ghrelin, leptin, and serotonin, can produce various hormones and neurotransmitters that regulate hunger and food intake. Disruption of the regulation can cause an increase in food consumption and weight gain, as mentioned in reference (59). The gut microbiota, aside from being linked to obesity, has also been connected to diabetes development. The gut bacteria play a critical role in regulating glucose metabolism, one of the fundamental mechanisms contributing to diabetes development, as explained in reference (60). Various pathways

have been discovered through which gut bacteria impact glucose metabolisms, such as the production of SCFAs that improve insulin sensitivity and the regulation of intestinal permeability that affects glucose absorption from the gut. Moreover, gut microbiota contributes to diabetes development through inflammation regulation, a hallmark of diabetes. Gut bacteria play a vital role in modulating the inflammatory response in the gut and systemic circulation (61, 62). Dysbiosis, or an imbalance in the gut microbiota, has been associated with increased pro-inflammatory cytokine levels that can cause insulin resistance and impaired glucose metabolism (63, 64) (Figure 3).

2.7 Environmental factors

Obesity and diabetes are complex conditions that various environmental factors can influence. These factors include dietary habits, physical activity, stress, pollution, and socioeconomic status. This response will explore how environmental factors can mediate obesity and diabetes. One primary ecological factor contributing to obesity and diabetes is dietary habits (65). The risk of developing obesity and diabetes can be heightened by consuming diets high in calories, fats, and sugars. Excess calories from these diets are stored as fat, leading to weight gain, and can also cause insulin resistance,

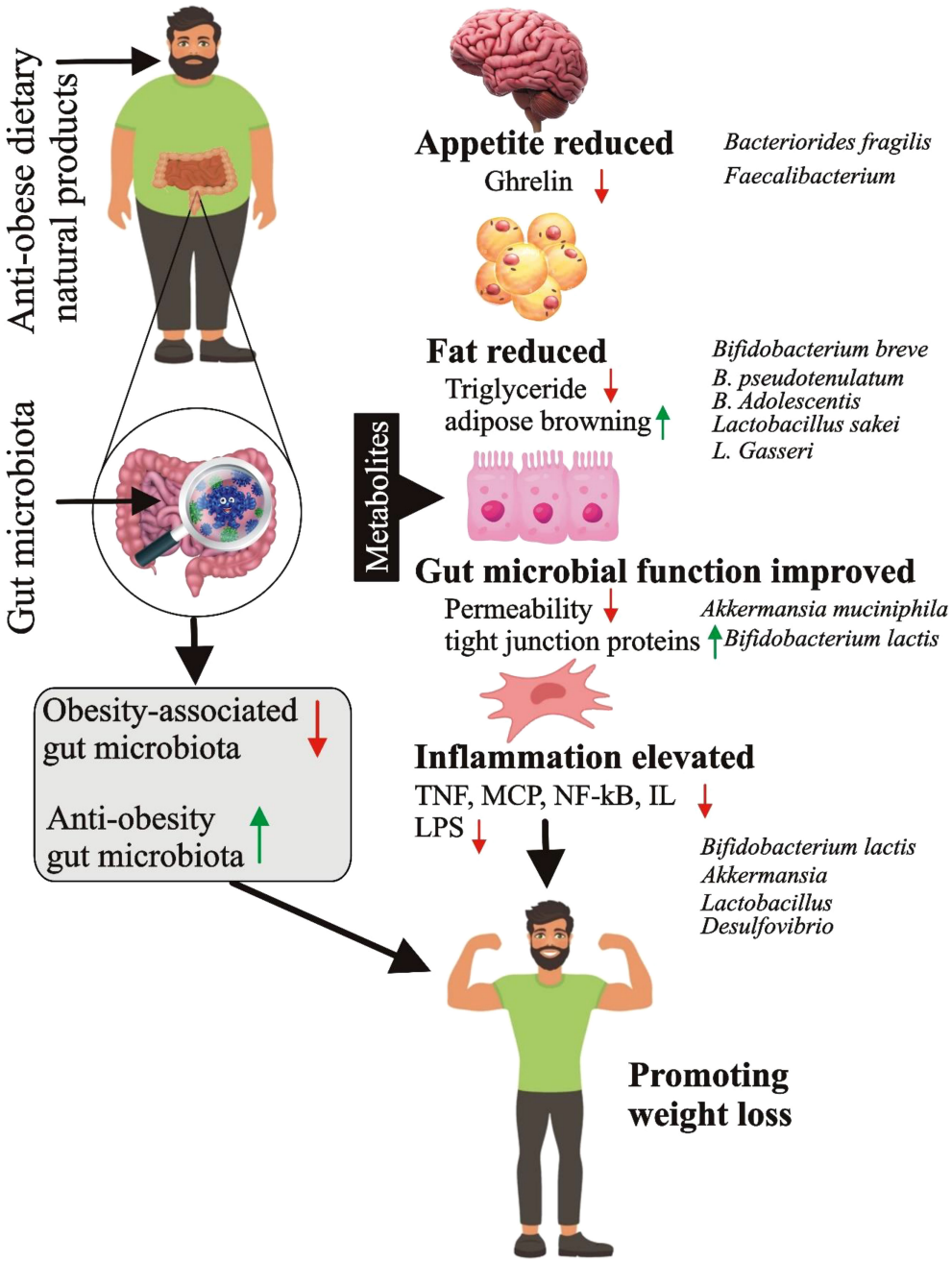

FIGURE 3
Mechanism of obesity mediation by gut microbiota (Figure credit: Meng-zhou Xie).

which impairs glucose uptake by cells and can lead to the development of diabetes (66).

Moreover, a lack of nutrient-dense foods, such as fruits and vegetables, can lead to deficiencies in essential vitamins and minerals, further exacerbating the risk of obesity and diabetes (67). Another environmental factor that can contribute to obesity and diabetes is physical activity (65). Sedentary lifestyles, such as spending extended periods sitting, can reduce metabolic rate, decreasing calorie burning and weight gain. Additionally, physical inactivity can increase insulin resistance, making it more difficult for cells to use glucose effectively, thereby increasing the risk of diabetes. Conversely, regular physical activity can improve insulin sensitivity, aid weight management, and reduce the risk of developing both obesity and diabetes (68). Stress is another environmental factor that can contribute to obesity and diabetes. Chronic stress can lead to releasing cortisol, a hormone that increases appetite and can cause weight gain.

Moreover, cortisol can impair glucose uptake by cells, leading to insulin resistance and increasing the risk of diabetes. Therefore, effective stress management techniques, such as mindfulness, exercise, and social support, can help reduce the risk of developing obesity and diabetes (69, 70). Environmental factors such as pollution and socioeconomic status can also play a role in developing obesity and diabetes. Insulin resistance and the onset of diabetes have been associated with air pollution. In contrast, poverty, limited availability of healthy food choices, and unsafe physical activity environments can contribute to obesity and diabetes (71–73).

3 Role of Traditional Chinese Medicine (TCM)

Traditional Chinese Medicine (TCM) has a long history of use for treating various health conditions, including obesity and diabetes. TCM adopts a comprehensive perspective towards well-being, perceiving the body as an intricate network of interrelated components that both internal and external factors can influence. TCM uses a combination of modalities, including herbal medicine, acupuncture, dietary therapy, and lifestyle changes, to promote balance and harmony within the body and restore optimal health (73–75). Obesity is a growing health problem worldwide, and TCM has been used for centuries to address this condition. In TCM, obesity is seen as a result of imbalances within the body, such as dampness and phlegm accumulation, qi stagnation, and spleen and stomach deficiency (76). TCM practitioners will first evaluate the patient's constitution and identify any underlying imbalances. Then, they will develop a personalized treatment plan that may include a combination of acupuncture, herbal medicine, and dietary therapy (77) (Table 1)

3.1 Acupuncture

For thousands of years, acupuncture has been a traditional Chinese medicine method to address various conditions, such as diabetes and obesity. This technique entails the insertion of thin needles into particular locations on the body, with the belief that it enhances the flow of energy or Qi, bringing about equilibrium within the body (86–88). In tackling obesity, acupuncture has shown promise in clinical studies by effectively decreasing body weight and body mass index (BMI). The needles are inserted into specific points on the body, including the ears, stomach, and spleen, which are thought to regulate appetite and metabolism. Acupuncture may also help to reduce inflammation in the body, which can contribute to weight gain and obesity-related health problems (89, 90).

TABLE 1 Summary of Traditional Chinese medicine in treating obesity and diabetes.

Traditional Chinese Medicine	Medicine Formula	Function in Treating Obesity	Function in Treating Diabetes	References
Rhizoma coptidis	Huang Lian	Reduces body weight by inhibiting fat accumulation and improves insulin resistance	Lowers blood glucose levels by improving insulin resistance	(78)
Semen cassiae	Jue Ming Zi	Reduces body weight by promoting lipid metabolism and reducing lipid absorption	Lowers blood glucose levels by improving insulin sensitivity and promoting insulin secretion	(79)
Fructus crataegi	Shan Zha	Reduces body weight by reducing lipid accumulation and improving digestion	Enhancing insulin sensitivity and decreasing insulin resistance results in a decrease in blood glucose levels	(80)
Radix puerariae	Ge Gen	Reduces body weight by increasing energy expenditure and reducing fat accumulation	Enhancing insulin sensitivity and stimulating glucose uptake results in a reduction of blood glucose levels	(81)
Folium mori	Sang Ye	Reduces body weight by inhibiting fat accumulation and improving lipid metabolism	Enhances insulin sensitivity and encourages the secretion of insulin, resulting in a reduction of blood glucose levels	(82)
Rhizoma polygonati	Huang Jing	Reduces body weight by reducing fat accumulation and improving lipid metabolism	Enhancing insulin sensitivity and stimulating insulin secretion can result in a reduction of blood glucose levels.	(83)
Radix astragali	Huang Qi	Reduces body weight by increasing energy expenditure and reducing lipid accumulation	Enhances insulin sensitivity and facilitates glucose uptake, resulting in a reduction in blood glucose levels	(84)
Radix ginseng	Ren Shen	Reduces body weight by improving energy metabolism and reducing fat accumulation	Lowers blood glucose levels by improving insulin sensitivity and promoting insulin secretion	(85)

Similarly, acupuncture can also be a helpful treatment for diabetes. By enhancing insulin sensitivity and decreasing insulin resistance, acupuncture has the potential to regulate blood sugar levels. The needles are typically inserted into points on the hands, feet, and ears, connected to the pancreas and other organs involved in blood sugar regulation (91). Furthermore, acupuncture can help to alleviate some of the symptoms of diabetes, such as neuropathy and nerve pain. Acupuncture has demonstrated the ability to trigger the production of endorphins, which are natural chemicals in the body that alleviate pain. This could potentially enhance diabetes management and decrease the likelihood of complications by minimizing discomfort and promoting overall health (92, 93). Acupuncture deforms connective tissue and increases the release of different molecules in acupoints as part of its anti-inflammatory impact, further activating the NF-κB, MAPK, and ERK pathways in mast cells, fibroblasts, keratinocytes, and monocytes/macrophages. Acupuncture-activated acupoints have somatic afferents that send sensory signals to the spinal cord, brainstem, and hypothalamus neurons. Acupuncture stimulates multiple neuro-immune pathways after information integration in the brain, such as the hypothalamus-pituitary-adrenal axis, which ultimately acts on immune cells via the release of critical neurotransmitters and hormones, the vagus-adrenal medulla-dopamine, the cholinergic anti-inflammatory, and sympathetic pathways (94–96).

3.2 Herbal medicine

Traditional Chinese Medicine (TCM) has been used for thousands of years to treat various health conditions, including obesity and diabetes. In TCM, obesity is viewed as a result of an imbalance in the body's energy or "qi," while diabetes is seen as a disorder of the body's "yin" and "yang." TCM practitioners use a variety of herbal medicines to address these imbalances and help promote weight loss and better blood sugar control (97, 98). One commonly used herb in TCM for treating obesity and diabetes is ginseng. Ginseng has been found to have anti-obesity and anti-diabetic effects, as it can help reduce insulin resistance, improve glucose metabolism, and increase energy expenditure (99).

Additionally, it has been shown to positively affect the gut microbiota, which can also contribute to weight loss (100). Another popular herb used in TCM for treating obesity and diabetes is bitter melon. Compounds present in bitter melon aid in regulating blood sugar levels and enhancing insulin sensitivity. It also has been found to have a mild appetite suppressant effect, which can aid in weight loss (101). Cinnamon is also commonly used in TCM for its anti-diabetic properties. It can help reduce fasting blood sugar levels, improve insulin sensitivity, and reduce inflammation. Cinnamon has also been shown to positively affect lipid metabolism, which can contribute to weight loss (102). In addition to these herbs, TCM practitioners may also recommend other lifestyle modifications, such as dietary and exercise, to help address obesity and diabetes. For example, TCM dietary guidelines often emphasize consuming nutrient-dense whole foods, such as vegetables, fruits, and whole grains, while limiting the intake of refined sugars and processed foods (103). In low-impact activities like tai chi and qigong, exercise can also help improve energy flow and promote overall health. While TCM may not cure obesity and diabetes, it can provide a valuable adjunct to conventional medical treatments. By addressing underlying imbalances in the body's energy and promoting healthy lifestyle habits, TCM can help patients achieve better weight management and blood sugar control. As always, anyone considering herbal medicines should consult a qualified TCM practitioner or medical professional to ensure the treatment is safe and appropriate for their needs (104).

3.3 Dietary therapy

For centuries, Traditional Chinese Medicine (TCM) has relied on dietary therapy to address various health conditions, such as obesity and diabetes. TCM views the body as a whole and focuses on restoring balance and harmony between different organ systems. In TCM, obesity and diabetes are seen as imbalances in the body's energy, or Qi, and can be treated through changes in diet and lifestyle (105, 106). In TCM, obesity is often associated with excessive dampness and phlegm in the body, which an unhealthy diet and lack of exercise can cause. The dietary therapy for obesity in TCM involves reducing the intake of fatty, greasy, and sweet foods while increasing the consumption of cooling foods that can help disperse dampness, such as bitter melon, lotus leaf, and green tea. Eating smaller, more frequent meals is also recommended, and avoiding eating late at night (107). Regular exercise, especially low-impact activities like walking and tai chi, is also recommended to help increase circulation and burn fat. Diabetes, on the other hand, is seen as a deficiency of Qi and Yin in the body. Qi refers to the body's energy, while Yin refers to the body's moisture and nourishment. In TCM, diabetes is often treated with dietary therapy, acupuncture, and herbal medicine (108). The dietary treatment for diabetes in TCM involves reducing the intake of sweet and greasy foods while increasing the consumption of foods rich in Qi and Yin, such as yams, sweet potatoes, and lotus seeds. Eating smaller, more frequent meals and avoiding raw or cold foods are also recommended. Regular exercises, such as brisk walking or cycling, are also advised to help improve circulation and regulate blood sugar levels (109). Overall, TCM dietary therapy for obesity and diabetes focuses on achieving balance and harmony in the body rather than simply treating the condition's symptoms. By making healthy nutritional and lifestyle choices and receiving acupuncture and herbal medicine treatments, individuals can help restore their body's natural balance and improve their overall health and well-being (110).

3.4 Qi gong and tai chi

Qi Gong and Tai Chi are traditional Chinese practices that can be used as alternative therapies for treating obesity and diabetes. These practices are based on the principles of Traditional Chinese Medicine, which views the body as a complex system of interdependent parts that must be harmonious for optimal health. Qi Gong and Tai Chi are gentle exercises incorporating breathing

techniques, mindfulness, and gentle movements to improve overall health and well-being (111–114). Obesity is a primary global public health concern linked to many health complications, such as diabetes. Qi Gong and Tai Chi are effective in helping individuals manage their weight and reduce the risk of developing obesity-related (115). These practices promote weight loss by improving metabolism, reducing stress, and increasing physical activity. One of the primary ways that Qi Gong and Tai Chi help with obesity is through stress reduction. Chronic stress significantly contributes to weight gain, as it can cause hormonal imbalances that increase appetite and decrease metabolism (116). Qi Gong and Tai Chi help to reduce stress by promoting relaxation, improving sleep quality, and reducing anxiety and depression.

Additionally, Qi Gong and Tai Chi are low-impact exercises that individuals of all fitness levels can practice. These practices can improve cardiovascular health, muscle strength, and flexibility, contributing to weight loss and overall health. Diabetes is a metabolic disorder that affects millions of people worldwide. While there is no cure for diabetes, lifestyle changes, including exercise, can help manage the disease. Qi Gong and Tai Chi effectively manage diabetes by controlling blood glucose, reducing inflammation, and improving cardiovascular health (117–120). One of the primary ways that Qi Gong and Tai Chi help manage diabetes is by improving blood glucose control. These techniques aid in managing blood sugar levels by enhancing insulin sensitivity and diminishing insulin resistance.

Moreover, the practice of Qi Gong and Tai Chi can potentially decrease inflammation, which is crucial in the onset and advancement of diabetes (121). Qi Gong and Tai Chi are effective traditional Chinese medicine options for managing obesity and diabetes. These practices promote overall health and well-being by reducing stress, improving physical activity levels, and helping to manage chronic diseases. Additionally, Qi Gong and Tai Chi are safe and gentle exercises that individuals of all ages and fitness levels can practice (122).

3.5 Massage and bodywork

Massage and bodywork therapies are integral to Traditional Chinese Medicine (TCM), a holistic healthcare system that focuses on restoring the balance of the body's vital energy or Qi. TCM offers a range of therapeutic options for treating various health conditions, including obesity and diabetes (123). Obesity is a metabolic disorder caused by an imbalance between energy intake and expenditure, leading to excessive accumulation of body fat (124). TCM views obesity as a result of Qi stagnation, dampness accumulation, and spleen and stomach weakness. Massage and bodywork therapies can help to regulate Qi flow, improve digestion, and promote lymphatic drainage, which can help to reduce body fat and improve metabolic function. Some typical massage and bodywork techniques used in TCM for obesity include acupressure, cupping, and Guasha (125). Acupressure involves applying pressure to specific points on the body, called acupoints, which correspond to different organs and systems. By stimulating these points, acupressure can help to regulate the function of the spleen and stomach, promote digestion, and reduce food cravings. Cupping is a technique in which cups are placed on the skin to create a suction effect (126). This can help to stimulate blood flow and lymphatic drainage, which can help to eliminate excess fluids and toxins from the body. Gua sha involves using a smooth-edged tool to scrape the skin, which can help to improve circulation, reduce inflammation, and promote healing (127).

Diabetes is a condition affecting metabolism marked by elevated blood sugar levels, which can result in various complications such as nerve damage, kidney disease, and cardiovascular disease (128). TCM views diabetes as a result of Qi deficiency, Yin deficiency, and dampness accumulation. Massage and bodywork therapies can help to tonify Qi and Yin, regulate blood sugar levels, and improve circulation (125). Some standard massage and bodywork techniques used in TCM for diabetes include acupressure, moxibustion, and foot reflexology (129). Moxibustion involves burning a herb called mugwort over specific acupoints, which can help to notify Qi and improve circulation. Foot reflexology is the practice of exerting pressure on particular points on the feet that correspond to various organs and systems within the body. By stimulating these points, foot reflexology can assist in regulating blood sugar levels and enhancing overall well-being (130–133).

3.6 Tauroursodeoxycholic acid (TUDCA)

A naturally occurring hydrophilic bile acid called tauroursodeoxycholic acid (TUDCA) has been used for generations in CM. In chemical terminology, TUDCA is a taurine conjugate of ursodeoxycholic acid (UDCA). This drug has been accepted by the Food and Drug Administration (FDA) for use in treating primary biliary cholangitis in modern pharmacology (134). Recent research studies indicate that TUDCA's functioning mechanisms extend beyond hepatobiliary conditions. Due to its cytoprotective effect, TUDCA has been demonstrated to have potential therapeutic applications in various disease models, including neurodegenerative diseases, obesity, and diabetes. TUDCA was identified as a chemical chaperone due to the mechanisms underlying its cytoprotective action, mostly associated with regulating the unfolded protein response (UPR) and reducing ER stress. In addition, TUDCA has been shown to reduce oxidative stress, control apoptosis and reduce inflammation in numerous *in-vitro* and *in-vivo* models of different diseases (135, 136).

3.7 Therapeutic effects of western medicine

The weight-related effects of drugs used to treat T2D vary; some show a beneficial effect on weight loss, some have weight-neutral effects, and some result in a gain in weight. Examining the currently available drug profile is crucial when weight loss is a priority to identify prospective areas for improving blood-glucose control and weight management. Table 2 discusses several classes of drugs, including metformin, SGLT2 inhibitors, and GLP-1 agonists, and how they affect weight loss in individuals with T2D (137, 138).

TABLE 2 Summary of diabetes pharmacological treatments and their effect on weight loss.

Medication/Drug	Dose	Change in weight (kg)	Negative effects
*SGLT2 inhibitors			
Canagliflozin	100 to 300 mg daily/orally	~1 to 2 kg weight loss	Risk of amputation Risk of bone fracture Increased LDL level Risk of volume hypotension Genitourinary infections risk
Ertugliflozin	5 to 15 mg daily/orally		
Dapagliflozin	5 to 15 mm daily/orally		
Empagliflozin	10-25 mg daily/orally		
Metformin	500-100 mg (IR; tablets)/twice a day) 2000 mg (ER; tablets/daily)	~1 to 8 kg weight loss	Lactic acidosis B12 deficiency Diarrhoea, nausea, vomiting
*GLP1 Agonists			
Lixisenatide	10-20 mcg SC daily	~3 to 10 kg weight loss	Risk of thyroid C-cell tumor Risk of pancreatitis Injection site reactions Vomiting, nausea
Dulaglutide	0.75-1.5 mg SC weekly		
Exenatide	ER: 2 mg SC weekly IR: 5-10 mcg SC twice daily		
Semaglutide	Tablet: 3-14 mg daily Injection: 0.25-2.4 mg SC weekly		
Liraglutide	0.6-1.8 mg Titrate daily 0.6 mg SC daily		

*SGLT2; sodium-glucose cotransporter 2, GLP-1; glucose-like peptide-1, IR; immediate release, ER; extended release.

4 Hormone associated with obesity

4.1 Insulin

Insulin is an essential hormone responsible for regulating blood sugar levels within the body. After we consume food, carbohydrates are broken down into glucose, which is absorbed into the bloodstream. The pancreas produces insulin, which facilitates the transportation of glucose from the bloodstream to cells, where it can be utilized as energy or stored for future use (139). However, in individuals with obesity, their bodies may become less responsive to the effects of insulin, leading to elevated glucose levels in the bloodstream and an augmented likelihood of developing T2D (140). One of the main ways that insulin resistance contributes to obesity is through its effects on fat cells. Insulin helps to regulate the storage and breakdown of fat in the body. When insulin levels are high, fat cells store glucose as fat. When insulin levels are low, fat cells break down stored fat to release energy. However, in people with insulin resistance, fat cells become less responsive to insulin and are less able to take up glucose and store fat. As a result, more glucose remains in the bloodstream, leading to higher insulin levels and increased fat storage (141).

Another way that insulin resistance can contribute to obesity is through its effects on appetite and metabolism (142). Insulin helps regulate hunger and satiety by signaling to the brain that the body has enough to eat. When insulin levels are high, the brain receives signals to stop eating and start using stored energy. However, the brain may become less responsive to these signals in people with insulin resistance, leading to increased appetite and overeating (143). Insulin resistance can also affect the body's metabolism or the rate at which it burns calories. When insulin levels are high, the body tends to store energy in the form of fat. However, in people with insulin resistance, the body may be less able to use stored fat for energy and instead rely on glucose as a fuel source. This can lead to lower metabolic rates and decreased calorie burning, challenging losing and maintaining a healthy weight (144).

4.2 Omentin

Omentin is a hormone that is primarily produced by adipose tissue, which is the tissue that stores fat in the body. It belongs to a group of hormones known as adipokines, which regulate metabolism and inflammation. Omentin is associated with obesity and metabolic disorders, and its levels in the body are altered in individuals with these conditions (145). Research has shown that omentin is essential in regulating insulin sensitivity, which is the body's ability to respond to insulin and use glucose for energy. Insulin resistance, which is the impaired ability of cells to respond to insulin, is a common feature of obesity and is a risk factor for T2D (146). Omentin has been found to improve insulin sensitivity in animal studies, and lower levels of omentin have been observed in individuals with insulin resistance. In addition to its role in insulin sensitivity, omentin regulates inflammation in the body (147). Inflammation is a natural response of the immune system to injury or infection, but chronic inflammation is associated with various health conditions, including obesity, diabetes, and cardiovascular disease. Research has indicated that omentin possesses anti-inflammatory properties, and evidence suggests that heightened inflammation within the body is associated with lower levels of omentin (148). An interesting finding is that omentin levels seem to be influenced by the location of adipose tissue.

Specifically, subcutaneous adipose tissue, located just beneath the skin, has been observed to produce greater amounts of omentin compared to visceral adipose tissue surrounding internal organs. This implies that how body fat is distributed could impact omentin levels and their impact on metabolism (149).

4.3 Leptin

Leptin, a hormone synthesized by adipose tissue or fat cells, significantly regulates body weight and metabolism. The amount of leptin released into the bloodstream is directly proportional to the body's fat stores. Its primary function is communicating with the brain, inducing decreased appetite and increased energy expenditure (150). In people with obesity, there is often a condition called leptin resistance, in which the body becomes less responsive to the effects of leptin. This can lead to a cycle of overeating and weight gain, as the brain doesn't receive the signal to decrease appetite or increase energy expenditure. Leptin resistance is thought to develop due to chronic overeating and high circulating leptin levels over an extended period. This leads to a desensitization of the receptors that respond to the hormone (151). Interestingly, while leptin resistance is commonly associated with obesity, not all people with obesity have leptin resistance, and not all people with leptin resistance are obese. Evidence suggests that other factors, such as genetics, inflammation, and environmental toxins, may play a role in developing leptin resistance (152, 153). In addition to regulating appetite and energy expenditure, leptin has other bodily functions, such as immune function, fertility, and bone metabolism. Therefore, disruptions in leptin signaling can have far-reaching effects on overall health and well-being (154).

4.4 Acylation stimulating protein (ASP)

ASP is a hormone found to regulate energy metabolism and adipose tissue physiology. ASP is produced primarily by adipocytes and is secreted into the circulation in response to food intake, especially dietary fat. Once in the bloodstream, ASP binds to its receptor, C5L2, expressed in adipocytes, muscle cells, and other tissues, and initiates various cellular responses (155). One of the main functions of ASP is to promote the uptake and storage of dietary fat in adipose tissue. ASP can encourage the production of fatty acids, or lipogenesis, and increase the absorption of fatty acids by adipocytes. This can lead to the enlargement of adipose tissue depots and potentially contribute to the development of obesity (156). Research has indicated that obese individuals have higher levels of ASP in their bloodstream than those who are lean. Additionally, ASP has been linked to glucose homeostasis and insulin sensitivity regulation. Some studies have demonstrated that ASP can facilitate glucose uptake in muscle cells and adipocytes and improve insulin sensitivity in these tissues (157). However, the impact of ASP on glucose metabolism appears to depend on the circumstances, as some studies suggest that ASP may hinder glucose tolerance and contribute to insulin resistance. The precise mechanisms underlying the effects of ASP on energy metabolism and glucose homeostasis are not fully understood (158). It is thought that ASP may act in concert with other hormones and signaling pathways, such as insulin and adipokine leptin, to regulate these processes. Evidence suggests that ASP may directly affect the hypothalamus, a brain region crucial in regulating energy balance (159).

The processes by which ASP increases triacylglycerol production are now firmly established. Stimulating the last (and most likely rate-limiting) enzyme involved in triacylglycerol production, diacylglycerol acyltransferase, has two distinct implications. The second is an increase in glucose-specific membrane transport. Increased diacylglycerol acyltransferase (EC 2.3.1.20) activity increases fatty acid incorporation into triacylglycerol and, as a result, adipocytes' rate of fatty acid intake. The increase in specific membrane glucose transport, an additional effect of ASP, is also of considerable significance. In human skin fibroblasts, human adipocytes, and L6 myotubes, ASP increases glucose transport (160).

4.5 Ghrelin

Ghrelin is a hormone produced mainly by the stomach, although small amounts are also secreted by other organs such as the pancreas and small intestine (161). It stimulates appetite and promotes weight gain, making it an essential hormone in regulating energy balance and body weight. Ghrelin acts on the hypothalamus, a region in the brain that controls food intake and energy expenditure, as well as other areas involved in reward and motivation. The release of ghrelin is influenced by various factors such as fasting, stress, and sleep deprivation (162). It is secreted in higher amounts during fasting or calorie restriction periods, which may explain why people often experience intense hunger. Ghrelin levels also increase in stress response, which may contribute to overeating and weight gain in some individuals who use food as a coping mechanism (163).

Lack of sleep has also increased ghrelin levels, possibly contributing to the link between sleep deprivation and obesity (164). Ghrelin is believed to promote weight gain by several mechanisms. First, it increases appetite and food intake, increasing energy surplus and weight gain. Second, it slows down metabolism and reduces energy expenditure, which can also contribute to weight gain. Third, it promotes fat accumulation in adipose tissue by stimulating the release of growth hormones, insulin, and other hormones involved in fat storage (165). Studies have shown that ghrelin levels are often higher in obese individuals than those of average weight. This suggests that ghrelin may play a role in developing obesity and related metabolic disorders (166). However, the relationship between ghrelin and obesity is complex, and further research is needed to understand its role in this context entirely (167).

4.6 Peptides YY (PYY)

The endocrine cells in the gastrointestinal tract's ileum and colon secrete a hormone called Peptide YY (PYY), which plays a

vital role in controlling appetite and satiety by being released after food intake. PYY belongs to the pancreatic polypeptide family and is structurally similar to neuropeptide Y (NPY) and peptide YY2 (PYY2) (168). Several studies have shown that PYY levels are altered in individuals with obesity. In particular, it has been observed that obese individuals have lower levels of PYY compared to normal-weight individuals. This suggests that PYY may be involved in the pathophysiology of obesity (169). The exact mechanisms through which PYY regulates body weight are still not fully understood. One of the main ways PYY influences appetite and food intake is by acting on the hypothalamus, which is part of the brain that regulates energy balance. PYY activates neurons in the hypothalamus that suppress appetite and promote satiety (21). This leads to a reduction in food intake and an increase in feelings of fullness. In addition to its effects on appetite, PYY has also been shown to influence energy expenditure. Studies have demonstrated that PYY can increase energy expenditure by stimulating the sympathetic nervous system, which regulates metabolic processes such as thermogenesis and lipolysis (170). This suggests that PYY may be involved in regulating body weight through its effects on both food intake and energy expenditure. The role of PYY in treating obesity has been investigated in several clinical studies. One approach involves using PYY analogs, synthetic molecules that mimic the effects of endogenous PYY. Studies have demonstrated that these analogs can decrease food consumption and facilitate weight loss in both human subjects and animal models (171).

5 Recently developed treatments for obesity

Obesity is a chronic disease characterized by an excessive accumulation of body fat, which can lead to a range of health problems, including diabetes, heart disease, and stroke. While diet and exercise are the primary means of managing obesity, several recently developed treatment options can help individuals lose weight and maintain a healthy lifestyle.

5.1 Bariatric surgery

Bariatric surgery is a weight loss surgery that involves altering the digestive system to help individuals struggling with obesity loses weight. Obesity is a chronic condition affecting millions worldwide and can lead to various health complications such as heart disease, T2D, and sleep apnea (172). Bariatric surgery is often recommended for individuals with a body mass index (BMI) of 40 or higher or those with a BMI of 35 or higher with at least one obesity-related medical condition. There are different bariatric surgery procedures, each with its benefits and risks. The most common types include gastric bypass, sleeve gastrectomy, adjustable gastric banding, and biliopancreatic diversion with a duodenal switch (173). In gastric bypass surgery, the surgeon creates a small stomach pouch and reroutes the small intestine, limiting the amount of food consumed and absorbed by the body. Sleeve gastrectomy involves removing a portion of the stomach to reduce its size. In contrast, adjustable gastric banding involves placing an inflatable band around the top part of the stomach to restrict food intake (174). Bariatric surgery is an effective procedure that requires careful consideration and preparation. Before surgery, patients undergo a comprehensive evaluation to determine their suitability for the process and identify any underlying medical conditions that may affect the outcome (175). Patients must also undergo extensive counseling and education to help them understand the procedure's risks and benefits and prepare them for the changes they must make to their lifestyle after the surgery. Bariatric surgery is not a magic solution to weight loss (176). While the surgery can help individuals lose significant weight, it requires a commitment to long-term lifestyle changes, including healthy eating habits and regular exercise. Patients undergoing bariatric surgery must also be monitored closely by their healthcare provider to ensure they meet their weight loss goals and address complications (177). The desire to participate in hormonal changes following bariatric surgery arises from two fundamental observations: (a) the weight loss seems to arise from reductions in appetite and food intake, implying that the surgical procedure interferes with the normal regulation of appetite and food intake, and (b) the reversal of T2D occurs a few days after surgery before any significant weight loss has occurred, implying that mechanisms other than weight loss are involved (178).

5.2 Endoscopic sleeve gastroplasty (ESG)

Endoscopic sleeve gastroplasty (ESG) is a relatively new, minimally invasive procedure for weight loss in people with obesity. The process involves using an endoscope, a thin tube with a camera, and surgical instruments attached to it to reduce the stomach size by creating a sleeve-like shape (179). This limits the amount of food the stomach can hold, leading to a feeling of fullness and reduced hunger. The procedure is usually done on an outpatient basis, and patients are given general anesthesia. Once the patient is sedated, the endoscope is inserted into the mouth and down the throat to reach the stomach. The surgeon gathers and folds the stomach tissue using sutures into a narrow tube shape, creating a sleeve-like structure (180). The sutures are then tightened to hold the sleeve in place. ESG typically takes 60 to 90 minutes, and patients are usually discharged on the same day. Most patients can return to normal activities within a few days after the procedure, although a liquid diet is generally recommended for the first week or so. ESG is effective for weight loss in people with obesity (181). Studies have shown that patients typically lose between 15% and 20% of their excess body weight within 12 months after the procedure. ESG has also improved various health markers, including blood pressure, cholesterol levels, and blood sugar control. However, ESG is not without risks. Complications can occur, although they are rare. These can include bleeding, infection, and perforation of the stomach or esophagus. Patients may also experience nausea, vomiting, and abdominal pain in the first few days after the procedure. ESG is not appropriate for everyone with

obesity (182). It is generally recommended for people with a body mass index (BMI) between 30 and 40 who cannot lose weight through diet and exercise alone. People with certain medical conditions, such as inflammatory bowel disease or previous surgeries on the stomach or intestines, may not be candidates for ESG (183).

5.3 Duodenal-jejunal bypasses liner

The duodenal-jejunal bypass liner (DJBL) is a non-surgical weight loss treatment for obesity that involves the insertion of a temporary liner into the small intestine. The liner works by restricting the absorption of nutrients from food, leading to a reduction in calorie intake and weight loss. During the DJBL procedure, a flexible tube with a balloon at one end is inserted through the mouth and into the small intestine (184). Once in place, the balloon is inflated, creating a barrier that prevents food from coming into contact with the first part of the small intestine, called the duodenum. By bypassing the duodenum and restricting nutrient absorption, the DJBL promotes weight loss and helps improve metabolic conditions such as diabetes and high blood pressure (185). The DJBL is a reversible procedure and can be removed after six months. During this time, patients are advised to follow a structured diet and exercise program to maximize weight loss results. The DJBL is intended for patients with a body mass index (BMI) of 30 or higher who cannot lose weight through diet and exercise alone. Studies have shown that the DJBL can be an effective weight loss tool, with patients losing an average of 20-25% of their excess body weight during the six-month treatment period (186).

Additionally, the DJBL has been shown to improve metabolic conditions such as diabetes and high blood pressure, with some patients experiencing remission. Like any medical procedure, the DJBL does carry some risks, including nausea, vomiting, and abdominal pain. In rare cases, the DJBL can lead to more severe complications such as bowel obstruction, bleeding, or perforation. Patients considering the DJBL should discuss the risks and benefits with their healthcare provider to determine if it is the proper weight loss treatment for them (187).

5.4 GLP-1 receptor agonists

GLP-1 receptor agonists are primarily used to treat T2D but have also shown efficacy in managing obesity. These medications imitate the effects of GLP-1, a gut hormone that helps regulate appetite and blood glucose levels, resulting in a decrease in hunger and an increase in fullness, leading to reduced food intake and subsequent weight loss (188). Furthermore, they offer additional benefits by improving glycemic control and decreasing cardiovascular risk factors in individuals with obesity and T2D (189). One of the most commonly used GLP-1 receptor agonists for treating obesity is liraglutide, administered once daily by subcutaneous injection, leading to an average weight loss of 5-10% of initial body weight (185). Semaglutide, another GLP-1 receptor agonist administered once weekly by subcutaneous injection, is even more effective, resulting in an average weight loss of 15-20% of initial body weight (190). However, GLP-1 receptor agonists can cause side effects, including nausea, vomiting, and diarrhea, which can be minimized by starting with a low dose and gradually titrating. Additionally, they may increase the risk of pancreatitis and thyroid tumors, but the overall risk is low (191).

Semaglutide is a medication recently approved by the US Food and Drug Administration (FDA) for treating obesity in adults. It is a glucagon-like peptide-1 (GLP-1) receptor agonist, miming the action of a naturally occurring hormone called GLP-1 (185). The intestine releases GLP-1 in reaction to food consumption, and it triggers the pancreas to secrete insulin, thus aiding in regulating blood sugar levels. Semaglutide has been found to have a dual action of regulating blood sugar levels and inducing weight loss. Semaglutide works by activating the GLP-1 receptor in the brain, which results in decreased appetite and increased feelings of fullness or satiety (192). This leads to a reduction in food intake, resulting in weight loss. Moreover, it has been demonstrated that semaglutide decelerates the process of gastric emptying, which refers to the speed at which food exits the stomach and moves into the small intestine. This prolongs the feeling of fullness, which helps to reduce calorie intake and promote weight loss (193).

In clinical trials, semaglutide is effective in promoting weight loss in adults with a body mass index (BMI) of 30 or higher, which is considered obese, as well as those with a BMI of 27 or higher who have at least one weight-related health condition, such as T2D or high blood pressure (176). In one study, participants who received a once-weekly injection of semaglutide lost an average of 15% of their body weight over 68 weeks, compared to a 2.4% weight loss in the placebo group. Semaglutide is typically administered once a week via subcutaneous injection. The recommended starting dose is 0.25 mg per week, gradually increasing to 2.4 mg per week over 16 weeks (194). The medication should be combined with a reduced-calorie diet and increased physical activity for optimal results. Like any medication, semaglutide may cause side effects. The most common side effects reported in clinical trials include nausea, diarrhea, vomiting, and constipation. In rare cases, semaglutide may cause inflammation of the pancreas, which can be severe and requires immediate medical attention (195).

5.6 Digital health interventions

Digital health interventions use digital technologies to support and improve health outcomes. In the case of obesity treatment, digital health interventions can be an effective tool to help individuals achieve and maintain a healthy weight. These interventions can include a range of technologies, such as mobile apps, wearable devices, online programs, and virtual coaching (180). One of the key benefits of digital health interventions for obesity treatment is their ability to provide personalized support and feedback. Many digital health programs use algorithms to track an individual's progress, provide personalized feedback, and adjust

their plan accordingly. For example, a digital health app may use data on an individual's weight, physical activity, and dietary habits to provide personalized recommendations on achieving their weight loss goals (196). Another advantage of digital health interventions is their accessibility. Individuals can access digital health programs from anywhere, which can be particularly beneficial for individuals with busy schedules or limited access to traditional healthcare resources. Additionally, digital health interventions can often be more cost-effective than conventional obesity treatments, making them more accessible to a broader range of individuals. Several studies have demonstrated the effectiveness of digital health interventions for obesity treatment (197). A systematic review of 23 randomized controlled trials found that digital health interventions were associated with significant body weight, BMI, and waist circumference reductions. Additionally, individuals who used digital health interventions reported high satisfaction with the programs. Despite the potential benefits of digital health interventions for obesity treatment, some challenges are associated with these programs (198). For example, some individuals may struggle to engage with the technology or find the programs overwhelming. Additionally, digital health interventions may not be appropriate for individuals with complex medical needs or require more intensive treatment (199).

6 Conclusion

Obesity and diabetes are complex metabolic disorders with multifactorial causes that require comprehensive management strategies. Traditional Chinese Medicine (TCM) offers a promising approach to preventing and treating these conditions, with a long history of use and a growing body of scientific evidence to support its effectiveness. TCM treatments, such as acupuncture, herbal remedies, and dietary interventions, may target various mechanisms underlying obesity and diabetes, including inflammation, oxidative stress, insulin resistance, and gut microbiota dysbiosis. However, further research is needed to elucidate the precise mechanisms of action and optimize the use of TCM to manage these disorders and ensure the safety and quality of TCM products. Given that most T2D is caused by obesity, it makes sense to favor treatment techniques that encourage weight loss. It is also necessary to consider the usage of specific 'anti-obesity' drugs to supplement an individual's attempts to improve their lifestyle. The combination of obesity/diabetes medications and glucose-lowering agents, as well as the usage of some pharmaceuticals in any category for both purposes, blur the line between obesity and diabetes therapy. SGLT2i and GLP-1 RAs, for example, are already available glucose-lowering medicines that induce modest weight loss and are anticipated to play a larger role in diabetes care in the future, especially given the positive findings of their usage in recent cardiovascular outcome trials. Novel obesity-specific medications, on the other hand, offer potential in diabetes management, and, as a result, their use in diabetes treatment appears likely to increase over time.

Author contributions

All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Comparison of the efficacy and safety of 10 glucagon-like peptide-1 receptor agonists as add-on to metformin in patients with type 2 diabetes: a systematic review

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Purpose: This study aimed to perform a network meta-analysis to objectively evaluate the efficacy and safety of 10 Glucagon-like peptide-1 receptor agonists (GLP-1RAs) in combination with metformin that is approved for use worldwide in patients with type 2 diabetes and to provide evidence-based support and reference for the selection of clinical treatment.

Methods: Three databases (PubMed, Embase, and Cochrane Library) were searched from their respective inception until September 30, 2022. Only randomized controlled trials comparing the efficacy and safety of GLP-1RAs for treating type 2 diabetes (T2D) were included. The 10 GLP-1RAs are exenatide (including exenatide twice daily and once weekly), liraglutide, lixisenatide, dulaglutide, PEX168, semaglutide (subcutaneous and oral semaglutide), tirzepatide and albiglutide.

Results: 34 RCTs with 10 GLP-1RAs and 12993 patients were included in the Network Meta-Analysis (NMA). According to the NMA, tirzepatide 15 mg, semaglutide 1.0 mg, PEX168-200μg, oral semaglutide 14 and dulaglutide 1.5 mg reduced HbA1c by -2.23%, -1.57%, -1.12%, -1.10%, -1.09% and body weight by -11.33 kg, -5.99 kg, +0.40 kg, -3.95 kg, -1.87 kg, respectively. There was no significant difference in the rate of adverse events for tirzepatide 15 mg, oral-semaglutide 14 mg, and semaglutide 1.0 mg. PEX168-200μg, tirzepatide 15mg, and oral semaglutide 14mg had Surface Under the Cumulative Ranking (SUCRA) values greater than placebo, and only tirzepatide 15mg and oral semaglutide 14mg were significantly different from placebo in the rate of serious adverse events. All GLP-1RA did not lead to increased incidence of hypoglycemia. Albiglutide 30mg and semaglutide 1.0mg significantly differed from placebo in Adverse Event (AE) withdrawal. Finally, the sensitivity analysis and publication bias analysis results indicate that the study results are reliable.

Conclusion: This study's results showed that GLP-1RAs were effective in lowering HbA1c and reducing body weight without increased incidence of

hypoglycemic reactions. In addition, this study may provide reference and evidence-based medical evidence for clinicians to select GLP-1RAs in patients with T2D and high body mass index (BMI). Based on the NMA results, tirzepatide 15mg and semaglutide 1.0mg may be preferred.

KEYWORDS

glucagon-like peptide-1 receptor agonists, safety, efficacy, systematic review, randomized controlled trials, type 2 diabetes

1 Introduction

As people's diets and lifestyles change, diabetes prevalence is increasing, with type 2 diabetes accounting for 90-95% of all cases of diabetes. Diabetes imposes a heavy economic burden on individuals and society, with the annual cost of diagnosing diabetes estimated at $327 billion in 2017 in the US (1). Diabetes prevalence is significantly higher in people who are overweight or obese. Studies have shown that the higher the BMI, the higher the risk of type 2 diabetes (2–4), and patients with pre-diabetes often have associated cardiovascular risk factors, including hypertension and dyslipidemia, and may be at increased risk of cardiovascular disease (5–7).

The American Diabetes Association (ADA) Medical Standards of Care for Diabetes 2022 Edition recommends metformin as the first-line treatment and GLP-1RAs as part of the treatment regimen for patients with T2D in combination with atherosclerotic cardiovascular disease or high-risk factors, renal disease, or heart failure (8). GLP-1RA has been shown to improve hemoglobin A1c (HbA1c), reduce body weight, and have cardiovascular benefits, and should be more widely used in clinical practice (9–15).

In 2019, 48% of diabetes deaths occurred before age 70. Elevated blood glucose contributes to about 20% of deaths from cardiovascular disease (16). In 2021, approximately 537 million adults (aged 20-79) worldwide had diabetes, and an estimated 6.7 million adults will have died from diabetes or its complications (17). However, in the CAPTURE study (18) (a multinational, cross-sectional study of cardiovascular disease prevalence in adults with type 2 diabetes across 13 countries), results showed that the prevalence of cardiovascular disease in Chinese patients with T2DM was 33.9%, of which 94.9% was atherosclerotic cardiovascular disease (ASCVD). Only 1.5% of Chinese patients with T2DM and combined ASCVD were treated with GLP-1RAs with cardiovascular benefit, a large gap between clinical practice and guideline recommendations (19, 20).

An NMA of metformin in combination of GLP-1RAs has not been previously performed. Therefore, we sought to objectively evaluate the efficacy and safety of 10 globally approved and marketed GLP-1RAs in combination with metformin for treating patients with T2DM through NMA and to provide evidence-based support and reference for the clinical selection of GLP-1RAs.

2 Methods

2.1 Registration

The Preferred Items report for this systematic review, the NMA for Systematic Reviews and Meta-Analyses (PRISMA) statement, and the PRISMA checklist is provided in Supplementary File 1. The protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) (registration number: CRD42023390347).

2.2 Data source

The Cochrane Library, PubMed, and Embase databases were searched from the creation of the databases to September 30, 2022, with no language restrictions. We used search terms including "diabetes mellitus, type 2", "type 2 diabetes", "glucagon-like peptide-1 receptor agonist", "GLP-1 receptor agonist", and "randomized controlled trial," including any of these terms in any field. The search strategy, including all search terms, is shown in Supplementary File 2.

2.3 Inclusion criteria

Inclusion and exclusion criteria were developed according to the principles of Participants, Intervention, Comparison, Outcomes, and Study (PICOS). Inclusion criteria: (1) Participants: Patients diagnosed with type 2 diabetes, HbA1c ≥7.0%, age ≥18 years, no diabetes-related complications, no gender or race restrictions, and drug treatment cycle ≥12 weeks (initial dose + maintenance dose). (2) Intervention: 10 GLP-1RAs ("exenatide (including exenatide twice daily and once weekly)" or "liraglutide" or "lixisenatide" or "dulaglutide" or "loxenatide" or "PEX168" or "semaglutide (including oral semaglutide)" or "tirzepatide" or "albiglutide") +/- other oral antidiabetic drugs (OADs) were taken in the treatment group. (3) Comparison: Placebo or another GLP-1RA in the control group. (4) Outcomes: Primary outcomes were HbA1c and rate of all adverse events. Secondary outcomes were weight loss, serious adverse events,

hypoglycemic events, and withdrawal due to adverse events. (5) Study: The study type was RCT.

2.4 Exclusion criteria

Exclusion criteria: (1) The article was republished, case report, review, conference, monotherapy, animal studies, retrospective studies, and data could not be extracted. (2) The patient's age <18, HbA1c<7.0%, and treatment cycle <12 weeks, presence of associated diabetic complications. (3) High-risk trials and type of non-randomized controlled trials.

2.5 Literature screening and data extraction

The Note Express software has been used to eliminate duplicates of articles, then read the titles and abstracts to exclude articles that did not meet the inclusion criteria, and finally read the full text according to the predefined inclusion and exclusion criteria to determine the final included studies, and extracted data from the included studies. Two investigators extracted data independently (Gu and Hu), and a third investigator resolved conflicting data (Chen). The extracted data include the following information: (1) study characteristics (e.g., year of publication, first author, mean age, sex, mean HbA1c (%), and total number of people included in the study). (2) Therapeutic interventions (e.g., drugs, dose, and cycle). (3) Clinical data (e.g., HbA1c reduction (%), weight loss (kg), number of adverse events, serious adverse events, withdrawals due to adverse events, and hypoglycemia events).

2.6 Risk of bias assessment

The risk of bias in the included studies was assessed independently by two investigators (Gu and Hu) using the software Review Manager 5.4.1 according to the Cochrane Risk of Bias Assessment Tool criteria, and a third investigator (Chen) adjudicated conflicting studies.

2.7 Analysis of data

Frequentist NMA was performed using software (Stata 16.1). The mean difference (MD) value was used to calculate continuous variables. Each effect size was expressed as a 95% confidence interval (95%_CI). The odds ratio (OR) value is used to calculate dichotomous variables (the number of adverse events), and a higher value means more adverse events, which means worse outcomes. We used the surface under the cumulative ranking (SUCRA) to rank the outcome of each treatment and finally expressed it as a percentage. For heterogeneity results of each outcome, we used software (Review Manager 5.4.1) to calculate, and the results are expressed in I^2 and p-value (if p-value for Q test < 0.10 or I^2 > 50% was defined as significant heterogeneity).

For the inconsistency test of the whole network meta, we used software (Stata 16.1) to test global inconsistency, local inconsistency (node splitting method), and closed-loop inconsistency (If the p-value is greater than 0.05, it means that the inconsistency is not significantly different). If the results are consistent (p>0.05), the results of the network meta are reliable. Meanwhile, we evaluated the included studies by drawing the network plots, funnel plots of outcome indicators, and risk of publication bias plots. In addition, a cluster analysis was performed to compare the effect of GLP-1RAs on efficacy (reduction in HbA1c) and safety (rate of adverse events). Finally, a sensitivity analysis is required if the included studies are high-risk.

3 Result

3.1 Inclusion process and study characteristics

The study's screening process and the patients' characteristics included are shown in Figure 1; Table 1. A total of 9816 articles were initially included by searching the databases according to the predefined search criteria, including PubMed (1702), Embase (1904), and the Cochrane Library (4293), further removing duplicates (7517) and reading the full text and abstracts (348). Finally, 34 studies with 11 treatments (involving 12993 patients) were included in the NMA. In the 34 included studies, 18 used metformin + GLP-1RA as the therapeutic agent, and 16 used metformin + GLP-1RA ± OAD (e.g., sulfonylurea, thiazolidinedione, and sodium-glucose cotransporter-2 inhibitors).

3.2 Quality assessment of included study

The risk of bias assessment plots and risk summary plots are shown in Figures 2, 3. Regarding the risk of bias, in the 34 included studies, all studies described in detail random sequence generation, incomplete outcome data, selective reporting, and the fact that they were all considered low-risk studies. In the allocation concealment bias, 10 (29.4%) studies and 1 (2.9%) study were classified as unclear and high risk because they were not reported, and an open random allocation table was used. Regarding blinding of patients and personnel and blinding of outcome assessment, 11 (32.4%) were considered high risk because they were not blinded, and 2 (5.9%) were regarded as unclear risk because they were not reported in the study. In other biases, all studies were not reported and were considered of unclear risk.

3.3 Results of network meta-analysis

The network plot is shown in Figure 4. In the 34 included studies, 34 (100%) studies with 12993 (100%) participants reported a reduction in HbA1c, 32 (94%) studies with 12906 (99%) for weight loss, 27 (79%) studies with 11608 (89%) for the rate of adverse events, 29 (85%) studies with 12558 (96%) for the rate of serious adverse

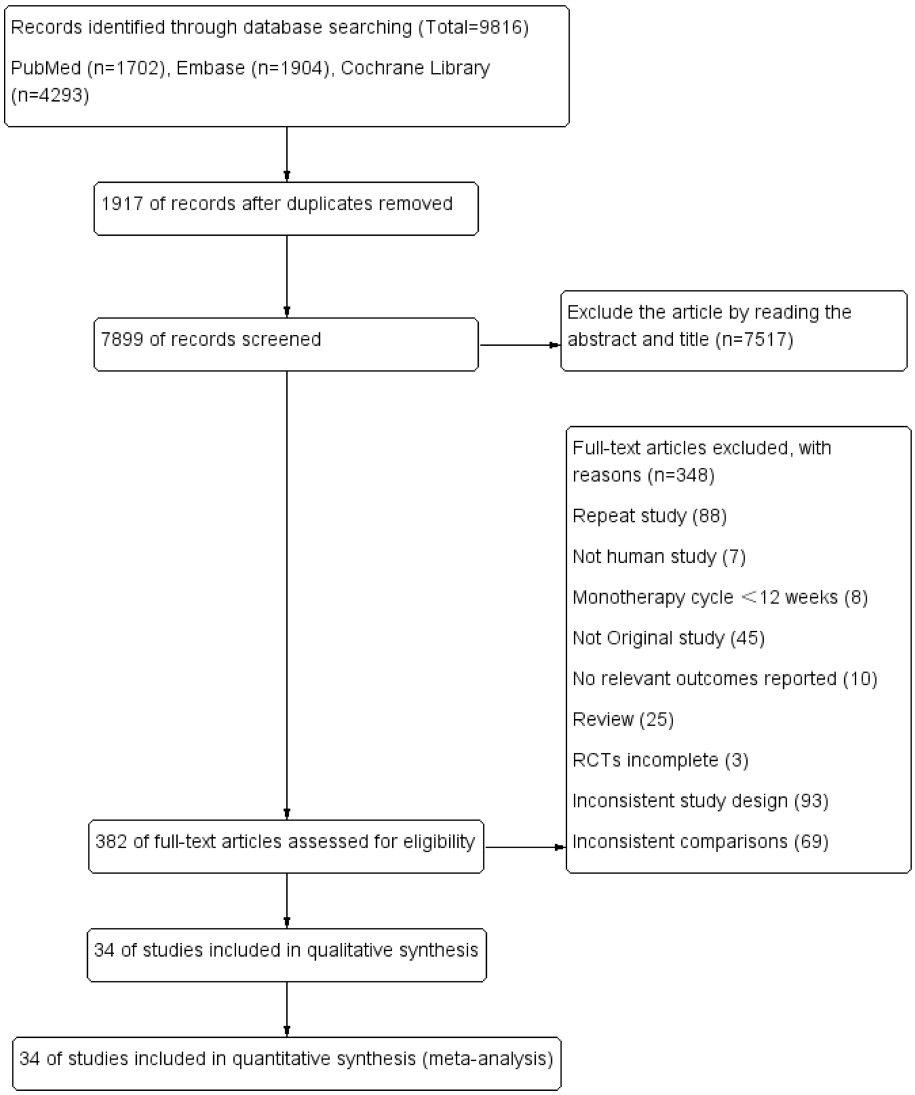

FIGURE 1
Flow chart of the study selection process.

events, 30 (88%) studies with 12522 (96%) for the hypoglycemic events, 30 (88%) studies with 12638 (97%) for the rate of withdrawals due to adverse events. Each evidence network has direct and indirect comparisons, with 15 closed loops for all outcome indicators except hypoglycemia, which has 10 closed loops.

3.4 Result of the inconsistency analysis

The results of the global inconsistency test are shown in Supplementary File 3 (Table 1). The p-values of the global inconsistency test results for the six outcome indicators of HbA1c reduction, weight loss, total adverse events, serious adverse events, hypoglycemic events, and AE withdrawal were 0.99, 0.96, 0.52, 0.28, 0.48, and 0.80, respectively, indicating that there was no inconsistency. In the local inconsistency test (node splitting method), there was inconsistency between the semaglutide 1.0 mg group and the placebo group for weight loss and AE withdrawal, and there were no significant inconsistent differences between the other group comparisons. ($p \geq 0.05$).

In the loop inconsistency analysis, there were four loops for decreased HbA1c and weight loss outcome indicators and one loop of inconsistency for serious adverse events; other closed loops are not significantly inconsistent ($p \geq 0.05$ or CI_95 include 0). The heterogeneity of each outcome indicator was calculated using I-squared; the results of the heterogeneity and loop inconsistency tests are shown in Supplementary File 4.

3.5 Decreased HbA1c (%)

The results of HbA1c reduction for 10 interventions are shown in Table 2. Compared with placebo, all 10 interventions were effective in reducing HbA1c (e.g., tirzepatide 15mg (MD=-2.23%,

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TABLE 1 Patient basic characteristics of included studies.

Study ID	Year± SD	N_{Total}	N_{Male}	Baseline (HbA1c (%))	Weeks	Combination Therapy Drugs	Intervention	HbA1c (%)		Weight (kg)		Safety				
								N_1	Mean ±SD	N_2	Mean ±SD	N_3	N_T	N_S	N_H	N_{AE}
01 Bo Ahrén 2014 (20)	54.3 (10.1)	403	185	8.1 (0.8)	104	Metformin	Albi 30mg	297	-0.63 (0.07)	296	-1.21 (0.24)	302	253	36	13	20
							Placebo	100	0.27 (0.11)	100	-1.00 (0.41)	101	80	13	5	5
02 P D Home 2015 (21)	54.5 (9.5)	386	205	8.2 (0.9)	52	Metformin+ SU	Albi 30mg	269	-0.55 (0.06)	269	-0.42 (0.24)	271	216	17	57	12
							Placebo	115	0.33 (0.08)	115	-0.40 (0.36)	115	80	7	13	6
03 Fei Gao 2020 (22)	52.8 (10.6)	354	204	8.5 (0.9)	24	Metformin	PEX168 200μg	175	-1.14 (0.08)	175	-0.40 (0.34)	178	85	5	1	2
							Placebo	179	-0.35% (0.08)	179	-0.80 (0.41)	182	78	3	1	3
04 Xiaoping Chen 2017 (23)	49.8 (10.9)	78	48	8.3 (1.2)	12	Metformin	PEX168 200μg	40	-1.36 (0.32)	NA		40	18	NA	0	NA
							Placebo	38	0.13 (0.32)	NA		38	10	NA	0	NA
05 Michael Nauck 2016 (24)	56.2 (10.3)	404	244	8.4 (0.7)	26	Metformin	Lixisenatide 20μg	191	-1.238 (1.01)	191	-3.69 (4.75)	202	129	7	5	15
							Liraglutide 1.8mg	194	-1.809 (0.92)	194	-4.24 (4.27)	202	145	12	3	13
06 Chang Yu Pan 2014 (25)	54.5 (10.3)	390	200	7.9 (0.8)	24	Metformin± SU	Lixisenatide 20μg	196	-0.83 (0.10)	196	-1.50 (0.27)	196	126	3	11	11
							Placebo	194	-0.47 (0.10)	194	-1.24 (0.27)	194	92	4	5	3
07 G B Bolli 2014 (26)	56.1 (9.3)	482	215	8.0 (0.8)	24	Metformin	Lixisenatide 20μg	308	-0.88 (0.10)	313	-2.65 (0.39)	322	223	12	7	22
							Placebo	158	-0.42 (0.10)	158	-1.63 (0.39)	160	105	4	1	4
08 Julio Rosenstock 2013 (27)	57.4 (9.9)	634	338	8.0 (0.8)	24	Metformin	Lixisenatide 20μg	295	-0.79 (0.05)	295	-2.96 (0.23)	318	221	9	8	33
							Exenatide 10μg	297	-0.96 (0.05)	296	-3.98 (0.23)	316	228	7	25	41
09 Bo Ahrén 2013 (28)	54.5 (9.2)	680	293	8.0 (0.9)	24	Metformin	Lixisenatide 20μg	483	-0.81 (0.07)	497	-2.02 (0.24)	510	354	13	19	32
							Placebo	164	-0.38 (0.08)	168	-1.64 (0.27)	170	102	2	1	2
10 Juan P Frias 2021 (29)	56.6 (10.4)	939	500	8.3 (1.0)	40	Metformin	Tirzepatide 15mg	464	-2.46 (0.05)	464	-12.40 (0.34)	470	324	27	8	40
							Semaglutide 1.0mg	461	-1.86 (0.05)	462	-6.20 (0.33)	469	301	13	2	19
11 Juan Pablo Frias 2018 (30)	58.7 (7.8)	158	75	8.1 (1.0)	26	Metformin	Tirzepatide 15mg	35	-2.4 (0.17)	35	-11.30 (0.88)	53	45	2	4	13
							Dulaglutide 1.5mg	47	-1.1 (0.15)	47	-2.70 (0.78)	54	40	3	2	6
							Placebo	41	0.1 (0.16)	41	-0.40 (0.81)	51	27	2	2	2
12 Tim Heise MD 2022 (31)	61.1 (7.1)	117	89	7.83 (0.72)	28	Metformin ±OAD	Tirzepatide 15mg	45	-2.06 (0.11)	45	-11.20 (0.90)	45	43	1	3	1
							Semaglutide 1.0mg	44	-1.52 (0.10)	44	-6.90 (0.90)	44	43	0	1	0
							Placebo	28	+0.19 (0.19)	28	0 (1.10)	28	22	2	0	3
13 Juan Pablo Frias 2020 (32)	56.6 (9.2)	82	51	8.4 (1.1)	12	Metformin	Tirzepatide 15mg	49	-1.9 (0.19)	49	-5.60 (0.79)	56	43	0	10	1
							Placebo	26	0.2 (0.21)	20	-0.50 (0.86)	26	13	0	0	1
14 Juan P Frias 2019 (33)	57.7 (9.8)	162	87	8.0 (0.8)	18	Metformin	Dulaglutide 1.5 mg	81	-1.23 (0.10)	81	-2.80 (0.39)	81	54	3	2	5
							Placebo	82	-0.44 (0.10)	82	-1.60 (0.39)	82	47	4	0	4

(Continued)

TABLE 1 Continued

Study ID	Year± SD	N_{Total}	N_{Male}	Baseline (HbA1c (%))	Weeks	Combination Therapy Drugs	Intervention	HbA1c (%)		Weight (kg)		Safety				
								N_1	Mean ±SD	N_2	Mean ±SD	N_3	N_T	N_S	N_H	N_{AE}
15 R S Weinstock 2015 (34)	53.66 (10.0)	481	236	8.1 (1.1)	26	Metformin	Dulaglutide 1.5 mg	301	-1.22 (0.05)	303	-3.18 (0.18)	NA				
							Placebo	176	0.03 (0.07)	177	-1.47 (0.24)	NA				
16 Kathleen M Dungan 2014 (35)	56.5 (9.3)	599	287	8.1 (0.8)	26	Metformin	Dulaglutide 1.5mg	279	-1.42 (0.05)	299	-2.90 (0.22)	299	185	5	26	18
							Liraglutide 1.8mg	272	-1.36 (0.05)	299	-3.61 (0.22)	300	189	11	17	18
17 Carol Wysham 2014 (36)	56 (10.0)	696	402	8.1 (1.3)	26	Metformin ±SLGT2	dulaglutide 1.5 mg	279	-1.51 (0.06)	279	-1.30 (0.29)	279	215	12	29	8
							Exenatide 10μg	276	-0.99 (0.06)	276	-1.07 (0.29)	276	198	15	44	9
							Placebo	141	-0.46 (0.08)	141	1.24 (0.37)	141	104	12	5	3
18 Richard Pratley 2019 (37)	56 (10.0)	711	370	8.0 (0.7)	26	Metformin ±SLGT2	Oral Semaglutide 14 mg	278	-1.2 (0.90)	278	-4.40 (4.40)	285	229	31	2	22
							Liraglutide 1.8 mg	272	-1.1 (0.90)	271	-3.20 (3.70)	284	211	22	7	17
							Placebo	134	-0.1 (0.70)	134	-0.60 (3.10)	142	95	15	3	3
19 Richard E Pratley 2018 (38)	55 (10.6)	699	333	8.2 (0.9)	40	Metformin	Semaglutide 1.0 mg	300	-1.78 (0.06)	300	-6.53 (0.28)	300	221	22	5	20
							Dulaglutide 1.5 mg	299	-1.37 (0.06)	299	-2.98 (0.27)	299	207	23	5	29
20 Andrew J Ahmann 2018 (39)	56.6 (24.4)	809	447	8.3 (1.1)	56	Metformin ±TZD/SU	Semaglutide 1.0 mg	404	-1.54 (0.06)	404	-5.63 (0.29)	404	303	38	33	38
							Exenatide 2.0 mg	405	-0.92 (0.06)	405	-1.85 (0.29)	405	309	24	33	29
21 Huub J van Eyk 2019 (40)	55 (11.0)	47	19	8.1 (0.9)	26	Metformin ±SU	Liraglutide 1.8 mg	22	−0.8 (1.00)	22	− 3.90 (3.60)	NA				
							Placebo	25	−0.6 (0.80)	25	− 0.60 (2.20)	NA				
22 Michael Nauck 2009 (41)	56.8 (9.4)	363	214	8.4 (0.9)	26	Metformin	Liraglutide 1.8 mg	236	-1.00 (0.07)	241	-2.79 (0.23)	242	158	16	9	31
							Placebo	120	0.09 (0.09)	121	-1.51 (0.31)	122	44	9	6	16
23 Bernard Zinman 2009 (42)	55 (11.0)	200	113	8.6 (1.2)	26	Metformin ±TZD	Liraglutide 1.8 mg	100	-1.56 (0.10)	100	-2.00 (0.30)	100	NA	7	8	17
							Placebo	100	-0.5 (0.10)	100	0.60 (0.30)	100	NA	12	5	6
24 D Russell-Jones 2009 (43)	57.6 (9.5)	244	187	8.3 (0.9)	26	Metformin ±SU	Liraglutide 1.8 mg	230	-1.33 (0.09)	230	-1.8 (0.33)	230	151	9	63	11
							Placebo	114	-0.24 (0.11)	114	-0.42 (0.39)	114	64	8	19	1
25 John B Buse 2009 (44)	56.3 (9.8)	464	241	8.2 (1.0)	26	Metformin ±SU	Liraglutide 1.8 mg	227	-1.12 (0.08)	231	-3.24 (0.33)	233	174	12	60	23
							Exenatide-10μg	226	-0.79 (0.08)	229	-2.87 (0.33)	231	182	6	78	31
26 Chieh-Hsiang Lu 2013 (45)	50.5 (9.0)	50	27	8.1 (1.0)	16	Metformin ±SU	Exenatide 10μg	26	-0.8 (0.60)	26	-2.00 (1.10)	26	13	NA	12	1
							Placebo	24	-0.1 (0.60)	24	-0.40 (1.00)	24	9	NA	1	0
27 G Derosa 2012 (46)	57.0 (7.5)	171	88	8.0 (0.7)	52	Metformin	Exenatide 10μg	81	-1.2 (0.20)	81	-6.40 (1.20)	NA				
							Placebo	82	-0.4 (0.10)	82	-2.30 (1.10)	NA				
28 Caroline M Apovian 2010 (47)	54.5 (10.0)	194	73	7.7 (0.9)	24	Metformin ±SU	Exenatide 10μg	96	-1.2 (0.15)	96	-6.20 (0.89)	96	NA	2	NA	4
							Placebo	98	-0.4 (0.15)	98	-4.00 (0.80)	98	NA	2	NA	5

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TABLE 1 Continued

Study ID	Year± SD	N_{Total}	N_{Male}	Baseline (HbA1c (%))	Weeks	Combination Therapy Drugs	Intervention	HbA1c (%)		Weight (kg)		Safety				
								N_1	Mean ±SD	N_2	Mean ±SD	N_3	N_T	N_S	N_H	N_{AE}
29 Yan Gao 2009 (48)	55 (9.0)	466	207	8.3 (1.0)	16	Metformin ±SU	Exenatide 10μg	234	-1.2 (0.10)	234	-1.20 (0.30)	234	134	3	83	23
							Placebo	232	-0.4 (0.10)	232	-0.10 (0.20)	232	84	4	21	3
30 David M Kendall 2005 (49)	55 (10.0)	488	281	8.5 (1.0)	30	Metformin ±SU	Exenatide 10μg	241	-0.8 (0.10)	241	-1.60 (0.20)	241	NA	12	67	22
							Placebo	247	+0.2 (0.10)	247	-0.90 (0.20)	247	NA	15	31	11
31 Ralph A DeFronzo 2005 (50)	52 (11.0)	226	135	8.2 (1.0)	30	Metformin	Exenatide 10μg	113	-0.8 (0.10)	113	-2.8 (0.50)	113	NA	3	6	8
							Placebo	113	+0.1 (0.10)	113	-0.30 (0.30)	113	NA	4	6	1
32 John B Buse 2013 (51)	57 (9.6)	911	499	8.5 (1.0)	26	Metformin ±SU	Exenatide 2.0mg	390	-1.28 (0.05)	404	-2.68 (0.18)	461	283	13	51	12
							Liraglutide 1.8mg	386	-1.48 (0.05)	398	-3.57 (0.18)	450	307	7	40	24
33 Kishore M Gadde 2017 (52)	53.4 (9.8)	242	126	8.4 (1.0)	28	Metformin	Exenatide 2.0mg	181	-1.13 (0.11)	181	-1.12 (0.26)	181	101	5	4	3
							Placebo	61	-0.40 (0.19)	61	0.15 (0.48)	61	29	2	2	3
34 Takashi Kadowaki 2009 (53)	57.8 (10.4)	78	53	7.9 (0.9)	12	Metformin ±SU	Exenatide 10μg	37	-1.4 (0.10)	37	-1.30 (0.30)	37	35	0	20	5
							Placebo	40	0.02 (0.10)	40	-0.70 (0.20)	40	26	0	4	1

N_{total}, The total number of people included in the study; N_{male}, total number of males; N_1, total number of decreased HbA1c (%); N_2, total number of weight loss; N_3, total number of safeties; N_T, total number of total adverse events; N_S, total number of serious adverse events; N_H, Hypoglycemic events; N_{AE}, total number of withdrew due to adverse events; SU, Sulfonylureas; TZD, Thiazolidinedione;. OAD, Oral Anti-Diabetic; SLGT-2, sodium-dependent glucose transporters 2.

95%_CI [-2.45, -2.01])). All 10 GLP-1RAs were significantly more effective than placebo in reducing HbA1c (p≥0.05), and tirzepatide 15mg and semaglutide 1.0mg were significantly more effective than the other GLP-1RAs.

The results of SUCRA of 10 interventions and cumulative probability plots are shown in Table 3 and Figure 5. According to the SUCRA results, the ranking of HbA1c reduction from highest to lowest was as follows: tirzepatide15mg>semaglutide1.0mg>PEX168-200μg>dulaglutide1.5mg>oral semaglutide14mg>liraglutide1.8mg>albiglutide30mg>weekly-exenatide2.0mg>daily-exenatide10μg>lixisenatide20μg>placebo.

3.6 Weight loss (kg)

The weight loss results for 10 interventions are shown in Table 4. Compared with placebo, all 10 interventions were associated with weight loss (e.g., tirzepatide15mg (MD=-10.93kg, 95%_CI [-11.89, -9.98]). Compared with the placebo, the other 8 GLP-1RAs were more effective in reducing weight (kg), except for albiglutide 30 mg and PEX-200 μg, which did not show significant changes in weight reduction. In addition, tirzepatide 15 mg and semaglutide 1.0 mg were significantly more effective than the other interventions in reducing weight.

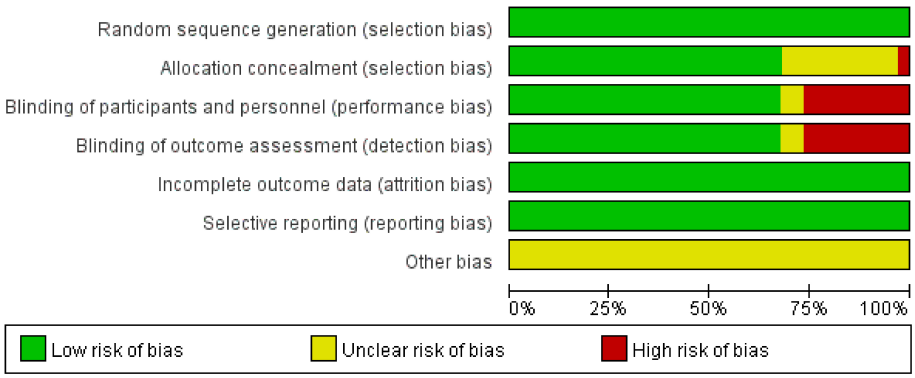

FIGURE 2
Risk of bias assessment plots.

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FIGURE 3
Risk of bias summary plots.

As shown in Table 3 and Figure 5, the SUCRA results show that the ranking of weight loss from highest to lowest was as follows: Tirzepatide15mg>Semaglutide1.0mg>Oral semaglutide14mg>Liraglutide1.8mg>Dulaglutide1.5mg>Daily exenatide10μg>Weekly exenatide2.0mg>Lixisenatide20μg>Albiglutide30mg>Placebo>PEX168-200μg.

3.7 The rate of adverse events

The results of the adverse events rate for 10 interventions are shown in Table 5. Tirzepatide 15 mg, oral semaglutide 14 mg, and semaglutide 1.0 mg did not differ significantly from placebo in the rate of adverse events, while the other seven GLP-1RAs did. Semaglutide1.0mg, tirzepatide15mg had a significantly higher rate of adverse events compared with the other GLP-1RAs, and oral semaglutide14mg had a significantly higher rate of adverse events compared with weekly exenatide2.0mg, albiglutide30mg, lixisenatide20μg, and PEX168-200μg. while all other head-to-head comparisons between GLP-1RAs were not significantly higher in terms of adverse event rates.

As shown in Table 3 and Figure 5, the SUCRA results show that the ranking of the rate of adverse events from highest to lowest was as follows: Tirzepatide15mg>Oral semaglutide14mg>Semaglutide1.0mg>Liraglutide1.8mg>Daily exenatide10μg>Dulaglutide1.5mg>Weekly exenatide2. 0mg>Albiglutide30mg>Lixisenatide20μg>PEX168-200μg>Placebo, this result indicates that tirzepatide15mg had the highest incidence of adverse events compared to all other interventions.

3.8 The rate of serious adverse events

The results of the serious adverse event rate for 10 interventions are shown in Table 6. Compared to the placebo, there was a significant difference in the incidence of serious adverse events between tirzepatide 15 mg and oral semaglutide 14 mg. In addition, the SUCRA values for PEX 168-200 μg, tirzepatide 15 mg, and oral semaglutide 14 mg were higher than placebo, meaning that PEX 168-200 μg, tirzepatide 15 mg and oral semaglutide 14 mg had the highest incidence of serious adverse events compared to placebo.

As shown in Table 3 and Figure 5, the SUCRA results suggest that the ranking of the rate of serious adverse events from high to low was as follows: PEX168-200μg>Tirzepatide15mg>Oralsemaglutide14mg>Placebo>Albiglutide30mg>Lixisenatide20μg>Liraglutide1.8mg>Semaglutide1.0mg>Daily-Exenatide10μg>Weekly-Exenatide2. 0mg>Dulaglutide1.5mg, it means that PEX168-200μg had the highest incidence of serious adverse events compared to all other interventions.

3.9 Hypoglycemic events

The results of hypoglycemic events for 5 interventions including sulfonylureas, and 10 interventions excluding sulfonylureas are shown in Tables 7, 8. There was no significant difference in hypoglycemic events when sulfonylureas were included in combination therapy with the 5 GLP-1RAs compared with placebo. There was no significant difference in hypoglycemic events in head-to-head comparisons. When sulfonylureas were

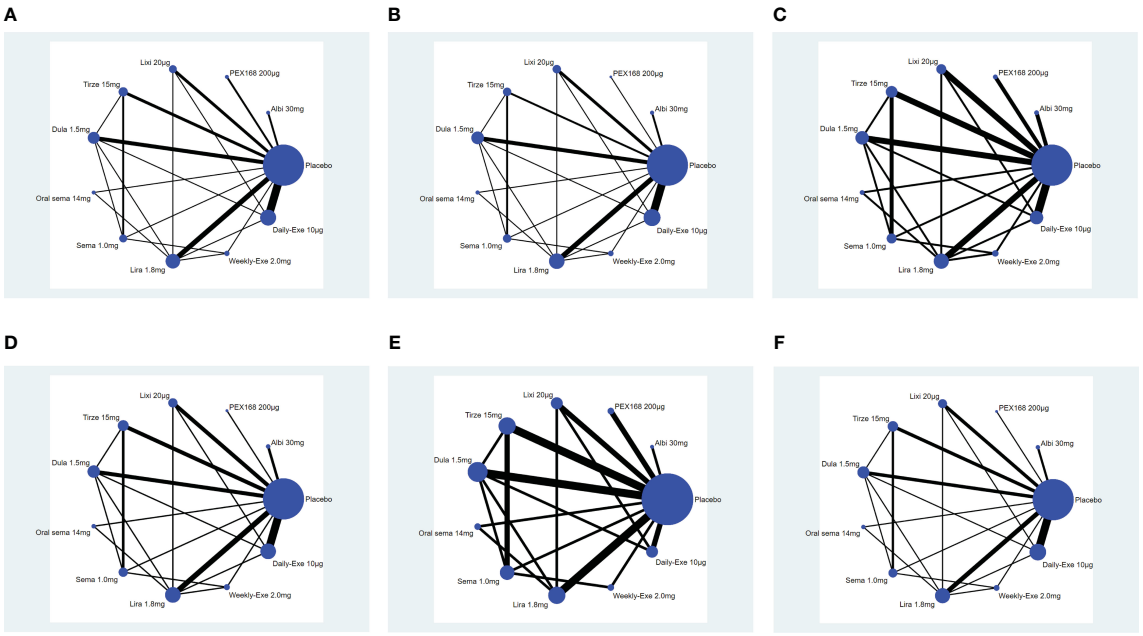

FIGURE 4
Network plot. **(A)** (Decreased HbA1c), **(B)** (Weight loss), **(C)** (The rate of adverse events), **(D)** (The rate of serious adverse events), **(E)** (Hypoglycemic events); **(F)** (AE withdrawal); (Note: Each node represents a specific intervention, the size of the nodes corresponds to the number of participants assigned to each treatment).

TABLE 2 Comparisons for the Δ HbA1c (%) of the 10 Interventions.

Tirze15mg										
-0.66 (-0.91, -0.41) *	Sema1.0mg									
-1.11 (-1.49, -0.73) *	-0.45 (-0.84, -0.06) *	PEX168200μg								
-1.15 (-1.39, -0.90) *	-0.48 (-0.74, -0.23) *	-0.03 (-0.39,0.32)	Dula1.5mg							
-1.13 (-1.58, -0.68) *	-0.47 (-0.93, -0.01) *	-0.02 (-0.52, 0.48)	0.02 (-0.41, 0.45)	Oralsema14mg						
-1.23 (-1.49, -0.96) *	-0.57 (-0.84, -0.29) *	-0.12 (-0.46, 0.23)	-0.08 (-0.30, 0.13)	-0.10 (-0.50, 0.30)	Lira1.8mg					
-1.34 (-1.71, -0.97) *	-0.68 (-1.06, -0.30) *	-0.23 (-0.66, 0.20)	-0.20 (-0.54, 0.15)	-0.21 (-0.71, 0.29)	-0.11 (-0.45,0.23)	Albi30mg				
-1.41 (-1.73, -1.09) *	-0.75 (-1.04, -0.45) *	-0.30 (-0.71, 0.11) *	-0.26 (-0.56, 0.04)	-0.28 (-0.75, 0.19)	-0.18 (-0.46,0.09)	-0.07 (-0.47, 0.33)	Weekly-Exe2.0mg			
-1.44 (-1.70, -1.18) *	-0.78 (-1.05, -0.51) *	-0.33 (-0.67, 0.01)	-0.30 (-0.51, -0.08)	-0.31 (-0.73, 0.11)	-0.21 (-0.41, -0.02) *	-0.10 (-0.43, 0.23)	-0.03 (-0.33,0.26)	Daily-Exe10μg		

(Continued)

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TABLE 2 Continued

Tirze15mg										
-1.77 (-2.07, -1.48) *	-1.11 (-1.42, -0.80) *	-0.66 (-1.03, -0.29) *	-0.63 (-0.88, -0.37) *	-0.64 (-1.08, -0.20) *	-0.54 (-0.78, -0.31) *	-0.43 (-0.79, -0.07) *	-0.36 (-0.69, -0.04) *	-0.33 (-0.55, -0.11) *	Lixi20μg	
-2.23 (-2.45, -2.01) *	-1.57 (-1.81, -1.33) *	-1.12 (-1.43, -0.81) *	-1.09 (-1.26, -0.91) *	-1.10 (-1.50, -0.70) *	-1.00 (-1.16, -0.85) *	-0.89 (-1.19, -0.59) *	-0.82 (-1.09, -0.56) *	-0.79 (-0.93, -0.65) *	-0.46 (-0.66, -0.26) *	Placebo

*Significant difference ($P < 0.05$).

not included among the drugs in combination therapy, there was a higher rate of hypoglycemic events with semaglutide 1.0 mg versus exenatide 2.0 mg and liraglutide 1.8 mg versus placebo, and no significant difference of hypoglycemic events in the other head-to-head comparisons. However, the hypoglycemic events (OR value) were significantly higher when the combination therapy included a sulfonylurea than when there was no sulfonylurea, suggesting that sulfonylureas can dramatically increase the incidence of hypoglycemia events. Meanwhile, there was no significant difference in the incidence of hypoglycemic events with or without sulfonylureas compared to placebo, suggesting that GLP-1RAs do not increase the incidence of hypoglycemic events.

As shown in Table 3 and Figure 5, the SUCRA results indicated that the ranking of the hypoglycemic events from high to low excluding sulfonylureas as follows: Tirzepatide15mg>Daily-Exenatide10μg>Dulaglutide1.5mg>Lixisenatide20μg>Semaglutide1.0mg>Weekly-Exenatide2.0mg>PEX168-200μg>Liraglutide1. 8mg>Placebo>Albiglutide30mg>Oral semaglutide14mg, and it suggested that tirzepatide15mg had the highest incidence of hypoglycemic events compared with all other GLP-1RAs.

3.10 AE withdrawal

The results of the AE discontinuation rate for 10 interventions are shown in Table 9. Compared to the placebo, the other eight GLP-1RAs were similar regarding adverse event withdrawal rates, except for a significant difference between albiglutide 30 mg and semaglutide 1.0 mg. Semaglutide 1.0 mg versus exenatide 2.0 mg, oral semaglutide 14 mg versus exenatide 10 μg, and lixisenatide 20 μg showed significant differences in adverse event withdrawal rates. Lixisenatide 20 μg significantly differed from dulaglutide 1.5 mg, tirzepatide 15 mg, lixisenatide 20 μg, and exenatide 10 μg. The other head-to-head comparisons between GLP-1RAs did not show a significantly higher rate of adverse event withdrawal.

As shown in Table 3 and Figure 5, the SUCRA results indicate that the ranking of AE withdrawals from highest to lowest was as follows: Daily exenatide10μg>Lixisenatide20μg>Oral semaglutide14mg>Tirzepatide15mg>Liraglutide1.8mg>Dulaglutide1.5mg>Albiglutide30mg>Placebo>Semaglutide1.0mg>PEX168-200μg>weekly-exenatide2.0mg, it suggested that daily-exenatide10μg had the highest incidence of AE withdrawal compared to all other interventions.

TABLE 3 The SUCRA (%) results of network meta of the 6 outcome indicators.

Treatment	Decreased HbA1c	Weight Loss	Total Adverse Events	Serious Adverse Events	Hypoglycemic Events	AE with-draw
Placebo	0	11.4	1.5	67.8	32	25.3
Albi 30mg	40.3	14.9	35.4	58.8	30	31.0
PEX168 200μg	66	7.2	28.9	81.5	40.2	22.7
Lixi 20μg	10.3	28.9	29.1	53.1	55.7	82.1
Tirze 15mg	100	100	95.3	73.8	93.7	70.1
Dula 1.5mg	65.2	56.1	50	20.3	69.3	51.2
Oral sema 14mg	62.7	79.5	78.2	71.9	6.8	82.2
Sema 1.0mg	89.7	89.9	71.6	33.2	48.9	24.6
Lira 1.8mg	54.3	64.3	61.7	41.4	39.2	63.6
Weekly-Exe 2.0mg	33.7	43.4	44.1	21.3	44.4	12.1
Daily-Exe 10μg	27.8	54.4	54.1	26.9	89.8	85.1

For efficacy outcome indicators, larger SUCRA values indicated better efficacy. For safety outcome indicators, larger SUCRA values indicated a higher incidence of adverse effects and poorer results.

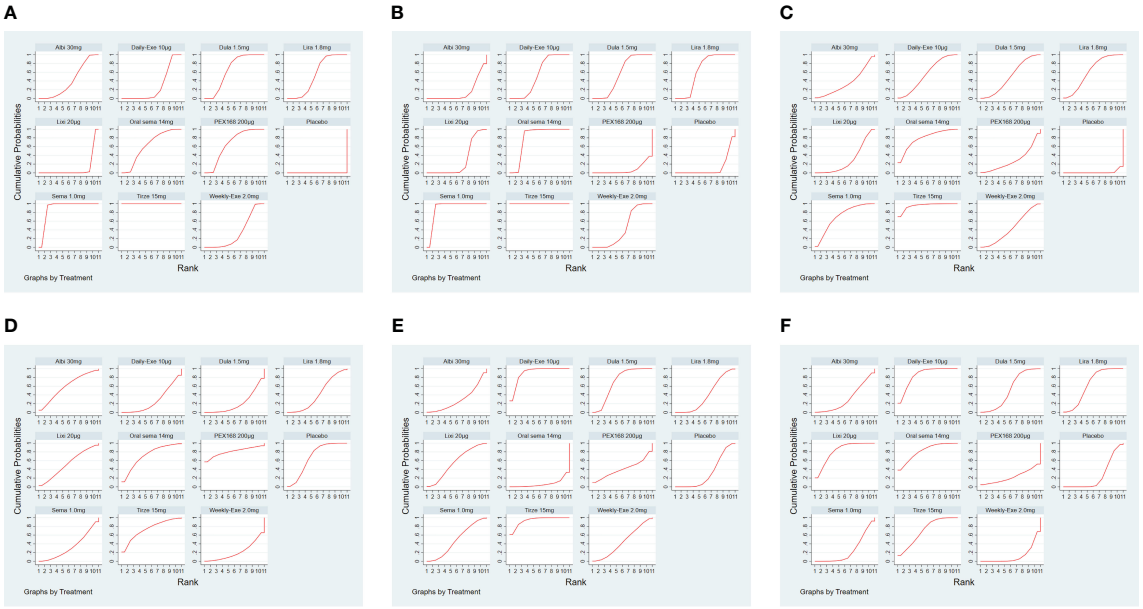

FIGURE 5
The ranking of GLP-1RA based on cumulative probability plots and SUCRA. **(A)** (Decreased HbA1c), **(B)** (Weight loss), **(C)** (The rate of adverse events), **(D)** (The rate of serious adverse events), **(E)** (Hypoglycemic events), **(F)** (AE withdrawal).

3.11 Cluster analysis

Cluster analysis was performed based on the SUCRA values for clinical efficacy (reduction in HbA1c) and safety (the rate of adverse events), and the results of the cluster analysis are shown in Supplementary File 3 (Figure 1). Compared with other interventions, 15 mg of tirzeptide had a significant advantage in efficacy, but it had a higher rate of adverse events than other GLP-1RAs. Semaglutide1.0mg, oral semaglutide14mg, liraglutide1.8mg, dulaglutide1.5mg, PEX168-200μg, albiglutide30mg, daily exenatide10μg, weekly exenatide2.0mg have a similar advantage in terms of efficacy and safety. Lixisenatide 20 μg has an efficacy advantage but not in security (the rate of adverse events).

3.12 Sensitivity analysis

Supplementary File 3 (Table 2) shows the sensitivity analysis results. Stata software was used to perform sensitivity analyses for the primary outcome indicator (HbA1c reduction) after excluding 10 high-risk studies (5, 8, 10, 12, 16, 19, 20, 25, 32, 33) according to results 3.2 Quality assessment of included studies. Supplementary File 3 (Table 2) shows that the network meta-analysis did not change significantly without inversion, indicating that the results were reliable.

3.13 Publication bias analysis

Stata software generated funnel plots for six outcome measures: HbA1c reduction, weight loss, total adverse events, serious adverse events, hypoglycemic events, and AE discontinuation. The funnel plots for efficacy and safety are shown in Figure 6. Figure 6 shows that the distribution of studies in the funnel plot is approximately symmetric, suggesting no publication or other bias between studies. However, in Figures 6A, B, there is heterogeneity in some studies with scatter plots outside the funnel plot, possibly due to minor sample effects.

4 Discussion

This review was based on 34 RCTs involving 12,993 patients with type 2 diabetes who had poor glycemic control on metformin and were randomized to 10 GLP-1RAs or placebo. In the 34 included studies, 18 used metformin + GLP-1RA as the therapeutic agent, and 16 used metformin + GLP-1RA ± OAD (e.g., sulfonylurea, thiazolidinedione, and sodium-glucose cotransporter-2 inhibitors). According to the NMA results, after treatment (82.4%≥ 24 weeks), all GLP-1RAs were superior to placebo in efficacy. In terms of HbA1c reduction, the most effective drug was tirzepatide 15 mg (-2.23%), followed by semaglutide 1.0 mg (-1.57%) and PEX 168200 (-1.12%). In terms of weight loss, tirzepatide 15 mg was the best (-11.3 kg), followed by semaglutide 1.0 mg (-5.99 kg) and oral semaglutide 14 mg (-3.95 kg). This result suggests that tirzepatide 15mg and semaglutide 1.0mg have a clear advantage in reducing blood glucose and weight in patients with T2D.

In terms of safety, tirzepatide 15mg (OR=1.13), oral semaglutide 14mg (OR=0.88), and semaglutide 1.0mg (OR=0.76) were more frequent than the other GLP-1RAs in the rate of adverse events (e.g., nausea, vomiting, and diarrhea, etc.). The OR values for tirzepatide 15mg were more significant than 1, indicating a higher risk of adverse events. In addition, tirzepatide 15mg, oral

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TABLE 4 Comparisons for the weight loss of the 10 interventions.

Tirze15mg										
-5.35 (-6.29, -4.40) *	Sema1.0mg									
-7.39 (-9.15, -5.62) *	-2.04 (-3.78, -0.30) *	Oralsema14mg								
-8.83 (-9.90, -7.75) *	-3.48 (-4.50, -2.46) *	-1.44 (2.94, 0.06)	Lira1.8mg							
-9.07 (-10.07, -8.06) *	-3.72 (-4.67, -2.78) *	-1.68 (-3.29, -0.07) *	-0.24 (-1.02,0.54)	Dula1.5mg						
-9.11 (-10.17, -8.05) *	-3.76 (-4.79, -2.74) *	-1.72 (-3.29, -0.16) *	-0.28 (-0.99,0.42)	-0.04 (-0.81, 0.73)	Daily-Exe10μg					
-9.51 (-10.75, -8.27) *	-4.17 (-5.25, -3.08) *	-2.12 (-3.87, -0.38) *	-0.69 (-1.69,0.32)	-0.44 (-1.53, 0.65)	-0.40 (-1.47, 0.67)	Weekly-Exe2.0mg				
-10.17 (-11.36, -8.98) *	-4.83 (-5.98, -3.67) *	-2.78 (-4.43, -1.14) *	-1.35 (-2.20, -0.49) *	-1.10 (-2.05, -0.15) *	-1.06 (-1.87, -0.25)	-0.66 (-1.85, 0.53)	Lixi20μg			
-10.82 (-12.27, -9.37) *	-5.47 (-6.90, -4.05) *	-3.43 (-5.29, -1.58) *	-1.99 (-3.23, -0.76) *	-1.75 (-3.02, -0.48) *	-1.71 (-2.91, -0.51) *	-1.31 (-2.77, 0.15)	-0.65 (-1.96, 0.66)	Albi30mg		
-10.93 (-11.89, -9.98) *	-5.59 (-6.50, -4.67) *	-3.55 (-5.05, -2.05) *	-2.11 (-2.69, -1.53) *	-1.87 (-2.51, -1.22) *	-1.82 (-2.32, -1.33) *	-1.42 (-2.39, -0.46) *	-0.76 (-1.49, -0.04) *	-0.11 (-1.21, 0.98)	Placebo	
-11.33 (-13.15, -9.52) *	-5.99 (-7.78, -4.19) *	-3.95 (-6.10, -1.79) *	-2.51 (-4.16, -0.86) *	-2.27 (-3.94, -0.59) *	-2.22 (-3.85, -0.60) *	-1.82 (-3.64, -0.00)	-1.16 (-2.87,0.54)	-0.51 (-2.41, 1.38)	-0.40 (-1.94, 1.14)	PEX168200μg

*Significant difference ($P < 0.05$).

TABLE 5 The results of the rate of adverse events for 10 interventions.

Tirze15mg										
0.25 (-0.50,1.01)	Oralsema14mg									
0.37 (-0.08,0.82) *	0.11 (-0.59,0.81) *	Sema1.0mg								
0.47 (-0.08,1.01)	0.21 (-0.34,0.77) *	0.10 (-0.36,0.56) *	Lira1.8mg							
0.52 (-0.06,1.09)	0.26 (-0.36,0.88) *	0.15 (-0.35,0.65) *	0.05 (-0.30,0.39)	Daily-Exe10μg						
0.55 (0.02,1.08)	0.30 (-0.33,0.92) *	0.18 (-0.25,0.61) *	0.08 (-0.27,0.43) *	0.03 (-0.35,0.42) *	Dula1.5mg					
0.60 (0.03,1.17)	0.34 (-0.32,1.01)	0.23 (-0.21,0.68) *	0.13 (-0.27,0.53) *	0.08 (-0.40,0.56) *	0.05 (-0.41,0.50) *	Weekly-Exe2.0mg				

(Continued)

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TABLE 5 Continued

Tirze15mg										
0.70 (-0.02,1.42)	0.44 (-0.31,1.20)	0.33 (-0.34,1.00)	0.23 (-0.34,0.80) *	0.18 (-0.40,0.77) *	0.15 (-0.45,0.75) *	0.10 (-0.55,0.75) *	Albi30mg			
0.72 (0.14,1.30)	0.46 (-0.15,1.08)	0.35 (-0.16,0.86) *	0.25 (-0.09,0.59) *	0.20 (-0.15,0.56) *	0.17 (-0.24,0.58) *	0.12 (-0.36,0.60) *	0.02 (-0.56,0.60) *	Lixi20μg		
0.77 (0.03,1.51)	0.52 (-0.27,1.30)	0.40 (-0.29,1.10)	0.30 (-0.30,0.91) *	0.25 (-0.37,0.87) *	0.22 (-0.41,0.85) *	0.17 (-0.51,0.85) *	0.07 (-0.67,0.81) *	0.05 (-0.56,0.67) *	PEX168200μg	
1.13 (0.61,1.65)	0.88 (0.31,1.44)	0.76 (0.32,1.21)	0.66 (0.40,0.93) *	0.61 (0.31,0.92) *	0.58 (0.25,0.91) *	0.53 (0.12,0.94) *	0.43 (-0.07,0.93) *	0.41 (0.13,0.70) *	0.36 (-0.18,0.90) *	Placebo

*Significant difference (P < 0.05).

TABLE 6 The results of the rate of serious adverse events for 10 interventions.

PEX16820μg										
0.39 (-1.29, 2.08)	Tirze15mg									
0.45 (-1.13, 2.03)	0.06 (-0.94,1.05)	Oralsema14mg								
0.54 (-0.92, 2.01)	0.15 (-0.68,0.99) *	0.09 (-0.49,0.68) *	Placebo							
0.59 (-0.98, 2.16)	0.19 (-0.82,1.21)	0.14 (-0.68,0.95) *	0.04 (-0.53,0.61) *	Albi30mg						
0.67 (-0.90, 2.24)	0.27 (-0.72,1.27)	0.22 (-0.55,0.99) *	0.12 (-0.43,0.68) *	0.08 (-0.71,0.88) *	Lixi20μg					
0.78 (-0.73, 2.29)	0.39 (-0.48,1.26)	0.33 (-0.23,0.89) *	0.24 (-0.13,0.60) *	0.19 (-0.48,0.87) *	0.11 (-0.48,0.70) *	Lira1.8mg				
0.90 (-0.70, 2.49)	0.50 (-0.15, 1.15)	0.45 (-0.37,1.26)	0.35 (-0.28,0.98) *	0.31 (-0.54,1.16)	0.23 (-0.59,1.04)	0.11 (-0.54,0.76) *	Sema1.0mg			
0.93 (-0.59, 2.45)	0.53 (-0.36, 1.43)	0.48 (-0.21,1.16)	0.38 (-0.03,0.80) *	0.34 (-0.36,1.04)	0.26 (-0.35,0.87) *	0.15 (-0.34,0.63) *	0.03 (-0.66,0.73) *	Daily-Exe10μg		
1.04 (-0.58, 2.66)	0.65 (-0.27, 1.56)	0.59 (-0.26,1.44)	0.50 (-0.19,1.18)	0.45 (-0.44,1.34)	0.37 (-0.46,1.21)	0.26 (-0.41,0.93) *	0.15 (-0.45,0.74) *	0.11 (-0.64,0.87) *	Weekly-Exe2.0mg	
1.01 (-0.54, 2.57)	0.62 (-0.17, 1.40)	0.56 (-0.18,1.30)	0.47 (-0.05,0.98) *	0.43 (-0.34,1.19)	0.34 (-0.38,1.07)	0.23 (-0.32,0.79) *	0.12 (-0.42,0.66) *	0.09 (-0.49,0.66) *	-0.03 (-0.73,0.68) *	Dula1.5mgs

*Significant difference (P < 0.05).

TABLE 7 The results of the hypoglycemic events for 5 interventions include sulfonylurea drugs.

Daily-Exe10μg					
0.37 (-0.82,1.56)	Weekly-Exe2.0mg				
0.61 (-0.13,1.35)	0.24 (-0.69,1.17)	Lira1.8mg			
0.71 (-0.78,2.20)	0.34 (-1.46,2.14)	0.10 (-1.45,1.64)	Lixi20μg		
0.78 (-0.43,2.00)	0.41 (-1.17,2.00)	0.17 (-1.11,1.45)	0.07 (-1.64,1.78)	Albi30mg	
1.52 (0.89,2.14)	1.15 (-0.04,2.34)	s0.91 (0.16,1.65)	0.81 (-0.54,2.16)	0.74 (-0.31,1.78)	Placebo

*Significant difference (P < 0.05).

semaglutide 14mg, and semaglutide 1.0mg did not show a significantly higher incidence of adverse events. The incidence of serious adverse events (e.g., cardiovascular events, severe gastrointestinal reactions, infections, etc.) was higher for PEX168 (OR=0.54), tirzepatide 15mg (OR=0.15), and oral semaglutide 14mg (OR=0.09) than for other GLP-1RAs, and tirzepatide 15mg and oral semaglutide 14mg will have serious adverse events but at a lower risk.

GLP-1RAs generally do not cause hypoglycemia in patients with type 2 diabetes who are not taking insulin (54). However, it is noteworthy that when the combination included sulfonylureas, hypoglycemia (OR) incidence was significantly higher with GLP-

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TABLE 8 The results of the hypoglycemic events for 10 interventions exclude sulfonylurea drugs.

Tirze15mg										
0.24 (-0.84,1.32)	Daily-Exe10μg									
0.77 (-0.25,1.79)	0.53 (0.02,1.03)	Dula1.5mg								
0.99 (-0.28,2.26)	0.74 (-0.05,1.53)	0.21 (-0.66,1.09)	Lixi20μg							
1.14 (0.16,2.13)	0.90 (-0.10,1.90)	0.37 (-0.55,1.29)	0.16 (-1.05,1.36)	Sema1.0mg						
1.21 (0.10,2.33)	0.97 (-0.13,2.06)	0.44 (-0.59,1.46)	0.22 (-1.13,1.57)	0.07 (-0.52,0.65) *	Weekly-Exe2.0mg					
1.42 (-1.08,3.91)	1.17 (-1.18,3.53)	0.64 (-1.70,2.99)	0.43 (-2.02,2.87)	0.27 (-2.19,2.74)	0.21 (-2.29,2.71)	PEX168200μg				
1.30 (0.22,2.38)	1.06 (0.41,1.70)	0.53 (-0.02,1.07)	0.31 (-0.59,1.21)	0.16 (-0.85,1.16)	0.09 (-1.00,1.18)	-0.12 (-2.46,2.23)	Lira1.8mg			
1.41 (0.41,2.42)	1.17 (0.59,1.75)	0.64 (0.08,1.20)	0.43 (-0.45,1.31)	0.27 (-0.67,1.21)	0.20 (-0.82,1.23)	-0.00 (-2.28,2.28)	0.12 (-0.43,0.66) *	Placebo		
1.56 (0.08,3.04)	1.32 (0.08,2.55)	0.79 (-0.44,2.01)	0.57 (-0.83,1.97)	0.42 (-1.02,1.86)	0.35 (-1.14,1.84)	0.14 (-2.38,2.67)	0.26 (-0.96,1.48)	0.15 (-0.94,1.24)	Albi30mg	
2.56 (0.71,4.41)	2.32 (0.67,3.96)	1.79 (0.17,3.41)	1.57 (-0.19,3.34)	1.42 (-0.40,3.23)	1.35 (-0.51,3.21)	1.14 (-1.64,3.92)	1.26 (-0.29,2.81)	1.15 (-0.44,2.73)	1.00 (-0.93,2.93)	Oralsema14mg

*Significant difference (P < 0.05).

TABLE 9 The results of the rate of AE withdrew for 10 interventions.

Daily-Exe10μg										
0.04 (-0.54,0.62) *	Lixi20μg									
-0.02 (-1.01,0.98) *	-0.05 (-1.09,0.98) *	Oralsema14mg								
0.26 (-0.64,1.16)	0.23 (-0.77,1.22)	0.28 (-0.94,1.50)	Tirze15mg							
0.35 (-0.17,0.87) *	0.31 (-0.29,0.91) *	0.36 (-0.51,1.24)	0.08 (-0.79,0.96) *	Lira1.8mg						
0.57 (-0.09,1.23)	0.54 (-0.24,1.31)	0.59 (-0.46,1.64)	0.31 (-0.46,1.08)	0.23 (-0.39,0.84) *	Dula1.5mg					
s0.97 (0.01,1.94)	0.93 (-0.09,1.96)	0.99 (-0.28,2.26)	0.71 (-0.48,1.89)	0.62 (-0.34,1.59)	0.40 (-0.64,1.44)	Albi30mg				
1.04 (0.58,1.50)	1.00 (0.43,1.58)	1.05 (0.11,2.00)	0.77 (-0.05,1.59)	0.69 (0.24,1.15)	0.46 (-0.13,1.06)	0.07 (-0.78,0.92) *	Placebo			
1.08 (0.26,1.91)	1.05 (0.12,1.97)	1.10 (-0.06,2.26)	0.82 (0.12,1.51)	0.73 (-0.04,1.51)	0.51 (-0.16,1.17)	0.11 (-1.02,1.24)	0.04 (-0.70,0.79) *	Sema1.0mg		
1.43 (-0.54,3.40)	1.39 (-0.61,3.39)	1.44 (-0.70,3.58)	1.16 (-0.92,3.25)	1.08 (-0.89,3.05)	0.85 (-1.16,2.86)	0.46 (-1.64,2.55)	0.39 (-1.53,2.31)	0.35 (-1.71,2.40)	PEX168200μg	
1.36 (0.54,2.19)	1.32 (0.41,2.24)	1.38 (0.24,2.52)	1.10 (0.21,1.99)	1.01 (0.27,1.75)	0.79 (0.01,1.56)	0.39 (-0.75,1.53)	0.32 (-0.43,1.08)	0.28 (-0.41,0.97) *	-0.07 (-2.13,1.99)	Weekly-Exe2.0mg

*Significant difference (P < 0.05).

1RAs, suggesting that sulfonylureas are an essential contributor to hypoglycemia (55). The 10 GLP-1RAs did not exhibit a significantly higher incidence of AE withdrawal.

Tirzepatide is a novel GIP and GLP-1 dual receptor agonist that the U.S. FDA approved for treating T2D in 2022. it has demonstrated potent HbA1c and body weight reduction. In a recently completed Phase 3, double-blind, randomized, controlled trial of once-weekly tirzepatide in the treatment of obesity, tirzepatide 15 mg achieved a mean percentage change in body weight of -20.9% (-21.9 kg) over 72 weeks of treatment, suggesting that tirzepatide 15 mg is also a potential weight loss agent (56). The result is a significant breakthrough in dual-targeted GLP-1 and GIP drugs that reduce HbA1c and body weight more effectively than GLP-1 RA. In addition, a phase 2 RCT demonstrated that retatrutide, a GIP, GLP-1, and glucagon receptor

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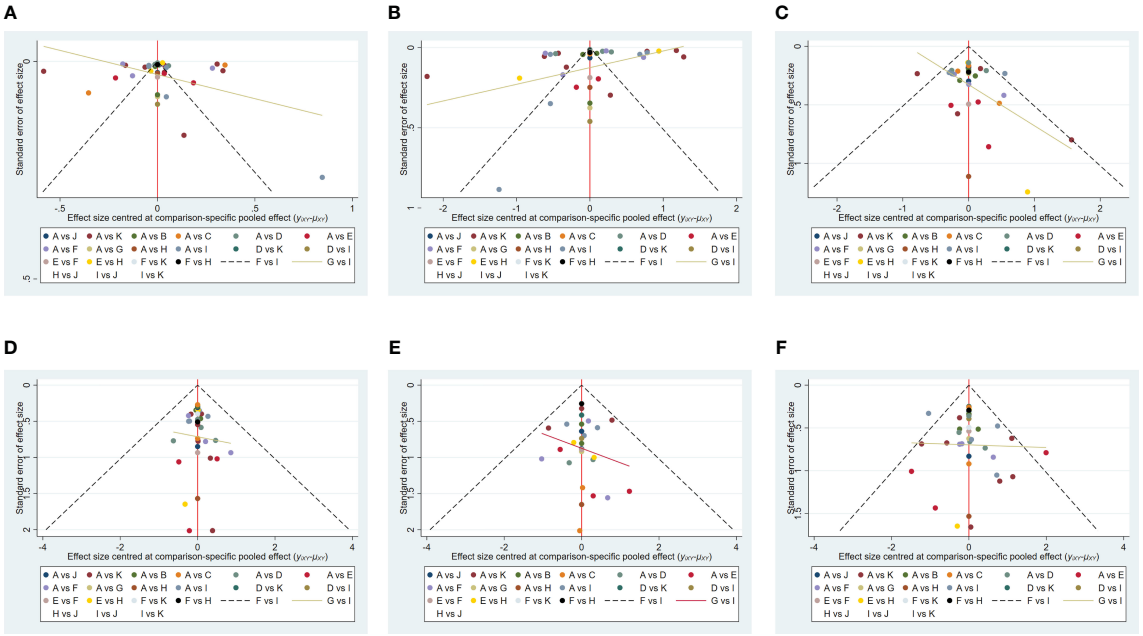

FIGURE 6
The funnel plots for efficacy and safety. **(A)** (Decreased HbA1c), **(B)** (Weight loss), **(C)** (The rate of adverse events), **(D)** (The rate of serious adverse events), **(E)** (Hypoglycemic events), **(F)** (AE withdrawal), (Note: A: Placebo; B: Albi30mg; C: PEX168-200μg; D: Lixisenatide20μg; E: Tirze15mg; F: Dulaglutide1.5mg; G: Oralsema14mg; H: Sema1.0mg; I: Lira1.8mg; J: Weekly-Exenatide2.0mg; K: Daily-Exenatide10μg).

agonist achieved clinically meaningful improvements in glycemic control (2.02% reduction in HbA1c with 24 weeks of treatment) and significant weight reduction (17.18kg reduction in weight with 36 weeks of treatment), and its safety profile was consistent with that of GLP-1 receptor agonists as well as GIP and GLP-1 receptor agonists (57). In the future, GIP, GLP-1, and glucagon tri-agonists are promising for patients with T2D and/or obesity.

Semaglutide1.0mg and oral semaglutide 14mg was inferior to tirzepatide in reducing HbA1c and body weight but had significant advantages over other GLP-1RAs. In addition, oral semaglutide offers excellent convenience to patients and improves compliance. The results were generally consistent with Xia L et al. 's (58) efficacy in lowering HbA1c and body weight. However, oral semaglutide treatment increases the incidence of nausea, vomiting, and diarrhea (Comparison with placebo or other OAD (e.g., Sitagliptin, empagliflozin)), and the oral route of administration is strongly associated with an increase in gastrointestinal adverse events (59). The higher AE withdrawal rate for oral semaglutide than for subcutaneous semaglutide and tirzepatide may be related to gastrointestinal adverse events.

The global and local inconsistency test results showed no significant inconsistency for each outcome indicator and two comparison groups. In the closed loop inconsistency test, the same four closed loops showed significant inconsistency in the two outcome indicators of HbA1c reduction and weight loss. The other closed loops were those for which there was no evidence of inconsistency (p >0.05 or CI_95 includes 0). In conclusion, the results for the inconsistency of the whole network are reliable.

In sensitivity analyses, the NMA results did not change significantly and were reliable. Overall, the risk of the included studies was low, the quality was good, and the NMA results were reliable. The publication bias results suggest that there may be some bias in the effectiveness due to differences in the drugs used and the effect of a small sample size. The heterogeneity results indicate a significant difference in efficacy, and the main reason for this was the difference in the use of background medication (metformin +/-OAD). In conclusion, heterogeneity does not significantly impact the NMA results.

T2D places a heavy burden on the body, causing various complications (cardiovascular and cerebrovascular complications, microvascular and neurological lesions, diabetic foot, etc.) that can reduce the quality of life and life expectancy (60). The anti-inflammatory effects of metformin and GLP-1RAs contribute to their beneficial effects, as T2DM is characterized by chronic low-grade inflammation. In addition to glycemic control, aggressive cardiac risk reduction is a priority for all patients with T2D. Evidence suggests (61, 62) that aggressive reduction of multiple risk factors (weight loss or maintenance, smoking cessation, blood pressure control, lipid-lowering, dietary modification, and exercise) reduces the risk of microvascular and macrovascular complications in patients with diabetes.

GLP-1RAs are safe and effective and can reduce weight and the risk of major cardiovascular disease (63). Semaglutide 2.4mg and liraglutide 3.0mg have been approved by the U.S. Food and Drug Administration (FDA) for weight management in people who are overweight or obese. A related weight loss meta-analysis (15)

showed that treatment with semaglutide 2.4 mg and liraglutide 3.0 mg for more than 20 weeks in combination with daily diet and exercise resulted in weight loss of 12.47 kg and 5.24 kg, respectively, in people with overweight and obesity. Tirzepatide 15 mg is expected to be approved shortly for weight control in adults with obesity or overweight (with >= 1+ obesity-related comorbidity). Liraglutide, dulaglutide, and semaglutide have been shown to have cardiovascular benefits, and the only other glucose-lowering agents for T2D that have been shown to have cardiovascular benefits are the SGLT-2 inhibitors empagliflozin, canagliflozin and dapagliflozin (64, 65). In clinical practice, selecting a GLP-1RA with cardiovascular efficacy, good glucose-lowering, and weight-loss (kg) effects, and a high safety profile based on the patient's medical condition and needs is critical. Based on efficacy and safety results, tirzepatide 15mg, semaglutide 1.0mg, and oral semaglutide 14mg are good options for patients with T2DM.

The main objective of this study is to, directly and indirectly compare the safety and efficacy of 10 GLP-1RAs in combination with metformin, to address the wide variety of medications encountered in clinical practice, and to provide evidence-based support and reference for the use of GLP-1RAs in clinical practice. Meanwhile, being overweight and obese are risk factors for diabetes, and this study evaluated the effect of weight loss with different GLP-1RAs and glucose lowering. However, there are limitations to this study: First, the range of drug treatment cycles is vast, mostly between 12 and 48 weeks, which affects the safety assessment results. Second, in this review, there were some studies where the background drug was metformin and others where it was metformin +/- OAD, which is the source of the heterogeneity in the efficacy assessment. Third, the populations included in this study include Caucasian, African and Asian populations. However, there are some differences in the physical condition of patients with type 2 diabetes from different regions or races, and their actual clinical efficacy is also biased. Finally, there were small samples of studies and high-risk studies in the included studies, which may make our results insubstantial and incomplete. Even though there are some limitations in this study, more and more large-sample, high-quality studies will gradually emerge over time so that our evaluation results will improve and become more convincing overall.

5 Conclusion

This study results showed that GLP-1RAs were effective in lowering HbA1c and reducing body weight without leading to increased incidence of hypoglycemic reactions. In addition, this study may provide reference and evidence-based medical evidence for clinicians to select GLP-1RAs in patients with T2DM and high BMI. Tirzepatide 15mg, semaglutide 1.0 mg, and oral semaglutide 14 mg may be preferred for treating patients with T2DM. Finally, there is an urgent need for more high-quality, large-sample randomized controlled trials to make our study results more comprehensive and reliable.

Author contributions

All co-authors made substantial contributions to this article and this version is published as the final version, agreeing to submit this study to the journal and taking responsibility for all aspects of the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1244432/full#supplementary-material

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Pharmaceutical efficacy of novel human-origin *Faecalibacterium prausnitzii* strains on high-fat-diet-induced obesity and associated metabolic disorders in mice

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Introduction: Obesity and related metabolic issues are a growing global health concern. Recently, the discovery of new probiotics with anti-obesity properties has gained interest.

Methods: In this study, four *Faecalibacte-rium prausnitzii* strains were isolated from healthy human feces and evaluated on a high-fat diet-induced mouse model for 12 weeks.

Results: The *F. prausnitzii* strains reduced body weight gain, liver and fat weights, and calorie intake while improving lipid and glucose metabolism in the liver and adipose tissue, as evidenced by regulating lipid metabolism-associated gene expression, including ACC1, FAS, SREBP1c, leptin, and adiponectin. Moreover, the *F. prausnitzii* strains inhibited low-grade inflammation, restored gut integrity, and ameliorated hepatic function and insulin resistance. Interestingly, the *F. prausnitzii* strains modulated gut and neural hormone secretion and reduced appetite by affecting the gut-brain axis. Supplementation with *F. prausnitzii* strains noticeably changed the gut microbiota composition.

Discussion: In summary, the novel isolated *F. prausnitzii* strains have therapeutic effects on obesity and associated metabolic disorders through modulation of the gut-brain axis. Additionally, the effectiveness of different strains might not be achieved through identical mechanisms. Therefore, the present findings provide a reliable clue for developing novel therapeutic probiotics against obesity and associated metabolic disorders.

KEYWORDS

***Faecalibacterium prausnitzii*, probiotics, anti-obesity, metabolic disorders, gut–brain axis, appetite, gut microbiota**

1 Introduction

Obesity has been deemed a worldwide epidemic. In recent decades, the prevalence of overweight and obesity has steadily increased in many areas (1). World Health Organization (WHO) claims that morbid obesity develops in prolonged obese individuals, and it is associated with various metabolic disorders, such as glucose intolerance, dyslipidemia, fatty liver, hypertension, insulin resistance, glucose intolerance, and even diabetes mellitus and some cancers (2–4). Obesity is exacerbated by environmental factors, such as high-fat, high-sugar, low-fiber diet, sedentary lifestyles, sleep deprivation, abuse of antibiotics, and aging (5, 6). Nowadays, the prevention and treatment of obesity have attracted many researchers to solve the global health problem (7).

Probiotics are single or several live bacterial species which can influence gut microbial activity directly or indirectly and enhance human health (8). Over the past few years, recent evidence has shown that probiotics are safe and have rapidly become a promising natural approach for remedying metabolic-related disorders (9). Probiotics assist the host by fostering gut microbiota equilibrium and enhancing immunological diseases, inflammatory bowel disease, type 2 diabetes, and obesity, according to growing research (10–12). Several *in vivo* studies and clinical trials revealed a possible causality between probiotic consumption and obesity. For example, supplements of different *Lactobacillus* and *Bifidobacterium* strains ameliorated HFD-induced weight gain, buildup of visceral fat, insulin resistance, hepatic steatosis, and expression of various pro-inflammatory cytokines (13, 14). Except for lactic acid bacteria, more researchers are paying attention to the relationship between the next-generation anaerobic strains and metabolic diseases, e.g., *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Roseburia* spp., *Akkermansia muciniphila* (15, 16). Although scientists and clinicians generally acknowledge the health benefits of probiotics, even within the same genus, different probiotic species can have varying impacts on fat buildup and obesity (17). Consequently, it is crucial to evaluate the efficacy of various probiotic strains from the same species in animal models.

According to reports, *F. prausnitzii*, which makes up more than 5% of the total bacterial population in the human gut microbiota and is one of the most prevalent anaerobic bacteria there, is a significant commensal bacterium (18). Patients with type 2 diabetes, obesity, and inflammatory bowel disease, which are characterized by food intolerance, insufficient calorie intake, and dysfunctional energy metabolism, inversely correlated with *F. prausnitzii* (19, 20). A high-fat diet (HFD) may make *F. prausnitzii* decreased. The treatment of *F. prausnitzii* in HFD-fed mice ameliorates hepatic and adipose inflammation (21, 22). Furthermore, *F. prausnitzii* abundance could increase after obese and type 2 diabetes individuals lose weight (23). Previous research has indicated that supplementary *F. prausnitzii* improves the gut permeability (24). Therefore, inflammation-related dysbiosis of the gut microbial community was treated with *F. prausnitzii* as an intervention method (25). Thus, *F. prausnitzii* may benefit human health, especially for chronic metabolic diseases.

The interactions between the brain and the gastrointestinal system are reflected in the gut-brain axis. In response to food intake, the brain gets neuronal and endocrine inputs from the gut, which are combined with signals from other organs to coordinate physiological responses (26). Many physiological processes involved in the gut–brain axis include appetite, satiety, metabolism of fat, insulin secretion/sensitivity and glucose regulation (27). The information of energy balance current state communicated from the gastrointestinal tract releases peptide hormones to the brain. Studies have documented the function of these gut hormone peptides to modulate appetite and energy expenditure through the vagus nerve and critical regions of brain activation implicated in energy homeostasis, such as the hypothalamus (26). Thus, it might be a therapeutic strategy for preventing and treating obesity.

Most comparative studies used *F. prausnitzii* type strain A2-165, which is isolated from the human intestinal tract (28). However, different strains within an identical species might initiate different host immunologic reactions (29). In the present study, a total of four novel strains of *F. prausnitzii* were initially isolated from human feces and utilized for a comparative evaluation. The *F. prausnitzii* type strain A2-165 and Orlistat were used as positive controls. The selected strains were employed to evaluate their efficacy in treating obese mice induced by a HFD, with the aim of establishing foundational evidence for potential future applications of *F. prausnitzii*.

2 Materials and methods

2.1 Bacterial strains and growth conditions

All of the *F. prausnitzii* was isolated from feces collected from healthy Koreans after obtaining informed consent from each subject. The genetic diversity of isolated *F. prausnitzii* were analyzed, and the detailed results are presented in Supplementary Section (Figure S1). This research was approved by the Institutional Review Board of Dongguk University, Ilsan Hospital (IRB# 2018-06-001-012). The reference strain A2-165 (Deutsche Sammlung von Mikroorganismen [DSM] 17677) was obtained from the Leibniz-Institute DSMZ-German Collection of Microorganism and Cell Cultures). The extremely oxygen-sensitive bacteria were grown in brain-heart infusion medium supplemented with 0.5% (w/v) yeast extract, 0.1% (w/v) cellobiose, 0.1% (w/v) maltose, and 0.05% (w/v) L-cysteine, at 37°C in an anaerobic chamber (atmosphere of 5% CO2, 5% H2, and 90% N2). 16S rRNA gene sequencing for the identification of *F. prausnitzii* was performed after polymerase chain reaction (PCR) amplification of a region of the 16S rRNA gene. For the animal experiments, all of the *F. prausnitzii* strains were cultured anaerobically in a soy-peptone based medium with some supplements and centrifuged at 12,000 × g for 5 min. They were

then adjusted to an end concentration of 1×10^8 CFU/150 µl using anaerobic PBS with 20% glycerol and stored at −80°C until use.

2.2 Animals and treatments

Seven-week-old male C57BL/6 mice were obtained from Orient Bio (Seongnam, South Korea). The mice were housed under controlled conditions (a 12:12-hr light-dark cycle, temperature of 20 ± 3°C, and humidity 55 ± 5%) with ad libitum access to water and a standard chow diet (FeedLab, Guri, South Korea) for 10 days of acclimatization. All experiments were approved by the institutional animal care and use committee (IACUC) of the Dongguk University (2020-11208) and conducted according to the guidelines of the National Rearch Council (Guide for the Care and Use of Laboratory Animals, 2011). Diets were purchased from Reseach Diets, Inc. (Nwe Brunswick, NJ, USA): normal diet (Nor) containing 10% calories from the fat with 3.85 kcal/g (19.2% protein, 67.3% carbohydrate, and 4.8% fat) and HFD containing 60% calories from the fat with 5.24 kcal/g (26.2% protein, 26.3% carbohydrate, and 34.9% fat).

After acclimatization, 72 mice were weighted and randomly divided into eight groups (n = 9) as follows: (1) Nor group; (2) HFD group; (3) Orilistat-treated HFD group (XEN); (4) *F. prausnitzii* type strain A2-165-treated HFD group (A2-165); (5) *F. prausnitzii* EB-FPDK3-treated HFD group (DK3); (6) *F. prausnitzii* EB-FPDK6-treated HFD group (DK9); (7) *F. prausnitzii* EB-FPDK11-treated HFD group (DK11); (8) *F. prausnitzii* EB-FPYYK1-treated HFD group (YK1). Mice had ad libitum access to diets and sterile water. Orlistat (Xenical®; Roche, Basel, Switzerland) was dissolved in sterile PBS at pH 7.4 and administrated orally to the Xen group (10 mg/kg/day). The live bacterial samples were resuspended in sterile PBS and then freshly administrated to the A2-165, DK3, DK9, DK11, and YK1 groups via oral gavage in sterile PBS with 20% glycerol at a dose of 1×10^8 CFU/150 µl per animal. The mice in the NOR and HFD groups were given only sterile PBS with 20% glycerol as a vehicle. The treatments were carried out 6 days per week for 12 weeks. The body weight of mice was assessed weekly, and food intake was recorded three times a week. Fecal samples were collected at weeks 0 and 12 post-treatment and kept at -80°C for further microbiome analysis

At the end of the experiment, mice were fasted for 12 h and blood was collected by cardiac puncture under anesthesia with a mixture of tiletamine-zolazepam (Zoletil 50, Virbac, Carros, France). Serum was separated by centrifuging at 2,000 × g for 15 min at 4°C and kept at −80°C until further biochemical analyses. The spleen, liver, large intestine, small intestine, and adipose tissues was removed, washed with ice-cold PBS, dried, and then weighed. Portions of the liver, large intestine, small intestine, adipose, and hypothalamus were removed and flash-frozen in the liquid nitrogen and stored at −80°C until further use. The other portions of the tissues were fixed with 4% paraformaldehyde (PFA) (Junsei, Tokyo, Japan) overnight at 4°C and then embedded in paraffin.

2.3 Serum biochemical analyses

Serum samples were analyzed for total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using commercial colorimetric assay kits (Asan Pham. Co., Seoul, South Korea). Insulin levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga, Yokohama, Japan). Insulin resistance was estimated by homeostasis model assessment of insulin resistance (HOMA-IR) index calculated as follows: HOMA-IR = [(fasting glucose (mg/dL) x fasting insulin (µU/mL))/405. Lower HOMA-IR values indicated better insulin sensitivity and vice versa.

2.4 Oral glucose tolerance test

Mice were fasted for 14h with free access to drinking water at week 11 of study period and administrated with glucose solution (2 g/kg body weight) by oral gavage. Blood glucose level was measured from the tail vein using an Accu-Chek Active blood glucose meter (Roche Diagnostics Corp, Rotkreuz, Switzerland) at 0, 30, 60, 90, and 120 min after injection. The glucose area under the curves (AUC) during the OGTT were calculated using GraphPad Prism 5.0 software (San Diego, CA, USA).

2.5 Histology analysis

PFA-fixed paraffin-embedded sections (4 µm) of the liver, intestine and mesenteric adipose tissues were placed on positively charged glass slides, de-waxed in xylene (Sigma Chemicals, St Louis, MO, USA), dehydrated in a gradual ethanol series, and stained with either hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA), Alcian blue (AB) solution (Abcam, Cambridge, UK) for goblet cells, or Oil Red O (Sigma-Aldrich) for fatty liver tissues according to the manufacturer's protocol. Tissues were examined under an inverted light microscope (Olympus, Tokyo, Japan). Representative images of the liver and mesenteric adipose tissue were taken from three individual liver and mesenteric adipose samples in each group at 4 x and 20 x magnifications, while for intestine AB stain, representative images were taken from six to seven individual samples in each group. The liver steatosis area and the Oil Red O-stained area were assessed on the images taken with Image-Pro Plus 6.0 software. The AB-positive area, villus length, and the number of the goblet cells were determined from image J software via images. The AB-positive goblet cells were quantified by counting the positive cells per crypt in all crypts per colon section, three sections per mouse. The villus length was measured from the base of the villus to the top in Five to six villi in section, three sections per mouse.

2.6 Fecal bacterial DNA extraction and sequencing analysis

DNA from fecal samples was extracted by the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) as previously described (30). The V1–V2 16S rRNA hypervariable regions were amplified using the Bio-Rad C1000 Touch thermal cycler (Hercules, CA, USA) with primers containing a unique 10-base barcode to tag each PCR product. The PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then sequenced using the Ion Torrent PGM system (Thermo Scientific, DE, USA).

Sequences below 300 bp in length and low-quality score below 20 were removed. All effective sequences were clustered into operational taxonomic units (OTUs) using SILVA rRNA gene database (http://www.arb-silva.de) with a threshold of 97% sequence identity. The Quantitative Insights into Microbial Ecology (QIIME) software was used to select the representative reads of each OTU and to calculate beta diversity. The clustering pattern of the microbial composition was presented by principal coordinated analysis (PCA) using UniFrac distance matrices. The differences between groups in the taxa with varying abundances were sassed by using the linear discriminant analysis (LDA) effect size (LEfSe). In LEfSe, a size-effect threshold of 2.0 on the logarithmic linear discriminant analysis (LDA) score and an alpha value of 0.05 for the factorial Kruskal-Wallis test among the classes were used. The microbiota sequencing data have been deposited to the NCBI Sequence Read Archive (SRA) database under PRJNA885263. SPSS software (version 19.0) was used to assess the relationship strength between relative abundance and obesity-related parameters by the two-tailed Pearson's correlation test.

2.7 RNA extraction and real-time PCR

Total RNA form liver, adipose, colon, jejunum, and hypothalamus tissues were isolated with TRIsureTM reagent (Meridian Life Science Inc, TN, USA) following the manufacturer's protocol. The A260/280 ratio confirmed RNA purity. One μg of total RNA was reverse-transcribed with an AccuPower RT PreMix with oligo-(dT)18 primer (Bioneer, Daejeon, South Korea). Quantitative real-time PCR was performed using the LightCycler® 480 Real-Time PCR. System (Roche, Mannheim, Germany) and SYBR® Green real-time PCR kit (Toyobo, Tokyo, Japan) according to the manufacturer's instruction. The GAPDH gene was used as a reference. The sequences of primers were shown in the Supplementary Table (Table S1) in the Online Repository.

2.8 Statistical analyses

All data are presented as means ± standard error of the means (SEM). GraphPad Prism 8.0 software (San Diego, CA, USA) was used to determine the statistical significance. Data were analyzed by an unpaired two-tailed Student's t-test for a two-group comparison or one-way ANOVA with Bonferroni-corrected *post hoc* tests for multiple comparisons unless stated otherwise. P-values less than 0.05 was considered statistically significant.

3 Results

3.1 Effect of *F. prausnitzii* administration on body weight gain, fat mass, and calories intake *in vivo*

Mice fed a 12-week high-fat-diet showed a 2.9 times increase in body weight gain ($p < 0.01$), 17% increase in calorie intake ($p < 0.05$), and 2.35 times increase in energy efficiency ($p < 0.01$) compared to the normal diet group (Figures 1A–C). However, the body weight gain was markedly reduced by the microbial strains compared to HFD, with reduced rates of A2-165: 40%; DK3: 37%; DK9: 31%; DK11: 36%; YK1: 44%; XEN: 44% ($p < 0.05$ or $p < 0.01$, Figure 1B). Furthermore, all these treated groups showed a significant decrease in calories intake compared to the HFD group (Figure 1D). The increased liver weights by HFD feeding also resulted in significant reductions in the other treatment groups (Figure 1E). The total visceral fat mass weight of the HFD mice (Figure 1F) was 4.79 times greater than the normal group ($p < 0.01$). This effect was explained mainly by a reduction in mesenteric and subcutaneous fat upon treatment with all four selected pasteurized *F. prausnitzi*i strains: DK3, DK9, DK11, and YK1. By contrast, only DK3, DK11 and YK1 reduced the epididymal fat significantly (Figures 1G–I). In addition, the relative liver and epididymal weights did not show a significant difference among all groups, while other the tissue mass as relative to body weight also showed a similar pattern with absolute organ mass (Figure S2).

3.2 *F. prausnitzii* prevented obesity and improved glucose homeostasis in HFD-fed mice

The impact of the *F. prausnitzii* strains treatment on glucose homeostasis and insulin sensitivity in mice was determined by oral glucose tolerance tests (OGTT) (Figure 2A). HFD-fed mice showed the 54% higher in fasting glucose level ($p < 0.01$), the 69% higher in 30 minutes' glucose level ($p < 0.01$), and the 64% higher in the OGTT area under the curve (AUC) ($p < 0.01$) compared with normal low-fat diet food mice (Figures 2B–D). The fasting glucose level was changed significantly by the strains A2-165, DK3, and YK1 treatment with reduction rate 20%, 19%, 19% respectively ($p < 0.01$), while other treatment groups showed a slight reduction compared to the HFD group (Figure 2D). The oral gavage glucose solution at 30 minutes recorded the highest glucose level. The HFD group had a significantly higher level than the other groups, which was decreased significantly by all treatments, with the decrease rates of XEN:18%; A2-165%; DK3:18%; DK9:18%; DK11:23%; YK1:31% ($p < 0.05$ or $p < 0.01$) (Figure 2C). In the OGTT, the AUC values also showed upregulation by HFD feeding compared to the normal group, while it was reduced significantly by treatment with XEN, A2-165, DK11, and YK1, with decrease rates of 13%, 16%, 19%, 23% respectively ($p < 0.05$ or $p < 0.01$); the other treatment groups showed a slight decrease compared to HFD group (Figure 2B). The serum insulin concentration was elevated significantly in response to HFD feeding, while it was reduced markedly by the microbial strains and XEN treatment, with reduction rate as XEN: 33%; A2-165: 46%; DK3: 41%; DK9: 42%; DK11: 38%; YK1: 42% ($p < 0.05$ or $p < 0.01$) (Figure 2E). Homeostatic model assessment for insulin resistance index (HOMA-IR) showed a significant increase in the HFD group and a noticeable decrease in the treatment groups compared to the HFD group, with decrease rates as XEN:44%; A2-165:57%; DK3:49%; DK9:47%; DK11:39%; YK1:47% ($p<0.01$) (Figure 2F).

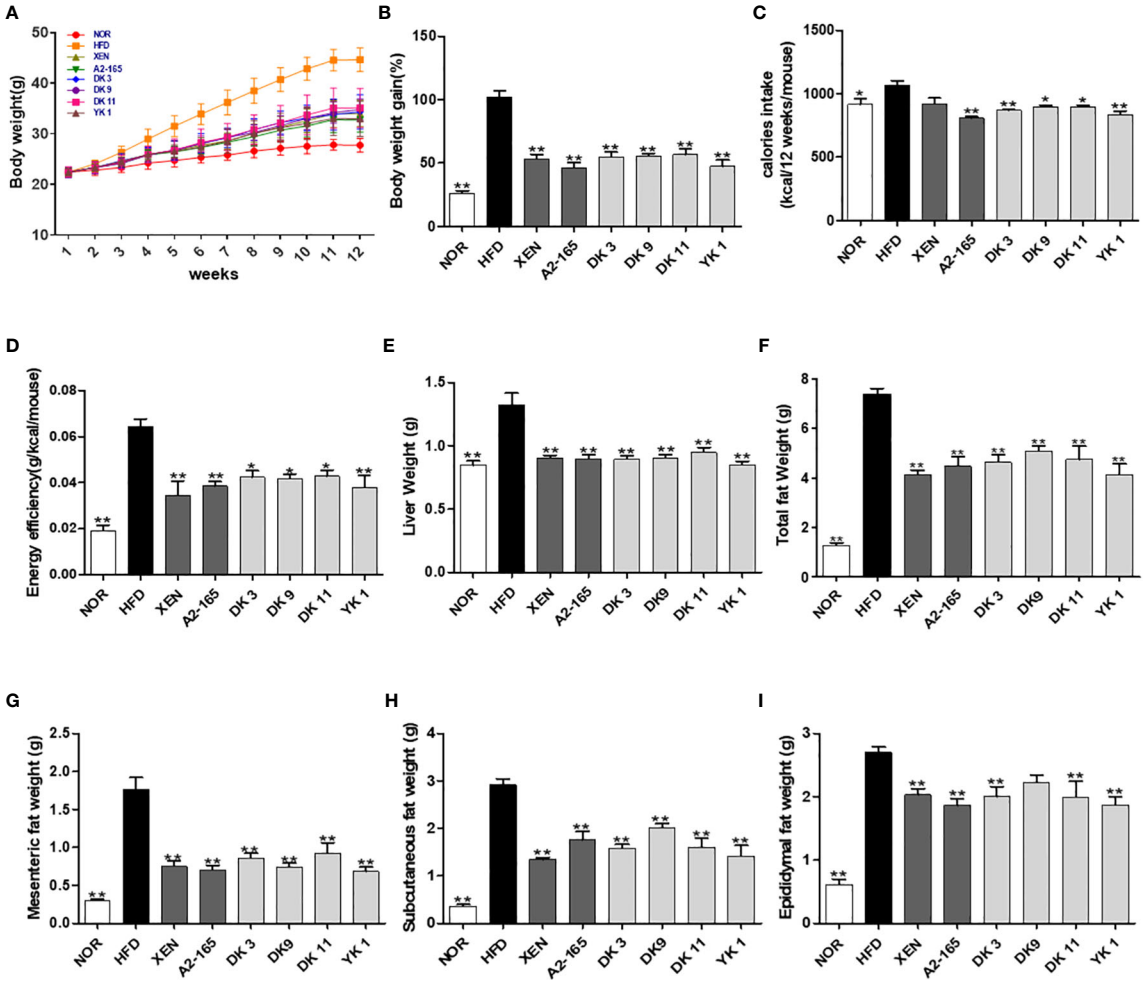

FIGURE 1
Effect of *F. prausnitzii* on the body weight, adiposity, organ weight, and caloric intake in mice. **(A)** Body weight measured weekly. **(B)** Total body weight gain. **(C)** The averaged calorie intake of each mouse and **(D)** energy efficiency (body weight gain/food intake). **(E)** Liver weight. **(F)** Representative weight of total fat (subcutaneous fat+epididymal fat+mesenteric fat), mesenteric fat **(G)**, subcutaneous fat **(H)**, and epididymal fat **(I)**. Data are represented as the mean ± SEM (n=9). The statistics were analyzed by one-way ANOVA. *p<0.05 and **p<0.01 versus the HFD group.

3.3 *F. prausnitzii* administration improved the lipid metabolism parameter of HFD mice

The obesity-associated serum biomarkers were further analyzed to investigate lipid metabolism in response to diet in HFD mice (Table 1). The total cholesterol (TC), triglyceride (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were increased significantly in the HFD group than in the normal group ($p < 0.05$ or $p < 0.01$). Moreover, the levels of TC all showed downward trends after treatment. Among them, the XEN and YK1 treatments caused a 22% and 28% reduction in TC levels compared to the HFD group, respectively ($p < 0.05$ or $p < 0.01$). In addition, the TG level showed similar results to the TC. The serum TG could be improved by all treatment ways and compared with the HFD group. The YK1 strain reduced the TG level by 42% compared to the HFD group ($p < 0.01$). All treatment groups could significantly improve the serum AST and ALT levels ($p < 0.05$ or $p < 0.01$), which are biomarkers of the hepatic function. These results showed that *F. prausnitzii* administration improved the lipid metabolism in serum.

3.4 *F. prausnitzii* administration reversed the HFD-induced effects on liver damage

For requirements for a specific article type please refer to the Article Types on any Frontiers journal page. Because the hepatic function is associated with HFD- induced obesity (31), the effects of *F. prausnitzii* supplement on HFD-induced hepatic function were next determined. Histological analysis of H&E staining and lipid staining on the liver sections (Figures 3A–D) confirmed the normal histology in normal low-fat diet-fed mice. In contrast, mice after 12 weeks of HFD developed a fatty liver phenotype, featuring a pale liver appearance caused by extensive fat accumulation, including cases of both macrovesicular and microvesicular steatosis. As expected, treatment with DK3, DK9, DK11, and YK1 resulted in

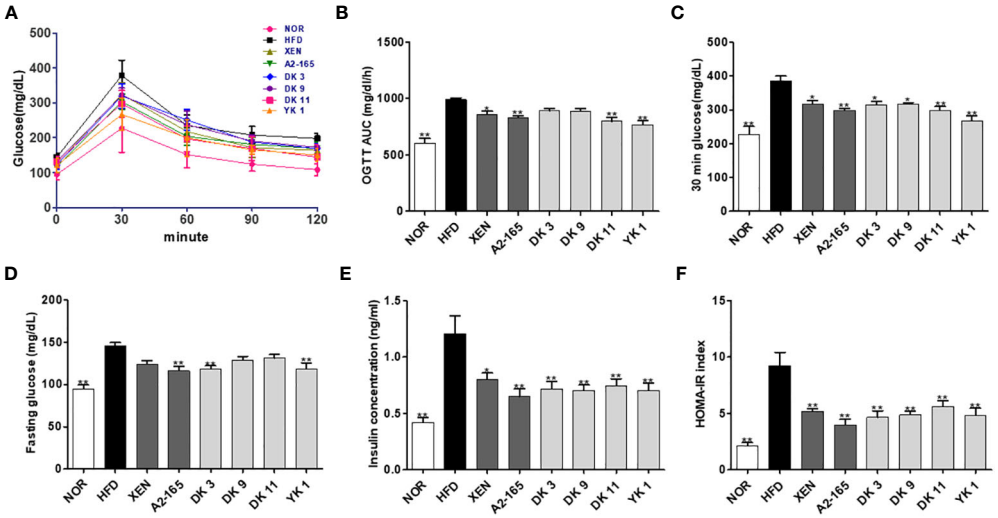

FIGURE 2
F. prausnitzii strains improved glucose homeostasis in HFD-induced obese mice. **(A)** Curve of oral glucose tolerance tests (OGTT). **(B)** Areas under the curve (AUC) of OGTT measured between 0 and 120min after glucose administration. **(C)** 30min glucose. **(D)** Fasting glucose. **(E)** Serum insulin and **(F)** HOMA-IR. Data are represented as the mean ± SEM (n=9). The statistics were analyzed by one-way ANOVA. *p<0.05 and **p<0.01 versus the HFD group.

markedly reduced vacuolation. The XEN and A2-165 groups also showed a slight improvement in fat accumulation and hepatic steatosis (Figures 3A, C). Moreover, the Oil Red O examination showed that the HFD-fed mice had 16.77 times more lipid droplets in the liver tissues compared to the normal group ($p < 0.01$). As shown in Figures 3B, D, the treatment groups showed obvious down-regulation of the lipid droplets compared to the HFD group, with reduction rates as XEN: 71%; A2-165: 72%; DK3: 64%; DK9: 66%; DK11: 70%; YK1: 73% ($p < 0.01$). In addition, the hepatic gene expression of ACC1, FAS, and SREBP1c in the normal group was significantly lower than in the HFD group ($p < 0.01$). In contrast, the expression of ACC1 mRNA was decreased significantly in the liver of the HFD-fed animals treated with all four selected pasteurized *F. prausnitzii* strains ($p < 0.01$). From the treatment, except DK9, other selected strains all significantly decreased the FAS and SREBP1c ($p < 0.01$). Among them, the hepatic expression of SREBP1c was significantly lower in the DK11 and YK1 groups than in the XEN group ($p < 0.05$) (Figure 3E).

On the other hand, the gene expression of (GLUT2), Glucose 6-phosphatase(G6Pase), and peroxisome proliferator-activated receptor-gamma (PPARγ) are associated with gluconeogenesis, but only G6Pase and PPARγ showed significant changes compared to the normal and HFD groups (Figure 3E). By treatment, in addition to DK3 and XEN, the other treatment methods upregulated the GLUT2 level markedly, but all treatments could inhibit G6Pase and PPARγ expression significantly. Consistent with the decrease in liver weight, the improvement of hepatic steatosis in histochemical and qPCR analysis showed that the *F. prausnitzii* treatment could ameliorate the hepatic steatosis and damage caused by the HFD and improve the hepatic function in mice.

3.5 Effect of *F. prausnitzii* administration on the adipokine profile of adipose tissue

For requirements for a specific article type please refer to the Article Types on any Frontiers journal page. Adipocyte hypertrophy is the major mechanism for the expansion of adipose tissue during the obesity development (32). To investigate the effects of *F. prausnitzii* administration on lipid accumulation in the adipose tissue of HFD-fed mice, three different parts of adipose tissue

TABLE 1 *F. prausnitzii* strains improved lipid metabolism parameter of the HFD mice.

Contents		NOR	HFD	XEN	A2-165	DK3	DK9	DK11	YK1
Serum	TC (mg/dL)	108.0 ± 17.63**	217.7 ± 9.45	170.0 ± 9.42**	175.6 ± 3.76**	182.0 ± 7.56*	182.7 ± 6.29**	189.7 ± 4.51*	157.8 ± 8.75**
	TG (mg/dL)	33.6 ± 4.71**	72.8 ± 10.86	58.0 ± 4.57	56.9 ± 6.53	60.9 ± 7.34	51.7 ± 4.69	56.6 ± 3.83*	42.56 ± 2.83*
	AST (IU/L)	33.9 ± 3.84*	52.3 ± 4.57	29.5 ± 3.53**	26.3 ± 4.66**	31.0 ± 4.61**	28.3 ± 2.04**	24.6 ± 2.42**	21.1 ± 1.43**
	ALT (IU/L)	9.1 ± 1.56*	17.3 ± 2.44**	7.2 ± 0.78**	7.9 ± 1.57**	8.8 ± 1.18**	8.2 ± 1.02**	9.4 ± 1.34**	5.1 ± 2.14**

The values are presented as the mean ± SEM. *p<0.05, **p<0.01 versus the HFD group; HFD, high-fat diet; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglyceride; TC, total cholesterol.

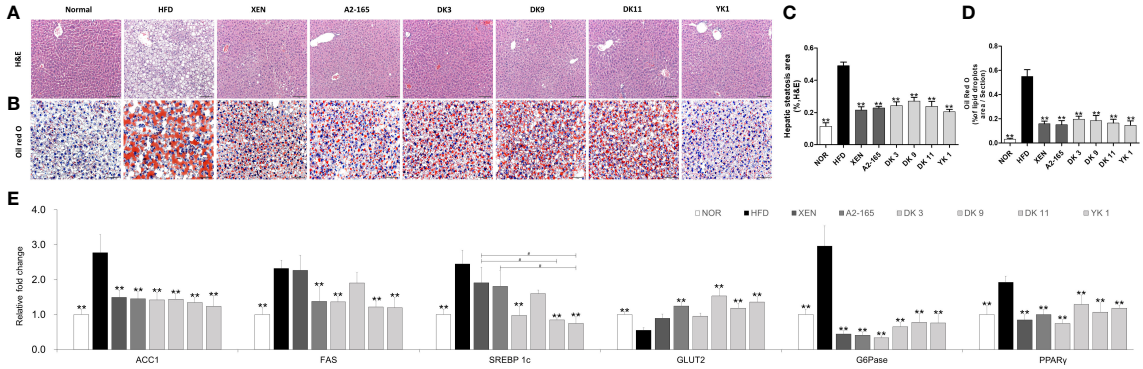

FIGURE 3
F. prausnitzii administration alleviated hepatic steatosis and improved hepatic function in mice. **(A)** Histological analysis of H&E stain on liver sections of mice (n=3 per condition, scale bar, 100μm). **(B)** Representative Oil Red O staining for fat deposition measurement in liver. **(C)** The liver steatosis area. The liver steatosis was characterized by micro- and macrovacuolization. **(D)** Oil Red O-stained fat deposition area. (n=3 per condition, scale bar, 50μm). Representative mRNA expression of **(E)** ACC1, FAS, SREBP1c, GLUT2, G6Pase, and PPARγ in liver tissue from mice (n=9 mice/group). Data are represented as mean ± SEM. The statistics were analyzed by one-way ANOVA. **p<0.01 versus the HFD group. #p<0.05.

samples were collected from the mice and the amount of fat was assessed. The fat mass weight and adipocytes size of mesenteric fat was examined by H&E staining.

As shown in the H&E staining pictures (Figure 4A), the results revealed up-regulation in the HFD group than the normal group of lipid accumulation in the mesenteric fat and a significant increase in the average fat cell size ($p < 0.01$). After treatment with different supplements, however, the fat cell size decreased significantly compared to the HFD group, with the reduction rates as XEN: 32%; A2-165: 27%; DK3: 36%; DK9: 33%; DK11: 35%; YK1: 39% ($p < 0.05$ or $p < 0.01$) (Figure 4B).

Obesity leads to adipose tissue dysfunction and vice versa (33). The expression levels of genes associated with lipogenesis or adipogenesis in mesenteric fat tissue were measured by RT-PCR to clarify the mechanism of the *F. prausnitzii* strains on HFD-induced obesity (Figure 4C). The expression levels of CD36 ($p < 0.01$), FAS ($p < 0.05$), and LDL ($p < 0.01$) mRNAs increased significantly after HFD feeding compared to the low-fat feeding group (normal group), but ACC1 showed an increasing trend in the HFD group. However, FAS and ACC1 levels were markedly reversed after all ways of treatments ($p < 0.01$). The selected strains, DK3 ($p < 0.01$), DK9 ($p < 0.01$), and YK1 ($p < 0.01$), resulted in markedly reduced CD36 mRNA expression, and DK3 ($p < 0.01$) and YK1 ($p < 0.01$) produced a significant decrease in the LDL levels. In addition, after the different treatments, the levels of the glucose and lipid metabolism regulation marker (aP2 and AKT), insulin-related markers (Adiponectin and IRS-1), and leptin, a hormone that facilitates the regulation of feeding and energy homeostasis (Figure 4C). A definite, insignificantly lower AKT, IRS-1, and Adiponectin mRNA levels were observed in the HFD groups compared to the normal groups. In contrast, the aP2 ($p < 0.01$) and leptin ($p < 0.01$) genes showed significantly higher expressions in the HFD group than in the normal group. After treatment, the mRNA levels of aP2 decreased ($p<0.05$ or $p<0.01$)., and except for the DK9 and DK11 groups, the leptin ($p < 0.01$) level also decreased significantly. Treatment with all four selected strains in the HFD groups, but not the other treatments, upregulated the expression of the Adiponectin gene significantly ($p < 0.05$ or $p < 0.01$). All the treatments except DK11 could increase the levels of AKT ($p < 0.05$ or $p < 0.01$), and the type strain A2-165

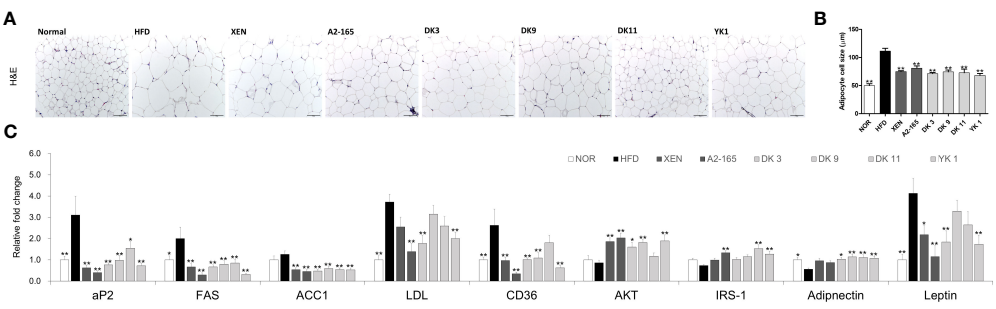

FIGURE 4
Effects of *F. prausnitzii* on adipokine profile of adipose tissue in HFD-fed mice. **(A)** Histological analysis (H&E staining) of sections of mesenteric fat tissues (n=3 per condition, scale bar, 100μm). **(B)** The average diameters of adipocytes in randomly chosen fields were measured and presented as pixels using Image-Pro Plus 6.0. **(C)** Ap2, FAS, ACC1, LDL, CD36, AKT, IRS-1, Adiponectin, and Leptin mRNA levels in adipose tissues (n=9). Data are represented as the mean ± SEM. The statistics were analyzed by one-way ANOVA or Student's t-test. *p<0.05 and **p<0.01 versus the HFD group.

($p < 0.01$), DK11 ($p < 0.01$), and YK1 ($p < 0.01$) could upregulate the IRS-1 mRNA expression level. Therefore, *F. prausnitzii* affects the adipose tissue metabolism.

3.6 *F. prausnitzii* treatment exerted anti-inflammatory effects in HFD-fed mice

The protective activity of pasteurized *F. prausnitzii* against HFD-induced inflammation in the colon was evaluated. This study examined the effects of *F. prausnitzii* in the HFD group on the gene expression of the following, which play vital roles in the inflammation and inflammatory signaling pathways: pro-inflammatory cytokines IL-1β, IL-6, and TNF-α; pro-inflammatory chemokine MCP-1; toll-like receptors TLR2 and TLR4, (Figures 5A, B). The colonic mRNA levels of IL-1β ($p < 0.01$), IL-6 ($p < 0.01$), TNF-α ($p < 0.05$), MCP-1 ($p < 0.01$), TLR2 ($p < 0.05$), and TLR4 ($p < 0.05$) were significantly higher in the HFD group than in the normal group. In particular, exposure of the HFD mice to all *F. prausnitzii* strains depleted the expression of IL-1β, IL-6, MCP-1, and TLR4 and suppressed the expression of the TNF-α gene in the colonic tissue, even though the latter change was insignificant. Treatment of the HFD-fed animals with XEN ($p < 0.01$), DK3 ($p < 0.01$), DK11 ($p < 0.01$), and YK1 ($p < 0.05$), but not the other treatments, decreased the expression of the TLR2 gene significantly.

3.7 *F. prausnitzii* treatment improved the intestinal barrier function in HFD-fed mice

Previously studies suggested that HFD feeding induced various dysbiosis of the intestines, which is associated with physiopathological changes, such as damaged mucus production and secretion, and injured the gut integrity and permeability of the intestinal epithelium (34). The intestine tissue was observed by AB staining to determine the effects of *F. prausnitzii* on the intestinal structure. As expected, HFD - induced obesity mice resulted in marked changes in the intestinal architecture (Figure 6A). In particular, a 55% decrease in the AB-stained area was observed in the HFD group compared with the normal group ($p < 0.01$), which is consistent with these results. The number of goblet cells ($p < 0.01$) and villus length ($p < 0.01$) were both lower than the normal group (Figures 6B–D). Nevertheless, the AB-positive area that represents the acidic mucins was improved markedly in four selected *F. prausnitzii* strain groups ($p < 0.05$ or $p < 0.01$) (Figure 6B). In support of the above results, a significant increase in the villus length was observed after treatment with A2-165 and all four selected strains compared to HFD control, with the increase rate as A2-165: 70%; DK3: 46%; DK9: 46%; DK11: 56%; YK1: 60% ($p < 0.05$ $p < 0.01$) (Figure 6D).

The *F. prausnitzii* supplement improved the mRNA expression of the intestine tissue and gut integrity (Figure 6E). Tight junctions connect the epithelial cells and play a key role in regulating the intestinal-barrier functionality (35). This study examined the expression levels of colonic mucin 2 (Muc2), ZO-1, Occludin, and JAM-A mRNAs in the intestine tissue by qRT-PCR. Compared to the untreated group (normal group), the mRNA expression of ZO-1 was inhibited significantly by HFD-feeding ($p < 0.05$). On the other hand, treatment of the HFD group with the XEN ($p < 0.05$), DK3 ($p < 0.05$), DK9 ($p < 0.05$), and DK11 ($p < 0.05$), but not A2-165 and YK1, increased the mRNA level of JAM-A significantly. The gene expression of occludin was upregulated significantly in the HFD group after treatment with DK9 ($p < 0.05$), DK11 ($p < 0.05$), and YK1 ($p < 0.05$) strains, but not the other regimens. Moreover, the gene expression of Muc2 was upregulated significantly by the oral DK9 ($p < 0.01$) and DK11 ($p < 0.01$) strains in the HFD-fed mice.

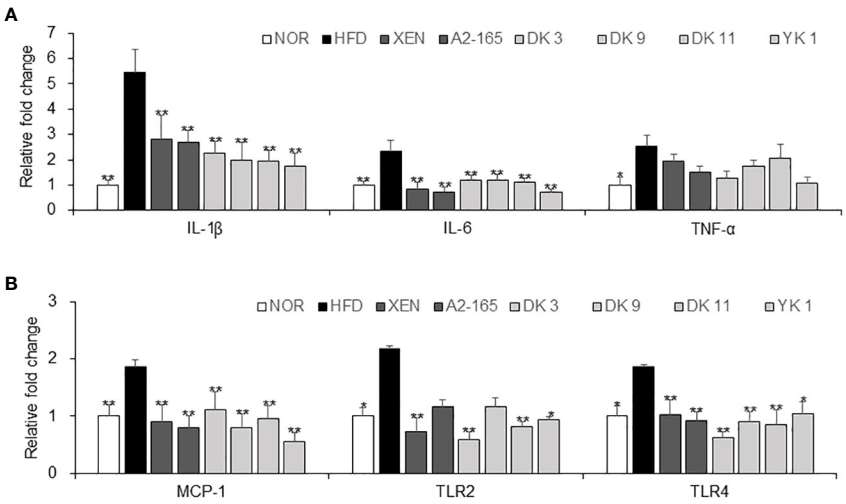

FIGURE 5
Anti-inflammatory effects of *F. prausnitzii* in the colon tissue. mRNA levels of inflammatory cytokines. **(A)** IL-1β, IL-6, and TNF-α, **(B)** MCP-1, TLR-2, and TLR4 in the colon tissue of each group were determined by real-time PCR. Data are represented as the mean ± SEM. The statistics were analyzed by one-way ANOVA or Student's t-test. *p<0.05 and **p<0.01 versus the HFD group.

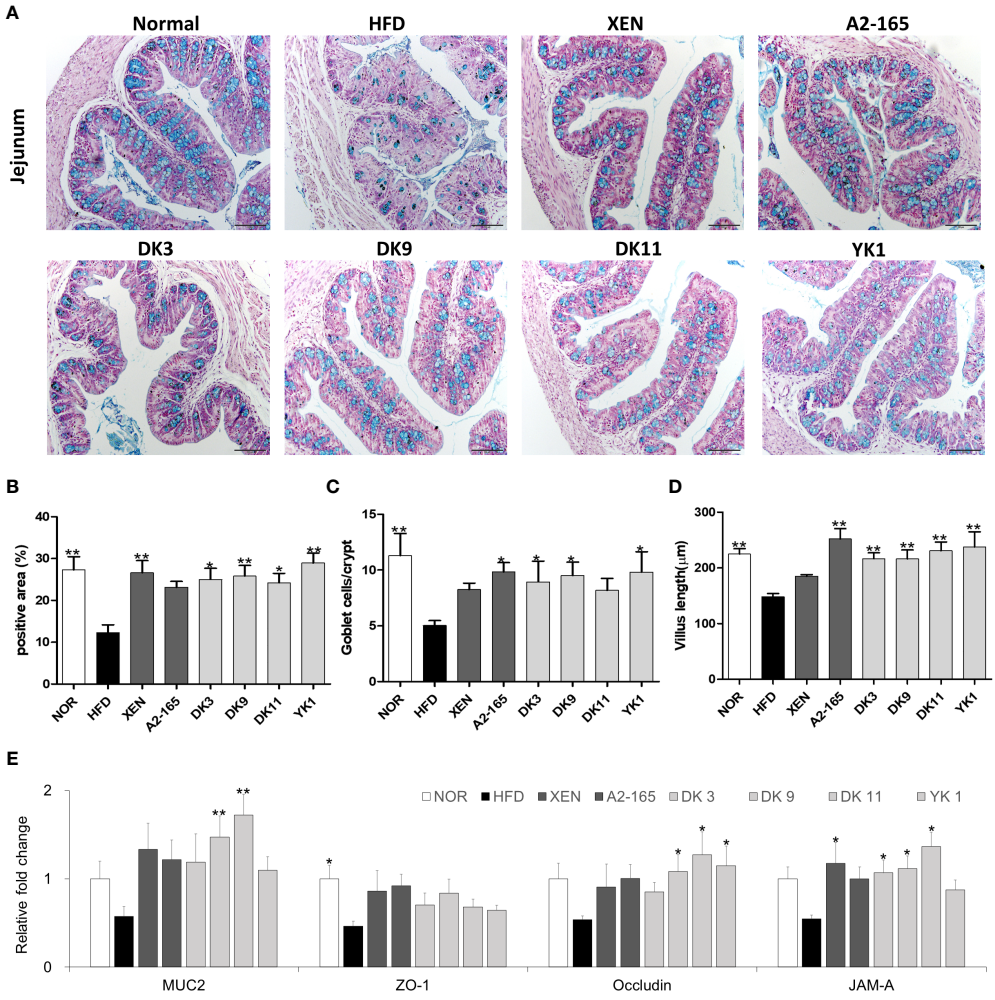

FIGURE 6
F. prausnitzii improved the functions and integrity of jejunum in HFD-fed mice. **(A)** Representative microscopic images demonstrating Alcian blue (AB)-staining of colonic tissue sections of mice from different experimental groups at a magnification of 200×, **(B)** the proportion of AB-positive area (%), **(C)** number of goblet cells, and **(D)** length of the villus in colonic tissue sections (n=3 per condition, scale bar, 100μm). Representative mRNA expression of **(E)** Muc2, ZO-1, Occludin, and JAM-A in intestine tissue from mice (n=9 mice/group). The statistics were analyzed by one-way ANOVA. *p<0.05 and **p<0.01 versus the HFD group.

3.8 *F. prausnitzii* modulated hormone secretion and regulated appetite by affecting the gut–brain axis

For requirements for a specific article type please refer to the Article Types on any Frontiers journal page. The gut–brain axis reflects the interactions between the gastrointestinal system and the brain in general. The brain receives both neural and endocrine inputs from the gut in response to food intake, which is integrated with signals from other organs to orchestrate physiological responses (26). Major integrating centers within the brain are the hypothalamic nuclei. Compared with the HFD-fed mice, food intake was decreased significantly by the *F. prausnitzii* strains, with the reduction rates as A2-165: 24%; DK3: 18%; DK9: 16%; DK11: 16%; YK1: 22%, ($p < 0.05$ or $p < 0.01$) (Figure S3). In this study, the hypothalamus, colonic, and small intestine tissue were used to provide evidence of gut-brain cross-talk involved in regulating food intake. Compared to the normal low-fat diet group, the gene expression of PYY ($p<0.05$), GPR120 ($p<0.05$), and GPR41 ($p<0.05$) of colonic tissues was markedly lower in the HFD group in response to feeding HFD. On the other hand, treatment of the HFD-fed animals with all the *F. prausnitzii* strains and XEN elevated the mRNA level of PYY significantly ($p < 0.05$ or $p < 0.01$). Similar to PYY, except for DK3, the other treatments could increase the GPR120 mRNA levels significantly ($p < 0.05$ or $p < 0.01$). Exposing the HFD group to type strain A2-165 and DK11, but not other treatments, upregulated the expression of the GLP-1 ($p < 0.05$) and GPR43 ($p < 0.05$) genes significantly. After treatment with DK9 ($p < 0.05$), the mRNA levels of GPR41 were also upregulated markedly compared to the HFD group (Figure 7A). In addition, the small intestine mRNA levels of CCK ($p < 0.01$) and PYY ($p < 0.05$) were significantly lower in the HFD group than in the normal group. In contrast, the gene expression of GIP ($p < 0.01$) and ghrelin ($p < 0.01$) was significantly higher in the HFD group than in the normal group. Exposure of the HFD group to all *F. prausnitzii* strains upregulated the mRNA level of CCK

markedly ($p < 0.05$ or $p < 0.01$). In contrast, the treatment of the HFD-fed mice with the *F. prausnitzii* strains except YK1 depleted the expression of Ghrelin genes significantly ($p < 0.05$ or $p < 0.01$). The small intestine expression of the PYY gene in the HFD group was upregulated significantly by XEN, A2-165, DK3, and DK11 ($p < 0.05$ or $p < 0.01$). Treatment of HFD-fed mice with A2-165 and YK1, but not other treatments, increased the CCK1R ($p < 0.05$) gene expression, DK3 and YK1 increased the GIP ($p<0.01$) gene expression compared with HFD group (Figure 7B). Furthermore, the *F. prausnitzii* supplement modulated the appetite-related hormone mRNA expression in the hypothalamus (Figures 7C, D).

The gene expression levels of GHSR, NPY, Leptin R, 5-HT1A, and AgRP in the hypothalamus were significantly higher in the HFD group than in the normal group ($p < 0.05$ or $p < 0.01$). On the other hand, treatment of the HFD group with XEN and the *F. prausnitzii* strains decreased the mRNA levels of GHSR, NPY, Leptin R, and 5-HT1A ($p < 0.05$ or $p < 0.01$). The gene expression of AgRP was down regulated significantly in the HFD group upon treatment with type train A2-165 ($p < 0.05$) and YK1 ($p < 0.01$), but not the other regimens. Significantly lower expression of the GIPR ($p < 0.05$) and POMC ($p < 0.01$) genes was observed in the hypothalamus of the HFD group vs. the normal group. A definite but insignificantly higher mRNA levels of Cart, 5-HT1B, and

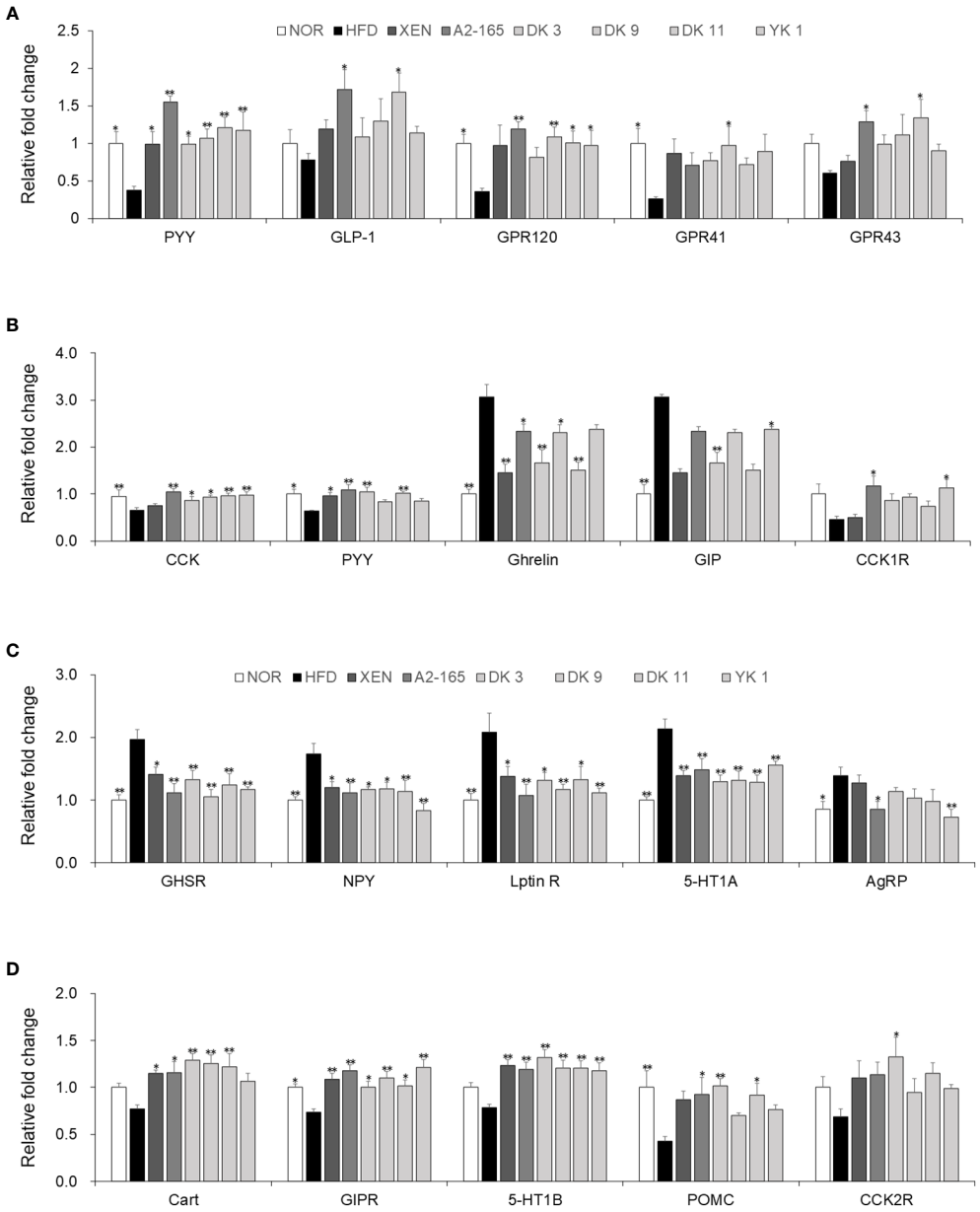

FIGURE 7
F. prausnitzii modulated hormone secretion and regulated appetite in HFD-fed mice. **(A)** PYY, GLP-1, GPR120, GPR41, and GPR43 mRNA levels in colonic tissues (n=9). **(B)** CCK, PYY, Ghrelin, GIP, and CCK1R mRNA levels in jejunum tissues (n=9). Representative mRNA expression of **(C)** GHSR, NPY, Leptin R, 5-HT1A, and AgRP and **(D)** Cart, GIPR, 5-HT1B, POMC, and CCK2R in hypothalamus tissue from mice (n=9 mice/group). The statistics were analyzed by one-way ANOVA. *p<0.05 and **p<0.01 versus the HFD group.

CCK2R were observed in the HFD group than in the normal group. The mRNA levels of GIPR ($p < 0.05$ or $p < 0.01$) and 5-HT1B ($p<0.05$) were increased significantly in the hypothalamus of the HFD-fed mice by all treatment ways. Cart gene expression in the HFD-fed mice was also upregulated significantly upon treatment with XEN and F. prausnitzii strains, except for the YK1 strain ($p < 0.05$ or $p < 0.01$). The POMC mRNA level in the HFD group was increased markedly by A2-165 ($p<0.05$), DK3 ($p<0.01$), and DK11 ($p < 0.05$). In addition, the expression of the CCK2R gene was increased significantly by the DK3 ($p < 0.05$) strain, but not the other treatments.

3.9 *F. prausnitzii* treatment modulated the intestinal microbiota in HFD-induced obesity mice

Several recent studies have provided significant evidence to reveal a strong association between obesity and gut microbiota (36). 16S rRNA gene analysis of fecal samples from the mice was performed to explore changes to the gut microbiota after *F. prausnitzii* administration on HFD-fed obesity mice. The bacterial composition was examined by taxonomy-based analysis. The results showed that compared to normal low-fat diet food mice, HFD induced a significant change in the populations of the intestinal microbiota (Figure 8).

First, principal component analysis (PCA) was performed. Figures 8A–D shows the PCA results of the different treated groups. The results showed the different clustering positions of the mice within and across different treatment ways. The fecal samples of the mice with HFD clustered together, whereas most of the samples from the normal chow-diet-fed mice comprised another group. This result indicates that HFD feeding could induce differences in the gut microbiota compared to the normal group. In particular, distinct segregation of the gut microbial communities was observed in the DK3, DK9, DK11, and YK1 groups compared to the normal and HFD groups (Figures 8A–D). Unlike with four *F. prausnitzii* groups, the gut microbiota communities of A2-165 were not significantly different with normal and HFD groups (Figure S4).

Further analysis of the 16S rRNA sequencing data showed that the relative abundance of the gut microbial taxa differed among the experimental groups. The relative abundance of DK3, DK9, DK11, and YK1 was assessed at the genus level to identify the specific taxa

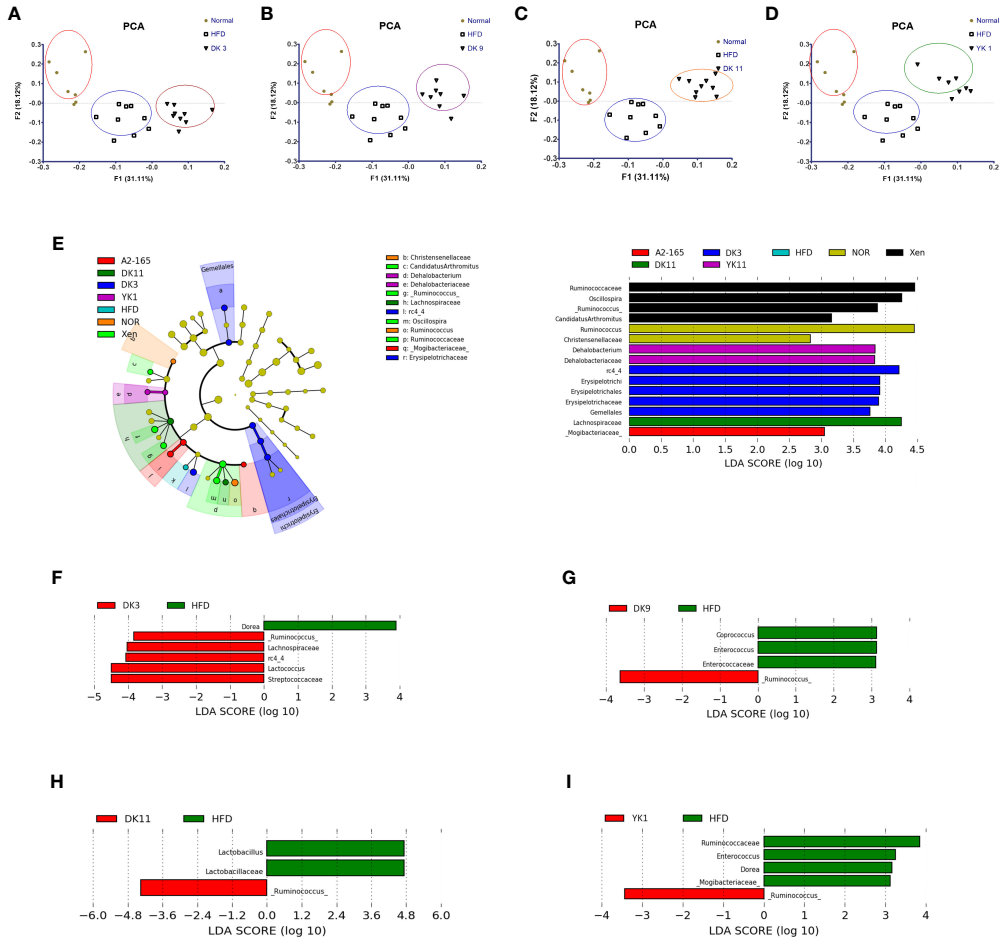

FIGURE 8
Effects of *F. prausnitzii* strains on gut microbiota composition. The gut microbiota was determined by 16S rRNA gene analysis of fecal samples from the mice. **(A–D)** Principal component analysis (PCA) of gut microbiota metagenomic samples. **(E)** Cladogram generated by the LEfSe analysis. The LEfSe plot shows enriched bacteria in all phenotypic categories. **(F–I)** LEfSe was used to identify bacteria represented differentially between DK3 and HFD **(F)**, DK9 and HFD **(G)**, DK11 and HFD **(H)**, and YK1 and HFD **(I)**. Only taxa meeting an LDA score threshold of two are listed.

related to selected strains supplementation (Figure S5). At the genus level, the relative abundances of *Ruminococcus* and *Lactococcus* were suppressed significantly in the HFD group compared to the normal chow diet group ($p < 0.01$). The *F. prausnitzii* strains could increase the abundance of *Ruminococcus, rc4-4, Parabacteroides, Lactococcus*, and *Bacteroides* significantly ($p < 0.05$ or $p < 0.01$). Furthermore, the increased proportions of sequences assigned to *Enterococcus* were significantly observed in the HFD group ($p<0.05$). In addition to *Dehalobacterium*, all the treatment groups significantly suppressed the increase in *Enterococcus* and *Coprococcus* ($p<0.01$). DK3, DK9, and DK11 markedly decreased the *Dehalobacterium* abundance ($p<0.05$). Overall, these results showed that administration of the selected strains *F. prausnitzii* could modulate the changes in these relative abundances of the gut microbiota induced by HFD-induced obesity.

3.10 Correlation between gut microbiota and obesity–related parameters

A correlation matrix was established using Pearson's correlation coefficient to completely investigate the relationships between levels of obesity biomarkers and abundance of gut microbiota (Figure 9). Intriguingly *Oscillospira* was positively correlated with body weight, fat weight, glucose metabolism related parameters, whereas *ruminococcus* showed negative correlation. On the other hand, *Lactobacillus* and *Dehalobacterium* were positively related with steatosis area (%) and Oil Red O-stained area (%) in liver, adipocyte cell size (μm) in adipose. Furthermore, it was indicated that rc4-4 shows a significant positive association with PYY levels in both colon and small intestine tissues, as well as a negative correlation with serum TC levels. Additionally, rc4-4 showed significant negative correlations with FAS and ACC1 levels in adipose tissues, and TLR2 levels in the colon.

4 Discussion

Obesity is linked to a number of complications, including specific anomalies in metabolism, such as hepatic steatosis, insulin resistance, hyperlipidemia, and some cancers (37, 38). Recent evidence has shown that probiotics have the potential to reduce obesity and improve metabolic parameters (39). *F. prausnitzii* is a major member of the gut microbiota in healthy adults. Accumulating evidence supports the health-promoting effects of *F. prausnitzii* (40), e.g., several metabolic-related parameters were improved in overweight and obese human subjects (22). A recent research suggested that the quantity of fecal *F. prausnitzii* is low in obese mice, and the abundance of *F. prausnitzii* could be increased by anti-obesity agents (41). Chronic HFD intake leads to obesity and hyperinsulinemia, hyperglycemia, and hypertension in rodents (42). Therefore, the HFD-induced mice model of obesity was used to compare the effect of pre-selected strains of *F. prausnitzii*.

In comparison to the normal group, the HFD group considerably outperformed it in terms of body weight gain, energy efficiency, calorie intake, weights of subcutaneous fat, epididymal fat, and mesenteric fat, as well as serum levels of TG and TC. The selected strains of *F. prausnitzii*, DK3, DK9, DK11, and YK1, reduced the body, liver, fat weight, and caloric intake compared to the HFD-fed animals. The fat reduction and lean mass increase showed that the *F. prausnitzii* treatment altered the body composition. In particular, only YK1 decreased the TC and TG serum levels compared to the other strains. Fat loss is related directly to glucose tolerance and insulin resistance (9). The four *F. prausnitzii* strains improved serum insulin and HOMA-IR. Moreover, DK11 and YK1 dramatically ameliorated the glucose tolerance rather than other strains. Therefore, these findings suggest that the HFD-induced obesity and associated metabolic abnormalities were improved by the selected *F. prausnitzii* strains.

The hepatic injury is associated with HFD-induced obesity (43). Probiotics have been found in numerous trials to enhance liver function (44). The disturbance of the glucose and lipid metabolism in the liver is linked to the pathogenesis of obesity, hepatic steatosis, and whole-body insulin resistance. FAS, ACC, and SREBP1 play essential roles in lipogenesis. FAS in the liver is a part of the lipogenic pathway (45), and SREBP1 mediates the induction of hepatic lipogenesis by insulin and glucose alteration (46). The *F. prausnitzi*i strains suppress hepatic lipogenic gene and protein

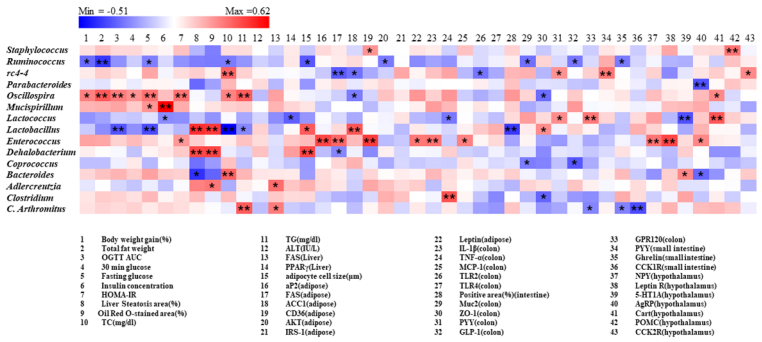

FIGURE 9
Correlation between gut microbiota and obesity related parameters. Pearson correlation results were used for heatmap establishment, with red indicating a positive correlation and blue indicating a negative correlation. "*" Denotes adjusted $p<0.05$ and "**" adjusted $p<0.01$.

expression, including FAS, ACC1, and SREBP1. These results are in accordance with the findings on biochemical index and histopathological staining. In particular, SREBP1c was significantly lower in DK11 than in the XEN group. In contrast, this value was much lower in the YK1 group than in the XEN and A2-165 groups. GLUT2 is the main glucose transporter in the hepatocytes of rodents and humans (47). As a direct target of PPARγ, hepatic GLUT2 aids in the transport of glucose into the liver (48). *Khiet Y. Trinh* et al. reported that increased-hepatic gluconeogenesis in obesity and T2DM is associated with insulin resistance, and the up-regulation of glucose-6-phosphatase (G6Pase) is crucial for the hepatic synthesis of glucose (49). Moreover, this study showed that *F. prausnitzii* stains, particularly DK9, reverse the levels of gluconeogenesis markers affected by the HFD treatment. Concomitantly with these data, a significant increase in liver weight and histological changes Page 13in hepatosteatosis was found, including hepatic steatosis area Page 13and marked oil droplets in HFD treated mice. In contrast, the *F. prausnitzii* strains reversed these elevations in keeping with the result from the mRNA levels of lipogenesis and gluconeogenesis markers.

Obesity leads to adipocyte hypertrophy and adipose tissue dysfunction. Adipocyte hypertrophy is the major mechanism for expanding the adipose tissue, and adipose tissue dysfunction leads to obesity (50). The size of the adipocytes and the adipose mRNA expression of the genes associated with insulin resistance were measured to clarify the mechanism of the *F. prausnitzii* strains on HFD-induced obesity. Adipocyte hypertrophy was identified as a major cause of adipose tissue expansion (51). The current investigation discovered that the *F. prausnitzii* strains decreased adipocyte size significantly in mesenteric fat tissue. Some studies have suggested that adipogenesis and lipolysis are complex processes involving several transcription factors (52), such as adipogenic differentiation-related genes aP2, lipogenesis genes FAS, ACC1, LDL, and insulin metabolism-related genes CD36, AKT, IRS, adiponectin, and leptin (53–56). In addition, the expression of the aP2, FAS, LDL, CD36, and leptin genes in the HFD-diet group was significantly higher than in the normal group. By contrast, the adiponectin mRNA level was lower than the normal group. aP2 is a lipid-binding protein that is upregulated during adipocyte differentiation, and the levels of circulating aP2 are dramatically elevated in dietary and genetic models of obesity (53). PCR showed that aP2 gene expression of adipose tissues is reduced by the treatment of *F. prausnitzii* in mice versus the HFD control. In the present study, gene over-expression of the lipogenesis markers, such as FAS, ACC1, and LDL, were significantly reduced by the *F. prausnitzii* strains in the adipose tissue. Therefore, *F. prausnitzii* strains potentially inhibited lipogenesis in adipose tissue, decreasing adipose accumulation in mice, which is consistent with fat mass reduction.

Leptin is mainly produced by adipose tissues, leading to insulin resistance development (57). CD36 is also associated with insulin resistance via modulation of lipid uptake (54). Of note, a significant expression reduction in the insulin resistance-related genes involved in *F. prausnitzii*-treated mice in accordance with the result of HOMA-IR. Furthermore, IRS-1 expression was decreased in type 2 diabetes and obesity subjects, and low IRS-1 expression causes a decrease in insulin-stimulated glucose uptake (58). Inhibition of Akt may cause insulin resistance because Akt is a major regulator of insulin action in muscle, fat, and liver (55). Adiponectin has a direct insulin-sensitizing action (59). Moreover, replenishing adiponectin reduced the insulin resistance and hypertriglyceridemia brought on by the HFD (56). Therefore, the reversal of the markers mentioned above by the *F. prausnitzii* strains inhibited adipogenic differentiation and lipogenesis and improved insulin resistance.

Increased levels of inflammatory cytokines contribute to the development of insulin resistance and obesity because this is linked to low-grade chronic inflammation (60). Previous studies reported that *F. prausnitzii* regulates the anti-inflammatory and pro-inflammatory cytokines to improve the inflammatory environment in the intestine (61). Pro-inflammatory cytokines such IL-1, IL-6, TNF-α, pro-inflammatory chemokine MCP-1, and toll-like receptors (TLR2 and TLR4) may be produced in different intestinal regions as a result of an HFD. These ultimately brought on the low-grade inflammation linked to metabolic diseases like insulin resistance, obesity, and others (39). Consistent with these findings, MCP-1, TNF-α, IL-6, IL-1β, TLR2, and TLR4 gene expression levels were substantially greater in the HFD group than in the normal group. Intestinal epithelial TLR signaling has a unique pathogenic function, according to *Everard* et al. TLRs abnormalities induce dysbiosis and predispose the individual to metabolic diseases (62). Therefore, TLRs play a crucial role in preserving intestinal and microbial homeostasis and contribute to the bowel inflammation brought on by the HFD and the ensuing metabolic abnormalities. The TLR2 and TLR4 gene expression was measured in the intestinal tissues to determine if *F. prausnitzii* affects the intestinal epithelial TLR signaling pathway. Based on the results, *F. prausnitzii* down regulated the gene expression of intestinal TLR2 and TLR4, which modulated the innate immunity of the intestinal epithelial cells. In accordance with these findings, these results demonstrated that the four *F. prausnitzii* strains had the ability to decrease the intestinal gene expression of IL-1, IL-6, and MCP-1.

The protective activity against an HFD-induced insult to intestinal integrity was evaluated because of the selected *F. prausnitzii* strains have anti-inflammatory characteristics. The anti-inflammatory process restored the gut barrier integrity in diet-induced obesity mice (63). AB staining of the mice intestine indicated that *F. prausnitzii* strains restored the damaged gut integrity caused by HFD. Goblet cells respond to microbial products to bolster the mucosal defense, which plays a key role in the host immunity and secretes mucin (64). Laura Wrzosek et al. reported that *F. prausnitzii* improves the establishment of epithelial homeostasis by modifying goblet cells and mucin glycosylation (65). The results showed that *F. prausnitzii* strains-increased goblet cells produce more mucin, thickening the intestinal barrier. The tight junction proteins modulate the intestinal permeability, including ZOs, JAMs, occludin, and claudins. These junction proteins, which firmly join epithelial cells, are essential for controlling the effectiveness of the intestinal barrier (66). Previous studies suggested that HFD feeding induced various intestinal

physiopathological dysbioses, such as alteration of the gut integrity and intestinal permeability (67). These results showed that *F. prausnitzii* is important in improving the gut barrier function. The data suggested that it could be a key mechanism through which *F. prausnitzii* restored gut barrier function and lessened gut permeability. In general, the gut-brain axis reflects the interaction that is present between the gastrointestinal system and the brain. In response to food consumption, the brain gets neuronal and endocrine inputs from the gut, which are combined with signals from other organs to coordinate physiological responses (26). The hypothalamic nuclei are major brain integrating centers. In this study, the hypothalamus, colonic and small intestine tissue were used to provide evidence of the gut–brain cross talk involved in regulating food intake. PYY is a peptide that enteroendocrine L cells release and plays a role in controlling appetite. Evidence has shown that PYY may affect body weight by reducing appetite and raising energy expenditure (68). L cells in gastrointestinal produce the incretin hormone GLP-1, exerts many biological functions, such as inducing satiety and slowing gastric emptying. The L cells of distal gut release PYY and GLP-1 after nutrient ingestion to reduce appetite (69). This study showed that *F. prausnitzii* strains could modulate the gene expression of PYY and GLP-1 in the jejunum and colon. Recently, some studies reported that several orphan G protein-coupled receptors (GPCRs), GPR41, GPR43, and GPR120, possess potential as fresh drug targets for disorders of metabolism, including T2D and obesity (70). Intestinal GPR120 is widely expressed and mediates GLP-1 secretion (71). Through the gut-brain neurological network, GPR41 expression in intestinal L cells, which release GLP-1 and PYY, also improves insulin sensitivity (70). GRP43 regulates appetite and PYY secretion and is expressed in enteroendocrine L cells (72). All selected strains of *F. prausnitzii* were used to treat the HFD-fed mice, and this treatment increased the mRNA levels of GPR41, GPR43 and GPR120 thereby affecting hormone secretion in the colon. According to reports, *F. prausnitzii* is one of the main butyrate producers in the intestine (73). Interestingly, when the butyrate binds to its receptors, GPR41 and GPR43, on L cells promote the synthesis of PYY and GLP-1 (74, 75).

Cholecystokinin (CCK) is a brain–gut peptide (76). The postprandial inhibition of stomach emptying and inhibition of colonic transit are the CCK motor effects. At the same time, CCK can activate direct vagal afferent fibers and modify the vagal mechanosensitive fibers to gastric and duodenal loads response properties (77). Currently study, a significant downregulation of the small intestine gene expression of CCK and CCK1R was found in HFD-fed mice. In addition, exposure of the HFD group to YK1 increased the mRNA levels of GIP in the jejunum, which can interact with GLP-1 to regulate energy absorption and food intake (78). In contrast, administration of DK3 and DK11 to the HFD group reduced the ghrelin gene expression in the jejunum. As seen in the current investigation, reversing the production of the aforementioned brain-gut peptide in the HFD-fed mice with *F. prausnitzii* supplementation may enhance ghrelin secretion and suppress appetite.

Furthermore, the *F. prausnitzii* strains modulated the mRNA expression of hormone secretion in the hypothalamus, and evidence of the gut–brain cross talk in controlling food intake was found. GHSR is a specific receptor of Ghrelin and exerts several physiological effects (79). In addition, NPY/AgRP neurons were activated by ghrelin (80). Thus, the levels of Ghrelin in the jejunum, as well as GHSR, NPY, and AgRP in the hypothalamus, are reduced significantly after administering the *F. prausnitzii* strains. The endogenous hypothalamic serotonin (5-HT) has been reported to be linked with the processes of satiety during meals and the final stage of post-meal satiety (81). In the present study, the *F. prausnitzii* strains modulated the mRNA expression of the 5-HT1A and 5-HT1B in the hypothalamus, which are the most directly 5-HT receptors implicated in feeding control. GIPR is expressed in the hypothalamus, which helps control food intake. Hypothalamic $Gipr^{+}$ neuron activation inhibits food intake in mice. In addition, the brain as a target organ is based on the GIP pharmacology (82). In the present study, *F. prausnitzii* strains increased the gene expression of GIPR in the hypothalamus and GIP in the jejunum. Hence, *F. prausnitzii* strains can simultaneously affect the secretion of appetite-related hormones in the small intestine and hypothalamus. Furthermore, the HFD group's exposure to the *F. prausnitzii* strains improved the expression of the Cart and POMC genes, which are anorexia peptides in hypothalamus.

Numerous evidence suggests a close association between obesity and gut microbiota, whereas probiotic therapy reduces obesity and intestinal dysbiosis by regulating the gut microbiota (39). 16S rRNA sequencing analysis of fecal samples was performed to investigate whether and how *F. prausnitzii* strains influence the gut microbiota. PCA analysis showed that *F. prausnitzii* affects the composition of the gut microbiota underwent clear modifications in the gut microbial structure compared to HFD-diet mice. As expected, *F. prausnitzii* affects the gut microbiota composition by increasing the presence of genus *Ruminococcus*, *rc4-4*, *Parabacteroides*, *Lactococcus*, and *Bacteroides* while decreasing *Dehalobacterium*, *Enterococcus*, and *Coprococcus* in HFD-fed mice. Evidence suggests that *Oscillospira* is associated with leanness or lower body mass index (BMI) for both adults and children. The increased abundance of *Oscillospira* was recently found to correlate with the health subjects (83). By supplementing *F. prausnitzii*, the *Oscillospira* abundance was increased significantly in the HFD group, which may explain part of the beneficial effects of the *F. prausnitzii* strains. As common probiotics, *Lactococcus* spp. are used to improve human and animal health. Recent studies have linked *Lactococcus lactis* to insulin resistance and systemic inflammation, exerting an anti-obesity effect (84). Herein, a higher abundance of *Lactococcus* was found in all *F. prausnitzii* strain treated groups than in the HFD group, which may assist in weight control. Gut *Parabacteroides* are important members of the human gut microbiota and have a lower abundance in those who have nonalcoholic fatty liver disease and obesity (85). *Parabacteroides* also has metabolic benefits in decreasing hepatic steatosis, hyperglycemia and weight gain (86). Obese individuals are associated with decreased *Bacteroides*, and an increased abundance of *Bacteroides* was recently found to correlate with benefits to health (35). In addition, the abundance of *Bacteroides* has been implicated in the appearance of diabetes-related auto-antibodies, according to

reports (87). YK1 strains increased the abundance of *Parabacteroides*, and YK1 and DK11 increased the *Bacteroides* abundance, suggesting that these strains alleviate hepatic steatosis and metabolic abnormal. *Dehalobacterium* showed a significant increase in HFD-induced obesity mice (88). Additionally, in a prior clinical investigation, compared to participants who were non-obese subjects, obese subjects had greater *Coprococcus* levels (89). In the present study, *F. prausnitzii* strains, DK3, DK9, and DK11, obviously changed the gut microbial communities at the genus level by decreasing the population of *Dehalobacterium* and *Coprococcus*. Previous studies have shown inconsistent results for the ratio of *Lactobacillus* and *F. prausnitzii*. For example, a recent study showed that consuming *Lactobacillus johnsonii La1* decreased the *F. prausnitzii* levels in healthy subjects (90). Meanwhile, potato fiber (FiberBind 400) improved Lactobacillus survival, while decreasing the abundance of *F. prausnitzii* (91). However, the Lactobacillus was increased while *F. prausnitzii* was decreased in the patients with inflammatory bowel disease as compared to healthy control (92). Taken together, these findings suggest that probiotics have strain-specific effects (61) and further research is needed to understand the underlying mechanisms.

While a prior study demonstrated that one strain of *F. prausnitzii* (ATCC 27766) has anti-obesity effects in the same animal model (22), our study used four different *F. prausnitzii* strains for a thorough effective comparison, all of which were newly isolated from the human gut. Even within the same species, different bacterial strains can exhibit varying degrees of differences in properties (93). In our previous study, we investigated how different strains of *Akkermansia muciniphila* (*A. muciniphila*) improve metabolic disorders in HFD-induced mice, and found that even within the same species, different bacterial strains can elicit different responses from the host (39). In the present study, the three *F. prausnitzii* strains increased the abundances of Ruminococcus, rc4-4, Lactococus and Oscillospira in different degrees, while DK11 decreased the relative abundance of Lactobacillus. The exact cause is still not fully clear, but differences in the gut microbiome emphasize the importance of our research on the distinctions between various strains. In addition, it has long been a conventional practice to study the interaction between microbes and hosts using mouse models. However, phylogenetic and metagenomics research has revealed that while mouse fecal, caecal, and human fecal samples have a large degree of overlap, they also differ significantly in abundance (94). Consequently, future studies should consider this limitation to accurately demonstrate the impact on humans.

It is worth noting that some specific gut microbiota is strongly correlated with obesity and its related metabolic disorders. For instance, *Oscillospira* exhibited a strong positive correlation with many obesity-related parameters, while the treatment with *F. prausnitzii* strains appeared to show a decreasing trend in its relative abundance. Rc4-4 displayed a strong negative correlation with the RNA expression of adipose FAS and ACC1 levels, as well as colon TLR2 levels. These correlation findings provide valuable insights into the associations between obesity biomarkers and the gut microbiota.

Overall, YK1 was found to be more effective in regulating glucose homeostasis, suppressing triglyceride levels, and inhibiting lipogenesis, as well as regulating hormone secretion in the hypothalamus. DK11 was found to have a better impact on glucose homeostasis, triglycerides, and the function of the intestinal barrier, as well as hormone secretion in the colon, but was less effective in suppressing lipogenesis compared to other strains. DK9 was found to have less efficiency in preventing liver damage, but had a greater impact on the intestinal tract. DK3 was found to have better effects in suppressing lipogenesis and regulating hormone secretion in the hypothalamus. Based on these results, it is speculated that YK1 and DK3 have a greater impact on the hypothalamus, with YK1 showing more significant inhibition of appetite-promoting hormones and DK3 releasing more appetite-suppressing hormones. Meanwhile, DK11 and DK9 have a more significant impact on the intestinal tract. DK11 may primarily affect hormone secretion in the colon and DK9 may primarily affect hormone secretion in the jejunum.

5 Conclusions

In conclusion, four novel human intestine-derived *F. prausnitzii* strains, DK3, DK9, DK11, and YK1, ameliorated HFD-induced obesity and related metabolic disorders and had better efficiency than type strain A2-165 and a comparison group XEN. These *F. prausnitzii* strains could inhibit low-grade inflammation, restore the gut integrity, improve hepatic injury and insulin resistance, modulate hormone secretion, regulate appetite, and inhibit lipogenesis by affecting the gut–brain axis. These observations show that four *F. prausnitzii* strains exerted functional differences surpassing the type strain. Moreover, *F. prausnitzii* makes a favorable contribution to the gut ecosystem due to a significant relationship between *F. prausnitzii* supplementation and gut microbiota composition. Therefore, all results provide a rationale for developing a treatment that uses different strains of *F. prausnitzii* to prevent or treat obesity and its associated metabolic disorders.

6 Institutional review board statement

This research was approved by the Institutional Review Board of Dongguk University (approval number: IRB# 2018-06-001-012).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA885263.

Ethics statement

The studies involving humans were approved by the Institutional Review Board of Dongguk University, Ilsan Hospital (IRB# 2018-06-001-012). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by All experiments were approved by the institutional animal care and use committee (IACUC) of the Dongguk University (2020-11208) and conducted according to the guidelines of the National Research Council (Guide for the Care and Use of Laboratory Animals, 2011). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

Conceptualization, HK and J-GS. Methodology, MY, Joo-HS, DL, and Ji-HS. Formal analysis, MY. Writing - original draft preparation, MY and J-HW. Visualization, MY and J-HW. Supervision, HK, JGS, XS, and Y-DN. Funding acquisition, HK, JGS, and Y-DN. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Faecalibacterium prausnitzii strains were obtained from Enterobiome Inc., and authors J-GS, J-HS 7th Author, S-NL, and DL are employees of Enterobiome Corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1220044/full#supplementary-material

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Intermuscular adipose tissue in obesity and related disorders: cellular origins, biological characteristics and regulatory mechanisms

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Intermuscular adipose tissue (IMAT) is a unique adipose depot interspersed between muscle fibers (myofibers) or muscle groups. Numerous studies have shown that IMAT is strongly associated with insulin resistance and muscular dysfunction in people with metabolic disease, such as obesity and type 2 diabetes. Moreover, IMAT aggravates obesity-related muscle metabolism disorders via secretory factors. Interestingly, researchers have discovered that intermuscular brown adipocytes in rodent models provide new hope for obesity treatment by acting on energy dissipation, which inspired researchers to explore the underlying regulation of IMAT formation. However, the molecular and cellular properties and regulatory processes of IMAT remain debated. Previous studies have suggested that muscle-derived stem/progenitor cells and other adipose tissue progenitors contribute to the development of IMAT. Adipocytes within IMAT exhibit features that are similar to either white adipocytes or uncoupling protein 1 (UCP1)-positive brown adipocytes. Additionally, given the heterogeneity of skeletal muscle, which comprises myofibers, satellite cells, and resident mesenchymal progenitors, it is plausible that interplay between these cellular components actively participate in the regulation of intermuscular adipogenesis. In this context, we review recent studies associated with IMAT to offer insights into the cellular origins, biological properties, and regulatory mechanisms of IMAT. Our aim is to provide novel ideas for the therapeutic strategy of IMAT and the development of new drugs targeting IMAT-related metabolic diseases.

KEYWORDS

intermuscular adipose tissue, obesity, insulin resistance, intermuscular adipogenesis, therapeutic strategy

1 Introduction

Obesity is associated with increased risks for diverse diseases, such as metabolic syndrome, type 2 diabetes, non-alcoholic fatty liver disease, and several cancers (1). Intermuscular adipose tissue (IMAT) is a unique adipose depot that expands between myofibers or adjacent muscle groups, which develops and progresses alongside the expansion of visceral and subcutaneous adipose tissue due to obesity (2). IMAT is distinct from the accumulation of lipids within myofibers, which is referred to as intramuscular lipids or intramyocellular lipids (3, 4). Imaging techniques have been increasingly used to noninvasively quantify IMAT, including computed tomography (CT) and magnetic resonance imaging (MRI) (5–7), and there is a good level agreement between IMAT assessment by MRI and histology (7). Several studies have suggested that IMAT poses a major threat to muscle metabolic disorders and physiological function, such as IR and muscle atrophy, in individuals with obesity, type 2 diabetes, and aging (5, 8, 9). Despite IMAT in the thigh being much less than subcutaneous adipose tissue (SCAT) in obese individuals, it is strongly correlated with IR (5). Additionally, IMAT in thigh muscle is independently associated with increased obesity-related heart failure risk after adjusting for cardiometabolic risk factors and other measurements of adiposity in humans (6). A separate study revealed that obesity-associated respiratory dysfunction in a mouse model was correlated with IMAT and collagen deposition within the diaphragm (10).

In recent years, several studies have suggested that IMAT adipocytes originate from muscle-resident stem/progenitor cells or other mesenchymal progenitors, resulting in the heterogeneity of intermuscular adipocytes with distinct metabolic characteristics (11, 12). For instance, human muscle-derived fibro/adipogenic progenitors (FAPs) *in vitro* give rise to white adipocytes that exhibit IR (13). Interestingly, one study reported the presence of brown progenitors in human skeletal muscle (14). Other researchers have demonstrated the existence of uncoupling protein 1 $(UCP1)^+$ brown adipocytes within IMAT in mice, providing a therapeutic target for obesity by acting on energy dissipation (15). These findings indicate that there is still ongoing debate regarding the cellular origins and metabolic properties of IMAT adipocytes.

Therefore, within this context, we will review recent studies to explain the cellular origins of IMAT adipocytes and regulatory mechanisms involved in intermuscular adipogenesis. This review aims to provide new insights and potential targets for addressing IMAT-related conditions such as obesity, type 2 diabetes, and related disorders.

2 Metabolic characteristics of IMAT

Multiple studies have shown that IMAT is a robust predictor of metabolic abnormalities, such as IR, in both younger and older adults (5, 16). Sachs et al. conducted the first direct sampling and analysis of IMAT in humans and they found that the conditioned media for cultivating IMAT obtained from obese individuals reduced the insulin sensitivity of myotube from donors *in vitro* (17). Similar to other adipose tissue depots, IMAT synthesizes and secretes various bioactive mediators, such as inflammatory cytokines, and extracellular matrix proteins, which can lead to local inflammation or systemic inflammation, ultimately leading to decreased insulin sensitivity in humans (2, 8, 17–19). Furthermore, Sachs et al. discovered that IMAT contains macrophages proportional to insulin sensitivity, and macrophage cytokine secretion within IMAT such as monocyte chemotactic protein 1 (MCP1), is negatively related to insulin sensitivity (17). In obese humans, macrophage and T cells markers were upregulated in skeletal muscle compared with lean humans (20). In addition, macrophages, T cells, and other immune cells that respond to skeletal muscle inflammation are mainly situated in IMAT in diet induced-obese mice (20). These macrophages exhibit polarization toward the proinflammatory M1 phenotype (20, 21), exacerbating skeletal muscle IR and metabolic disorders (Figure 1).

Due to its negative impact on whole-body metabolism, IMAT adipocytes have been extensively studied *in vivo* and *in vitro*. Multiple studies have shown that adipocytes derived from muscle-resident mesenchymal progenitors in IMAT share similar characteristics with white adipocytes (9, 10, 13, 22). Liu et al. discovered that muscle-derived non-Pax3 myogenic lineage cells differentiate into white-like adipocytes *in vitro* (23). Girousse et al. demonstrated that the mobilization of $CXCR4^+$ adipose stromal cells (ASCs) from SCAT toward skeletal muscle results in increased IMAT formation and subsequent impairment of glucose tolerance in mice (12).

Notably, different mouse strains exhibit differential susceptibility to diabetes and diet-induced obesity (15, 24). Almind et al. found that $UCP1^+$ brown adipocytes within IMAT are more prevalent in obesity-resistant 129S6/SvEvTac (Sv129) mice than in C57BL/6 (B6) mice (15). Gorski et al. further found that FAPs provide a likely source for intramuscular adipocytes expressing UCP1 in obesity-resistant Sv129 mice (25). Schulz et al. reported that a subpopulation of adipogenic cells residing in murine skeletal muscle can differentiate *in vitro* into brown-like adipocytes when stimulated with bone morphogenetic protein 7 (BMP7) (26). Similarly, Crisan et al. demonstrated the presence of brown progenitors in human skeletal muscle that can differentiate into brown adipocytes *in vitro*, and they also found increased expression of UCP1 mRNA in adult human skeletal muscle, which was further enhanced by PPARγ agonist treatment (14). Additionally, Liu et al. uncovered that transplantation of brown adipose progenitors into mouse skeletal muscles leads to ectopic adipose tissue formation (27). Moreover, induced brown adipose progenitors can develop into brown adipocytes in mouse muscles, resulting in increased energy expenditure (27). Cai et al. demonstrated that transplanted brown adipose tissue (BAT) into the *quadriceps femoris* muscle of *ob/ob* mice significantly improved glucose homeostasis, alleviated obesity, and exhibited brown adipocyte characteristics (28), indicating that skeletal muscle could provide a microenvironment for brown adipogenesis.

In summary, despite the detrimental effects of IMAT on metabolism, the presence of brown adipocytes within IMAT offers a potential avenue for treating obesity (Table 1). The

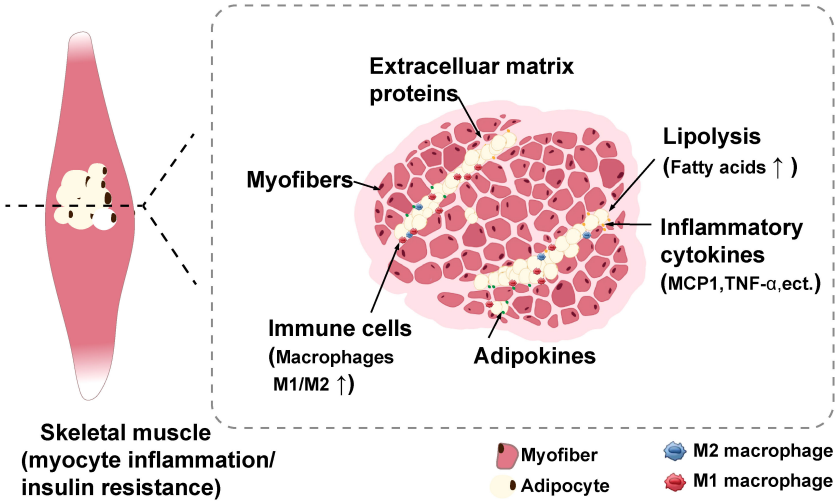

FIGURE 1
Schematic with potential mechanisms of myocyte inflammation and insulin resistance induced by IMAT (2). IMAT can synthesize and secrete numerous bioactive mediators such as inflammatory cytokines, adipokines, and extracellular matrix proteins to impart adverse effects, such as myocyte inflammation and insulin resistance. Moreover, these secretory inflammatory factors induces the recruitment of immune cells, particularly macrophages, which primarily infiltrate IMAT. IMAT, intermuscular adipose tissue. Created with BioRender.com.

skeletal muscle microenvironment provides for maintaining intermuscular brown adipogenesis, offering a promising therapeutic strategy for IMAT-related morbid obesity and diabetes.

3 Cellular origins of IMAT

Based on previous studies, it has been indicated that muscle-resident stem/progenitor cells and other adipose tissue depot progenitors are potentially involved in the formation of adipocytes within IMAT (11, 12, 15, 35).

3.1 Muscle satellite cells

Multipotent SCs have the ability to differentiate into adipocytes, ultimately contributing to IMAT formation (29, 30, 40). In the absence of the myogenic transcription factor MyoD/Myf5, myoblasts derived from SCs undergo adipogenic or osteogenic differentiation (29). De Coppi et al. suggested that human SCs marked with $CD44^{+}CD56^{+}HLA\text{-}ABC^{+}$ could differentiate into adipocytes when treated with rosiglitazone *in vitro* (30). Previous researches based on lineage tracing experiments have indicated that brown adipocytes can arise from Pax7/Myf5-expressing precursors in skeletal muscle (41–43). Seale et al. demonstrated that overexpression of *Prdm16* in myoblasts induces their differentiation into brown adipocytes *in vitro* (42). Yin and colleagues illustrated that the muscle-enriched microRNA-133 represses brown adipogenesis in skeletal muscle by targeting *Prdm16* mRNA in mice (43). Furthermore, Pasut et al. discovered that overexpression of the Notch1 intracellular domain (NICD1) in the *Pax7*-deficient SCs repressed both MyoD and microRNA-133, leading to brown adipocytes formation in regeneration muscle in mice (31). Thus, the regulation of satellite cell-derived brown adipocyte generation, targeting PRDM16 and microRNA-133, presents a crucial therapeutic target for combating obesity.

3.2 Fibro/adipogenic progenitors

Muscle-resident mesenchymal progenitors, specifically FAPs, are characterized by positive expression of platelet—derived growth factor receptor alpha (PDGFRα) and stem cell antigen-1 (Sca-1). These cells possess the ability to proliferate and differentiate into adipocytes (10, 11, 32, 34, 39). Camps et al. uncovered the presence of an interstitial $CD142^{-}$ cell subpopulation within the $Sca\text{-}1^{+}PDGFR\alpha^{+}$ population that undergoes adipogenic differentiation in skeletal muscle. They also discovered that the $CD142^{+}$ cell population could inhibit adipogenesis by secreting growth differentiation factor 10 (GDF10) (35). Arrighi et al. confirmed that the $PDGFR\alpha^{+}CD56^{-}$ muscle progenitors are identical to the $CD56^{-}CD15^{+}$ progenitors (13). Furthermore, they uncovered that adipocytes derived from FAPs exhibit a deficiency in UCP1 expression in both young and adult donors, and these adipocytes are insulin-resistant (13). Laurens et al. indicated that the $CD56^{-}CD15^{+}$ cell subpopulation isolated from the muscle of obese subjects differentiated into functional white adipocytes *in vitro*, which impaired insulin action and myofiber signaling (22). Collectively, the abovementioned studies suggest that FAPs and other $PDGFR\alpha^{+}$ progenitors have the potential for adipogenic differentiation.

3.3 Other muscle mesenchymal progenitors

Nonetheless, a subset of myeloid-derived cells characterized by $PDGFR\alpha^{-}CD68^{+}$ exhibited adipogenic potential (36). Lu et al. uncovered that PDGFRβ lineage cells from muscles undergo a fate

TABLE 1 The cellular origins and characteristics of IMAT adipocytes.

References	Cellular origins	Cellular marker	Study model	Cellular/metabolic characteristics
Asakura et al.(2001) (29)	Satellite cells	$MyoD^-Myf5^-$	Cell differentiation; *in vitro*	NA/NA
De Coppi et al.(2006) (30)	Human Satellite cells	$CD44^+CD56^+HLA\text{-}ABC^+CD3^-CD4^-CD45^-CD31^-$	Cell differentiation (with Rosiglitazone); *in vitro*	White adipocyte/NA
Pasut et al.(2016) (31)	Satellite cells	$Pax7^-$	Muscle regeneration; *in vivo*	$PRDM16^+$Brown adipocytes/ NA
Almind et al. (2007) (15)	Muscle resident cells	NA	Obesity (129S6/SvEvTac mice); *in vivo*	$UCP1^+$adipocytes/positive
Crisan et al. (2008) (14)	Human muscle cells	$CD45^-CD56^-CD146^-CD34^+$	Muscle cells/mice/human (with Rosiglitazone); *in vitro* and *vivo*	$UCP1^+$brown adipocytes/ positive
Uezumi et al. (2010) (32)	Mesenchymal progenitor	$CD31^-CD45^-SM/C\text{-}2.6^-$ $PDGFR\alpha^+$	Glycerol-induced fatty degeneration; *in vivo*	NA/negative
Laurens et al.(2010) (33)	Muscle-derived cells	$CD34^+CD56^-$	Human muscle cells/mice (glycerol-induced injury); *in vitro* and *vivo*	NA/negative
Schulz et al.(2011) (26)	Muscle resident progenitors (ScaPCs)	$Sca\text{-}1^+/CD45^-/Mac1^-$	Human or obesity- mice muscle cells; *in vitro*	Like-brown adipocyte/positive
Uezumi et al.(2014) (34)	Mesenchymal progenitor	$PDGFR\alpha^+$	Human skeletal muscle disease; *in vivo*	NA/negative
Laurens et al. (2016) (22)	Human muscle stroma-vascular fraction (SVF)	$CD56^-CD15^+$	Cell differentiation; *in vitro*	White adipocyte/negative
Camps et al.(2020) (35)	Interstitial cell	$Sca1^+PDGFR\alpha^+CD142^-$	Muscular dystrophy; *in vivo*	NA/negative
Xu et al. (2021) (36)	Myeloid-derived cells	$Pdgfra^+$; $Pdgfra^-/Cd68^+$	Glycerol-induced injury; *in vivo*	NA/NA
Lu et al. (2022) (37)	Muscle progenitors	$PDGFR\beta^+$	Aging; *in vivo*	NA/negative
Joe et al. (2010) (11)	FAPs	$Sca1^+CD34^+CD31^-$ $CD45^-\alpha7integrin^-$	Injury; *in vivo*	NA/positive
Arrighi et al.(2015) (13)	FAPs	$PDGFR\alpha^+CD15^+CD56^-$	Young and adult human muscle cells; *in vitro*	White adipocyte/negative
Buras et al.(2018) (10)	FAPs	$CD31^-CD45^-Sca1^+PDGFR\alpha^+$	Obesity mice; *in vivo*	Some $UCP1^+$; many $UCP1^-$ cells/negative
Farup et al.(2021) (38)	FAPs	$CD34^+$ $CD90^+$ $CD56^-$ $CD31^-$ $CD45^-$	Type 2 diabetic patients muscle cells; *in vitro*	NA/negative
Hogarth et al. (2019) (39)	FAPs	$Sca1^+PDGFR\alpha^+$	Muscular dystrophy/injury; *in vivo*	NA/negative
Girousse et al.(2019) (12)	Adipose Stromal Cells	$CXCR4^+$	Diet-induced obesity; *in vivo*	White adipocyte/negative
Liu et al. (2019) (27)	Brown adipose progenitors (BAPCs)	NA	Transplantation with or without VEGF; *in vivo*	$UCP1^+$ adipocytes/positive

NA, not available.

transition, contributing to the infiltration of adipose and fibrotic tissues in old mice (37). Studies found that a population of muscle cells expressing the surface protein CD34 can differentiate into adipocytes *in vitro* (14, 33). Liu et al. showed that muscle-derived $Pax3^-$ non-myogenic lineage cells differentiate into white-like adipocytes without UCP1 expression *in vitro* (23). These findings suggest that muscle-resident progenitor cells also have the potential for adipogenic differentiation under certain conditions.

3.4 Adipose stromal cells or progenitors

In addition to muscle-resident stem/progenitors, adipose stromal cells (ASCs) from SCAT can be released into circulation under the regulation of the chemokine CXCL12 and its receptor CXCR4 in mice (44). Girousse et al. demonstrated that $CXCR4^+$ ASCs released from SCAT, upon exposure to a high-fat diet or CXCR4 antagonist directly promoted ectopic adipocyte formation

in the muscle of mice, and subsequently impaired glucose tolerance in mice (12). In addition, one study has shown that there is a reservoir of brown progenitors, that is muscle cells expressing CD34, in human skeletal muscle, which can differentiate into brown adipocyte with a high level of UCP1 *in vitro* (14). Moreover, induced brown adipose progenitors can develop into brown adipocytes in the limb muscles of mice (27).

Unlike classical adipose tissue depots, IMAT adipocytes exhibit heterogeneity, which may be attributed to their potential stem/progenitor cell origins (Figure 2). The characteristics of adipogenic progenitors of IMAT adipocytes play a crucial role in determining the metabolic traits of adipocytes in IMAT, thereby impacting whole-body metabolism (Table 1).

4 Regulatory mechanisms of intermuscular adipogenesis

4.1 Transcriptional regulators

Similar to classical adipose depots, intermuscular adipogenesis is regulated by a complex transcriptional cascade network that involves CCAAT enhancer-binding protein (C/EBP) family proteins, peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulatory element-binding protein isoform 1c (SREBP1c), and fatty acid-binding protein (FABP4) (45, 46).

Apart from classical transcription regulators, Krüppel-like factor (KLF) family proteins play crucial roles in the differentiation of preadipocytes in livestock animals by interacting with C/EBPs and PPARγ. For instance, KLF4 inhibits the adipogenic differentiation of goat intermuscular preadipocytes *in vitro* by targeting C/EBPβ (47). KLF2 and KLF9 negatively regulate intermuscular adipogenesis (48, 49). KLF6 was the target gene of miR-22-3p and acted as an "on/off" switch in the differentiation of FAPs into adipocytes or myofibroblasts by regulating the matrix metallopeptidase 14 (MMP14) both *in vitro* and *in vivo* (50).

Fibroblast growth factors (FGFs) could also be potent regulators of adipogenesis in skeletal muscle. Basic FGF and FGF1 promote the differentiation of intramuscular adipocytes by regulating the expression of C/EBPα and PPARγ (51, 52). Sebastian et al. found that the conserved FGF2 increased IMAT formation in aged human skeletal muscle by inhibiting the adipogenic inhibitor SPARC (53). FGF21 negatively regulates the adipogenic differentiation of goat intermuscular preadipocytes *in vitro* by downregulating the expression of PPARγ and regulating the expression of numerous KLFs, including KLF3, 7, 9, 11, 14, and 16 (54).

4.2 Key signaling molecules

Previous studies have shown that multiple classical signaling pathways, such as the Hedgehog (Hh), Wnt, and Notch signaling pathways, can regulate IMAT formation in mouse models (55–57). Kopinke et al. demonstrated that Hh signals inhibit adipogenesis by regulating the expression of tissue inhibitors of metalloproteinase 3 (TIMP3) and MMP14 in a mouse model of injury-induced

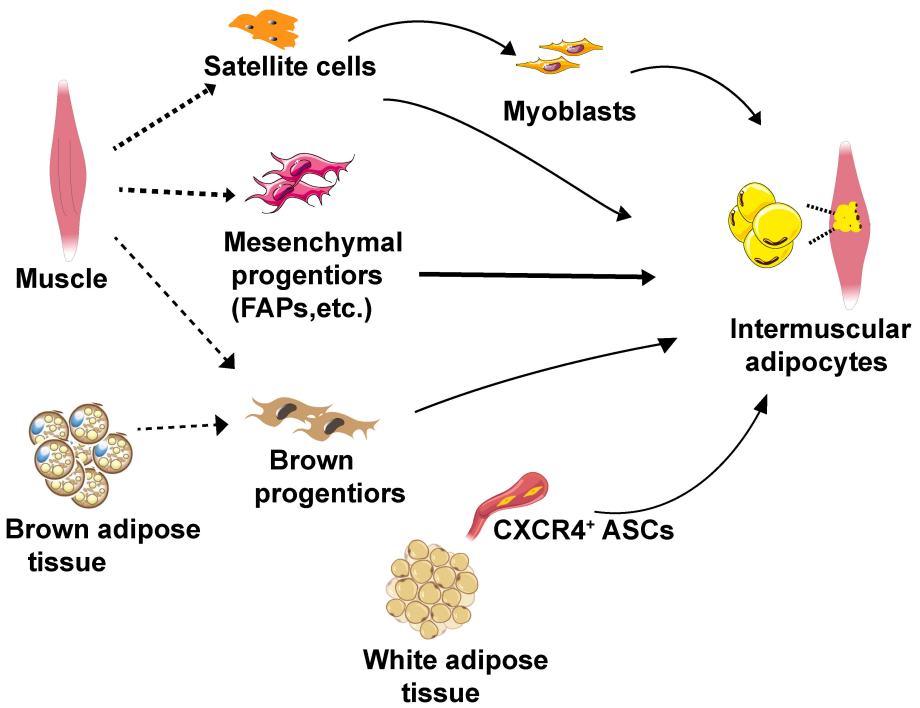

FIGURE 2
Schematic diagram of the cellular origins of IMAT adipocytes. IMAT adipocytes can potentially originate from muscle satellite cells, muscle-resident mesenchymal progenitors (specifically FAPs), CXCR4$^+$ ASCs and brown progenitors. IMAT, intermuscular adipose tissue. FAPs, fibro/adipogenic progenitors. ASCs, adipose stromal cells. CXCR4, chemokine CXCL12 receptor.

regeneration (55). Furthermore, other researchers uncovered a specific group of FAPs that were marked with glioma-associated oncogene homolog 1 (Gli1), which exhibited elevated Hh signaling and diminished adipogenic capability in a mouse model of muscle injury (58).

Wnt signals can act as a molecular switch controlling adipogenesis (59, 60). It has been suggested that Wnt10b inhibits adipogenesis by inhibiting PPARγ (60). Deceased Wnt10b signaling in myoblasts during aging induced adipose tissue infiltration in muscle (61). Similar results were observed in muscle SCs from obese Zucker rats (62). Reggio et al. identified Wnt5a as a noncanonical Wnt ligand that affects FAP adipogenesis by repressing PPARγ expression *in vitro* in a β-catenin-dependent manner (57). Brack and colleagues have shown that in a mouse model, the transition from Notch to Wnt signaling in myogenic progenitors is essential for effective muscle regeneration via glycogen synthase kinase 3 beta (GSK3β) (63). These findings potentially elucidate why, despite restoring the proliferative potential of Pax7$^{-/-}$ SCs, NICD1 causes differentiation into brown adipose tissue (31). In addition to its effect on SC fate, Marinkovic et al. observed that myotubes inhibit FAP adipogenesis via Notch signaling *in vitro* (56). They further demonstrated that synergistic cooperation between Notch and inflammatory signals inhibits adipogenic differentiation in *mdx* FAPs (56).

In addition, Li et al. found that HMG20A exerts inhibitory effects on adipogenesis in porcine muscle SVFs and C3H10T1/2 cells through its interaction with lysine-specific demethylase 1 (LSD1) (64). Mozzetta et al. found that histone deacetylase inhibitors (HDACis) repressed the adipogenic potential of FAPs and enhanced the myogenic differentiation of SCs in young dystrophic mice but not in old *mdx* mice (65). Moreover, Wosczyna et al. uncovered that miR-206 repressed the adipogenic differentiation of FAPs by targeting Runx1 to limit intramuscular fatty degeneration in mice injured muscle (66).

To summarize, exploring innovative approaches to modulate the destiny of intermuscular preadipocytes or FAPs to inhibit intermuscular adipogenesis will be beneficial for controlling IMAT formation in pathological conditions.

4.3 The impact of the skeletal muscle microenvironment on IMAT formation

Skeletal muscle is a complex and plastic tissue, which includes myofibers, SCs, FAPs, immune cells, endothelial cells (67). The interactions between muscle-resident cells and paracrine signals from the microenvironment regulate the expansion and differentiation of adipogenic progenitors, thereby controlling the development of IMAT.

4.3.1 The roles of myofibers in IMAT information

Previous studies demonstrated that the condition of myofibers affects IMAT accumulation (68, 69). Uezumi et al. found that myofibers strongly inhibit the adipogenic differentiation of PDGFRα^{+} cells in injured muscle in mice (32). Other studies showed variations in the adipogenic potential of preadipocytes in different muscles (23, 70). Liu et al. found that compared to the fast *extensor digitorum longus* (EDL) muscle, slow *soleus* (SOL) muscle contains more adipogenic progenitors in mice and these progenitors from SOL exhibits a higher propensity to form adipocytes *in vitro* (23), with the EDL muscle primarily consisting 80% type IIx and IIb fibers (glycolytic fibers) and the SOL muscle consisting 95% type I and IIa fibers (oxidative fibers) (71). In addition, Gu et al. showed that skeletal muscle-specific overexpression of PPARγ could significantly promote intramuscular fat deposition in the *longissimus dorsi* muscle but not in the *soleus* muscle in pigs (72). They further showed that overexpression of PPARγ in porcine muscle promotes the formation of slow oxidative fibers (72). These findings imply that myofiber type plays an important role in regulating intermuscular adipogenesis.

Studies have revealed that skeletal muscle-derived exosomes encapsulate the different myomiRs involved in local skeletal muscle tissue communication (73). They also found that the levels of these myomiRs within exosomes vary between skeletal muscles with different muscle fiber-type compositions (73). Chemello and colleagues showed differential expression profiles of microRNAs such as miR-206 and miR-499 between fast and slow myofibers (74). Wosczyna et al. uncovered that the adipogenic differentiation of FAPs was abrogated by miR-206 by repressing Runx1 translation in mice (66). Jiang et al. suggested that miR-499 hindered SCs adipogenic differentiation by reducing PRDM16 *in vitro* (75). Based on previous studies, we speculate that myofibers play a regulatory role in intermuscular adipogenesis.

4.3.2 Myokines regulate intermuscular adipogenesis

Skeletal muscle, as a secretory organ, secretes bioactive myokines, including myostatin (MSTN), IL-15, irisin and IL-6, which likely exert both local (paracrine) and long-range (endocrine) effects. Studies have highlighted the potential roles of myokines in mediating tissue crosstalk and modulating the process of intermuscular adipogenesis.

MSTN, a member of the TGF-β superfamily, is a secreted protein that is specifically expressed in skeletal muscle, and is associated with myogenesis and adipogenesis in muscle development and regeneration (76, 77). Reisz-Porszasz et al. observed that transgenic mice overexpressing *Mstn* in skeletal muscle exhibited reduced muscle mass and increased fat mass (78). Lin et al. showed that increased muscle development in *Mstn* knockout (KO) mice may be associated with reduced adipogenesis (79). Artaza et al. demonstrated that recombinant MSTN promotes the differentiation of C3H10T (1/2) multipotent mesenchymal cells into the adipogenic lineage while inhibiting myogenesis *in vitro* (76). Additionally, Feldman and colleagues showed that MSTN can serve as a substitute for dexamethasone in inducing adipogenesis in C3H10T (1/2) cells but not in 3T3-L1 preadipocytes, which indicates that MSTN plays a role in promoting adipogenesis in the specific early stage (77). It should be noted that the adipocytes induced by MSTN in cell cultures and

transgenic mice revealed the expression of markers associated with immature adipocytes, which exhibit favorable metabolic effects (77). However, inconsistent with previous findings, Liu et al. reported that the activated myostatin/SMAD4 signal promotes the expression of miR-124-3p, and inhibits adipogenesis by downregulating the expression of glucocorticoid receptor (GR) in porcine preadipocytes (80). Sun et al. suggested that MSTN inhibits intramuscular preadipocyte adipogenesis in a dose-dependent manner *in vitro* (81). Furthermore, they discovered that the culture supernatant from muscle tissue inhibits adipogenic differentiation of intermuscular preadipocytes *in vitro*. Zhang et al. indicated that MSTN inhibits the adipogenic differentiation of muscle SCs but not adipose-derived stem cells (82). Interestingly, Babcock and colleagues observed that the expression of MSTN and its receptor, activin receptor IIB (actRIIB), varied among different myofiber types in rat (83). They suggested that MSTN and actRIIB expression tends to be higher on IIx and IIb myofibers (I < IIa < IIx < IIb). Thus, depending on the context, MSTN can exhibit a dual role in the regulation of adipogenesis in skeletal muscle, either by inhibiting or promoting it.

IL-15 is a significant factor secreted by muscle fibers. It has been shown to inhibit the differentiation of porcine preadipocytes, specifically in the *longissimus dorsi* muscle, by suppressing the proliferation of preadipocytes in a dose-dependent manner *in vitro* (84). Another study revealed that the expression of IL-15 is negatively associated with fatty infiltration in injured human muscle (85). This study also found that IL-15 can stimulate the proliferation of FAPs and prevent the adipogenic differentiation of FAPs in injured muscle in mice (85). In addition, other myokines such as irisin (86, 87), IL-6 (88) and myonectin (89) also play an important role in the regulation of adipogenesis. These myokines are released by skeletal muscle in response to exercise and nutrients, suggesting that they may serve as potential therapeutic options for inhibiting IMAT accumulation.

4.4 Impact of trace elements on intermuscular adipogenesis

Dietary supplementation with trace elements, including vitamins and minerals, has the potential to regulate intermuscular adipogenesis by interacting with various regulatory factors.

4.4.1 Vitamin A and retinoic acid signaling

Previous studies have shown that RA, an active metabolite of vitamin A, is a nutritional regulator of adipose tissue biology (90, 91). Berry et al. found that RA inhibits adipocyte differentiation *in vitro* by upregulating the expression of the adipogenesis inhibitors Pref-1, Sox9, and KLF2, and suppresses diet-induced obesity in mice (91). Zhao and colleagues demonstrated that RA effectively suppresses adipogenesis of FAPs in a dose-dependent manner *in vitro* (92). RA supplementation proves to be beneficial for obesity-impaired muscle regeneration by inhibiting both adipogenic and fibrotic differentiation of FAPs in mice (92). However, other researchers showed that neonatal supplementation with vitamin A leads to an increase in intramuscular fat levels without increasing overall fat levels (93). Their findings revealed that RA promotes angiogenesis and increases the number of intramuscular PDGFRα$^+$ adipose progenitors *in vivo*, which subsequently leads to adipogenesis of intramuscular stromal vascular cells (SVCs) by activating VEGFA/VEGFR2 signaling (93). Therefore, during the early stage of IMAT development, changes in the muscle that impact extracellular matrix remodeling, along with the process of angiogenesis play a critical role (93, 94). Of note, it has also been shown that RA enhances adipocyte formation during the early stage but inhibits adipocyte hypertrophy at the terminal stage (93). While RA signaling inhibits white adipogenesis in murine cells through epigenetically inhibiting Zfp423 expression (95), it tends to downregulate ZFP423 in cattle SVCs, which aligns with the observation that RA downregulates the expression of adipogenic genes *C/EBPα* and *PPARγ* (93).

4.4.2 Vitamin D

Studies have suggested a close relationship between vitamin D status and fat infiltration in muscle. Gilsanz et al. showed that serum 25-hydroxyvitamin D (25-OHD) levels were negatively correlated with the muscle fat percentage independent of body mass or subcutaneous and visceral fat measured by CT in 90 postpubertal females (96). In a clinical study on elderly individuals, IMAT in thigh muscles was significantly associated with both low vitamin D levels and poor physical performance (97), indicating that vitamin D may impact the deposition of IMAT. Ryan et al. reported that higher physiological concentrations of 1,25-OH_2D_3 inhibit IMAT formation (98). Supplementation with vitamin D alone or in combination with calcium can inhibit the expression of C-reactive protein (CRP), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 (99), which partially explains the inhibition of IMAT formation in obese individuals. In addition, deficiency in vitamin D is associated with a decrease in the proportion and selective atrophy of type II (fast-twitch) fibers in elderly women (100), potentially altering the local microenvironment of muscles.

4.4.3 Mineral factors: Copper (Cu), Zinc (Zn) and iron

Apart from vitamins, the mineral content also influences the biological processes of IMAT formation in animal models (101). Afonso et al. discovered through muscle transcriptome analysis that Cu and Zn may have a negative regulatory effect on intermuscular adipogenesis in groups of Nelore steers (101). Moreover, studies have suggested that an increased iron burden plays a pivotal role in the development of sarcopenia in rats (102). Additionally, transferrin receptor 1 (Tfr1)-mediated iron homeostasis regulates skeletal muscle development, regeneration and metabolism (103–105). Ding et al. revealed that how the specific deletion of *Tfr1* in SCs impairs skeletal muscle regeneration with activation of ferroptosis in mice (105), whereas SC-derived myofibers play a critical role in regulating intermuscular adipogenesis and maintaining the skeletal muscle microenvironment.

Currently, the regulatory mechanisms underlying IMAT formation are primarily investigated in domestic animal and rodent models. Accumulating evidence has suggested that the regulation of intermuscular adipogenesis involves an intricate network, involving the proliferation and differentiation of adipogenic precursors, the skeletal muscle microenvironment and nutritional regulators (Figure 3).

5 Potential interventions and therapies for IMAT: future research directions

Due to the detrimental effects of IMAT infiltration in skeletal muscle, clarifying the etiology, quantity and metabolic characteristics of its development is attracting increasing attention. However, the special anatomical location of IMAT limits its accessibility and the ability to conduct in-depth mechanistic studies. Earlier studies have investigated the origin and potential molecular regulatory mechanisms of IMAT adipocytes in livestock and rodent models, offering insights for clinical interventions to mitigate IMAT infiltration. We reviewed previous studies and found that skeletal muscle-resident mesenchymal progenitors, including PDGFRα^{+}/Sca-1^{+} progenitors, and ASCs from other adipose depots serve as the primary source of IMAT, exhibiting characteristics similar to those of white adipocytes (12, 13, 22). Studies have demonstrated that inhibiting the proliferation and adipogenic differentiation of intramuscular FAPs can effectively impede the formation of intramuscular adipocytes. For instance, modulation of myokines, such as MSTN in the skeletal muscle microenvironment (80, 82, 83), and muscle fiber-derived miR-206, miR-499, can contribute to this inhibition (66, 75). In addition, researchers found that ASC trafficking is regulated by the CXCR4/CXCL12 axis, and pioglitazone intermittent treatment can prevent muscle ectopic fat deposition in high fat diet induced-obese mice (12).

Moreover, human skeletal muscle contains a reservoir of brown progenitors and provides a specialized microenvironment that supports intermuscular brown adipogenesis, which holds promise as a potential therapeutic target for obesity management (14, 27). However, although the expression of UCP1 is increased *in vivo* through PPARγ agonist treatment, the potential of adipocytes in the IMAT depot to serve as a fuel source for adjacent skeletal muscle remains unexplored in human subjects. Therefore, it will be a major challenge that how to facilitate intermuscular brown adipogenesis rather than white adipogenesis. Lineage tracing experiments have suggested that brown adipocytes in skeletal muscle can be derived from myogenic progenitors by modifying the expression of PRDM16 and miR-133 (42, 43). So it is necessary to investigate the potential molecular mechanisms of the transition from myogenic differentiation to brown adipogenic differentiation. In mouse models, the intermuscular brown adipocytes content was also affected by the species of mice, for example, more intermuscular brown adipocytes in obesity-resistant Sv129 mice than B6 mice (15, 25).

Additionally, in the context of obesity, the inflammatory response induces the recruitment of immune cells, primarily macrophages and T cells, which are predominantly located within the intermuscular adipose tissue. Moreover, macrophages undergo polarization into the proinflammatory M1 phenotype. Further research into the characteristics and potential molecular mechanisms of inflammatory cell infiltration in IMAT will also contribute to improving the management of metabolic disorders caused by IMAT.

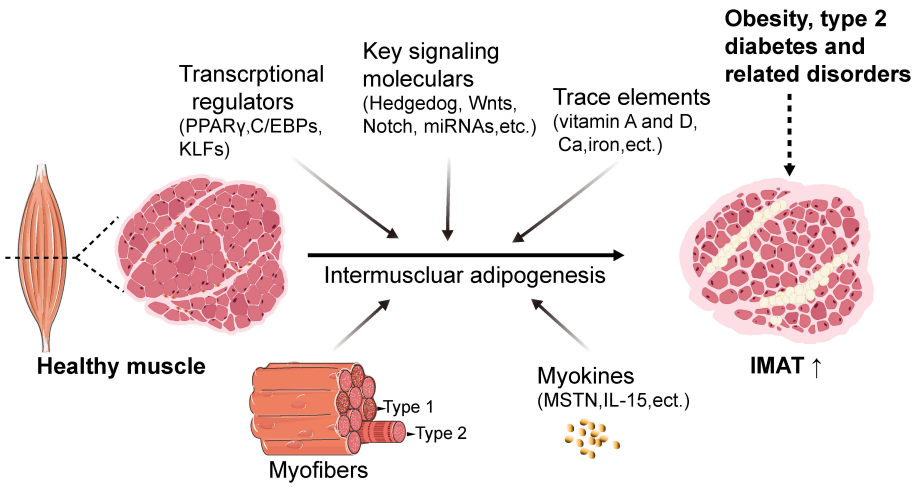

FIGURE 3
Schematic with proposed mechanisms of regulation of intermuscular adipogenesis. The regulation of intermuscular adipogenesis involves an intricate network cascade, which includes transcriptional regulators such as PPARγ, C/EBPs, and KLFs, as well as signaling molecules such as Wnt and Notch. Additionally, trace elements including vitamin A, vitamin D, calcium, and iron are involved. Furthermore, myofibers and myokines contribute to creating an essential microenvironment for intermuscular adipogenesis. IMAT, intermuscular adipose tissue. FAPs, fibro/adipogenic progenitors. C/EBPs, CCAAT enhancer-binding family proteins. PPARγ, peroxisome proliferator-activated receptor gamma. KLFs, Krüppel-like factor family proteins. MSTN, myostatin. Created with BioRender.com.

6 Conclusions

Up to now, our understanding of the unique biology of IMAT, including its cellular, molecular, and biochemical mechanisms, has been enhanced primarily through IMAT tissue biopsy and related methodologies. However, knowledge concerning specific components of IMAT cell composition, secretion factors, and their influence on other metabolic tissues is still in its infancy. To fully uncover the impact of this unique adipose tissue on human health and diseases, additional comprehensive investigations into the quantity and biology of IMAT are crucial. While there is much work to be done, unraveling the mechanisms of IMAT infiltration will be an exciting area of future inquiry.

Author contributions

TZ: Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. JL: Supervision, Writing – review & editing, Data curation, Methodology, Writing – original draft. XL: Conceptualization, Supervision, Writing – review & editing. YL: Supervision, Writing – review & editing, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

IMAT	intermuscular adipose tissue
IR	insulin resistance
SCAT	subcutaneous adipose tissue
FAPs	fibro/adipogenic progenitors
UCP1	uncoupling protein 1
MCP1	monocyte chemotactic protein 1
ASC	adipose stromal cell
BMP7	bone morphogenetic protein 7
PPARγ	peroxisome proliferator-activated receptor gamma
SC	satellite cell
NICD1	intracellular domain of Notch1
PDGFRα	platelet-derived growth factor receptor alpha
Sca-1	stem cell antigen-1
C/EBP	CCAAT enhancer-binding protein
SREBP1c	sterol regulatory element-binding protein isoform 1c
FABP4	fatty acid-binding protein
KLF	Krüppel-like factor
FGF	fibroblast growth factors
Hh	Hedgehog
MMP14	matrix metallopeptidase 14
EDL	extensor digitorum longus
SOL	soleus
MSTN	myostatin
IL	interleukin
RA	retinoic acid
SVC	stromal vascular cell
Cu	Copper
Zn	Zinc
Tfr1	transferrin receptor 1.

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Elderly rats fed with a high-fat high-sucrose diet developed sex-dependent metabolic syndrome regardless of long-term metformin and liraglutide treatment

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Aim/Introduction: The study aimed to determine the effectiveness of early antidiabetic therapy in reversing metabolic changes caused by high-fat and high-sucrose diet (HFHSD) in both sexes.

Methods: Elderly Sprague–Dawley rats, 45 weeks old, were randomized into four groups: a control group fed on the standard diet (STD), one group fed the HFHSD, and two groups fed the HFHSD along with long-term treatment of either metformin (HFHSD+M) or liraglutide (HFHSD+L). Antidiabetic treatment started 5 weeks after the introduction of the diet and lasted 13 weeks until the animals were 64 weeks old.

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Results: Unexpectedly, HFHSD-fed animals did not gain weight but underwent significant metabolic changes. Both antidiabetic treatments produced sex-specific effects, but neither prevented the onset of prediabetes nor diabetes.

Conclusion: Liraglutide vested benefits to liver and skeletal muscle tissue in males but induced signs of insulin resistance in females.

KEYWORDS

high-fat high-sucrose diet, diabetes mellitus, metabolomics, insulin resistance, sex differences

Introduction

Obesity, characterized by the accumulation of excessive fat tissue, is a major contributor to early disability and mortality, and its prevalence is reaching pandemic levels. Besides serving as energy storage, fat tissue is an active endocrine organ. Moreover, it can trigger systemic low-grade inflammation by secreting inflammatory cytokines (1). A causative relationship between obesity-related inflammation and insulin resistance has been established (2). Affected individuals cope with progressive insulin resistance by ever-increasing insulin secretion, up to the point where this adaptive strategy becomes insufficient and type 2 diabetes mellitus (DM2) develops (3). DM2 and obesity are associated with higher risks for many life-threatening conditions, including cardiovascular disease and unfavorable outcomes in patients diagnosed with the novel coronavirus disease (COVID-19) (4, 5). Therefore, an effort to decelerate or stop the progression of obesity-triggered metabolic syndrome in its early stages is warranted.

Metformin, the gold standard in the treatment of DM2, is implicated in the slowed progression of insulin resistance to DM2 (6) but is also discussed as a potential senescence therapy in apparently healthy people (7). In addition, liraglutide (a glucagon-like peptide 1 analog with euglycemic and weight-reducing effects) has been approved for clinical use in obese diabetic individuals (8). Some studies suggest that weight reduction alone might be sufficient to prevent the progression of initial insulin resistance to full-blown DM2 (9). However, since liraglutide has been mostly studied in previously diagnosed diabetic and obese patients, little is known about its preventive potential.

As obesity and DM2 are mainly caused by chronic caloric surplus (2), rodent dietary models of high-fat diet, high-fat and high-fructose diet, or high-fat and high-sucrose diet (HFHSD) exhibit characteristics observed in human metabolic syndrome (10), and the latter (HFHSD) is the closest to the modern Western diet. Although these diets can induce (pre)diabetes in rodents, most of the studies are not prolonged enough to adequately reflect the chronic setting in which dietary effects normally take place in humans (11–15).

In humans, DM2 predominantly develops in elderly populations. Chronic low-grade systemic inflammation, underlying both aging and obesity, may be the culprit behind many age-related conditions, including insulin resistance (16). Despite this, most HFHSD rodent studies were conducted on young adult animals (14, 15, 17–20). Furthermore, females and males differ in body composition, adipose tissue metabolism, weight gain susceptibility, as well as cardiometabolic and dysglycemic risks (21–23). Yet, the available HFHSD rodent studies have included either male or female animals (13, 14, 17, 18). Finally, the evaluation of dietary animal models warrants whole-body analyses, since obesity and DM2 influence the brain as well as peripheral tissues (1). Still, most available studies focused solely on either the central or the peripheral phenomena (10, 13, 15, 18).

This study was conducted on male and female aged rats to address the possibility of sex-specific effects using whole-body analyses. It assessed the consequences of a long-term, obesity-inducing diet as well as the potential of early and long-term pharmacologic interventions to prevent the development and progression of DM2.

Results

The experimental design included rats of both sexes (32 males and 32 females). When they were 45 weeks old (middle-aged), they underwent either a standard or HFHS diet (16 vs. 48 rats, experimental weeks 1–18). After 5 weeks of the HFHS diet, metformin or liraglutide medication was initiated, and it lasted a further 13 weeks (32 treated rats, experimental weeks 6–18). There were in total four experimental groups, each consisting of 16 rats (eight males and eight females): standard diet (STD), HFHS diet only (HFHSD), HFHSD and subsequent metformin medication (HFHSD+M), and HFHSD and subsequent liraglutide medication (HFHSD+L). At the end of the study, the animals were 64 weeks old (i.e., the onset of senescence) (24, 25). They ate a specific diet for 18 weeks (throughout the entire middle-age period), and those treated received medication for 13 weeks (Extended Data Figure 1).

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Senescence of females at the end of the study was proven by measuring estrogen values (6–12 pg/mL in all female rat groups).

Lack of weight gain in liraglutide-treated animals on HFHSD was accompanied by increased caloric intake and a loss of visceral fat in females

To explore the effects of the diet and medication (subsequently referred to as "intervention") on the obesity-induced features, body mass was measured (Extended Data Figure 2A), and the visceral adipose tissue was characterized in detail (Figures 1A, B; Extended Data Figure 2B). Diet and treatment had no influence on body weight. Significantly larger visceral adipocyte surface area were detected in the HFHSD and HFHSD+M animals compared to those in the STD groups, while animals treated with liraglutide did not significantly differ from the STD animals.

To get insight into the overall metabolic change reflected in the polyphagia as a symptom of diabetes, a normalized approximation of the weekly caloric intake for each group was calculated as the ratio of the whole-group caloric intake and the whole-group body mass. By using kcal instead of g of food and animal mass instead of the number of animals, we nullified the difference between the two diets and the loss of animals during the study. Unexpectedly, the females treated with liraglutide experienced an abrupt increase in caloric intake after the experimental week 13, reaching almost twofold higher values relative to other experimental groups (Figure 1C). To quantify the observed changes, marginal means of caloric intakes were estimated for the period prior to the intervention (weeks 1–5), of the early intervention (weeks 6–10), and the long-term intervention (weeks 10–18). As expected,

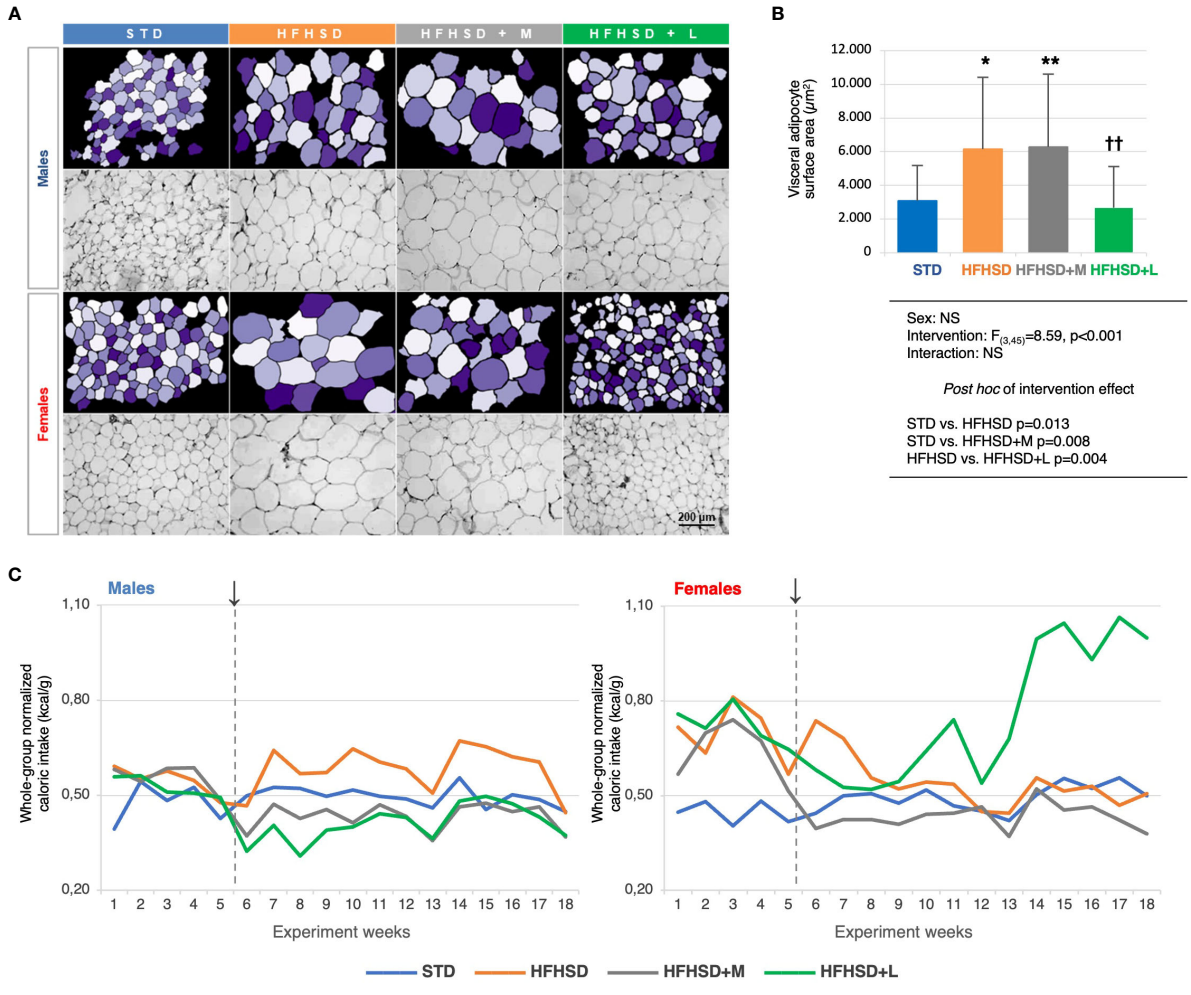

FIGURE 1
Liraglutide treatment in elderly rats on a high-fat high-sucrose diet reduced the surface areas of the visceral adipocytes, but triggered polyphagy in the female animals. **A)** Micrographs of the hematoxylin-eosin-stained visceral adipose tissue samples (magnification 200×) (bottom rows) and matching images obtained in CellProfiler (upper rows). **B)** Between-group comparison of average visceral adipocyte surface areas (µm2), two-way ANOVA and Games-Howell post hoc test for between-group comparisons: *compared to STD, †compared to HFHSD; *p < 0.05, **/††p < 0.01, NS – not significant. **C)** Whole-group caloric intake normalized for the whole-group body mass (kcal/g) per experimental week. ↓ – introduction of the antidiabetic drugs. Abbreviations: STD – standard diet group, HFHSD – high-fat high-sucrose diet group, HFHSD+M – HFHSD treated with metformin, HFHSD+L – HFHSD treated with liraglutide.

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metformin significantly decreased the caloric intake in both sexes, while liraglutide did it only in males. In females, liraglutide paradoxically increased the caloric intake after long-term intervention, indicating development of metabolic inefficiency (Extended Data Figure 2C).

Females tolerated acute hyperglycemic challenges less efficiently and exhibited decreased insulin sensitivity

Improved glucose tolerance and low glucose variability were expected to be the primary outcomes of antidiabetic treatment. Average values of areas under the curves (AUC) of glucose blood levels for each group during the glucose tolerance test (GTT) were calculated (Extended Data Figure 3A). In the experimental week 5, all HFHSD animal groups had significantly higher AUC values in comparison to the STD groups, revealing the decreased glucose tolerance. The same was observed in the experimental weeks 12 and 18 in the nonmedicated animals under HFHSD. In week 12, the AUC values of groups receiving medication approached STD group values, showing the acute benefits of antidiabetics. The males showed analogous results at experimental week 18; however, females of all groups (including the STD group) decreased glucose tolerance at this time point. With the onset of reproductive senescence, glucose tolerance worsened, particularly in the HFHSD female group, while the antidiabetic-receiving groups still benefited from the treatment. This result did not agree with the finding of polyphagia only in HFHSD+L females, especially because HFHSD females had by far the worst glucose tolerance of all the other groups.

To get more detailed insight into sex-based differences in glucose tolerance, we used mathematical modeling of GTT data (Figures 2A, B). Derived parameters describing curves explained group progression in glucose variability (0-, 5-, 12-, and 18-week time points). The females belonging to all groups reached significantly higher glucose concentrations during the GTT (maximal glucose concentration (mg/dL) (G_{max})) compared to the males and were slower in reestablishing normoglycemia than their male counterparts. Blood glucose set points described by G_0 followed by plasma glucose 2 h after load ($G(2)$), and fasting glucose ($G(0)$) were the best biomarkers of progressive metabolic failure. The G_0 parameter describes the base value to which the function returns; that is why we assumed that this parameter can be physiologically best translated into the centrally given glucose set point. In our case, we calculated it based on the value of the entire group. Figure 2A shows that STD males at the beginning of the study reach the G_0 value in just 1 h, while the females of the HFHSD +L group at the end of the study do not reach G_0 even in 3 h, so the value of the function period (T) is also the highest in them. According to plasma glucose 2 h after load ($G(2)$), all examined groups, except STD males, developed prediabetes (HFHSD and HFHSD+M males) or diabetes (all the rest) according to official DM2 diagnostic criteria (140–199 mg/dL for prediabetes and ≥ 200 mg/dL for diabetes) (26, 27). HFHSD+L females also met the diagnostic criteria for the fasting glucose dysglycemia biomarker (100–125 mg/dL). Mathematical modeling revealed five additional parameters that were the lowest (coefficient of oscillation amplitude decline (α), and initial speed of blood glucose increase ($G'(0)$) or the highest (the basic period of function (T), maximal speed of glucose concentration decrease (G'_I), and the moment at which G'_I is attained (t_I)) in HFHSD+L females reflecting changes in the glycemia regulation (Extended Data Figure 3B). An additional proof of the credibility of the mathematical model is that the AUC values obtained by mathematical modeling correlated well with the AUC values obtained from real measurements.

To identify hormones underlying the observed GTT changes, leptin, insulin, corticosterone, and adiponectin were measured at the endpoint of the study (Figure 2C; Extended Data Figure 3C). As expected, the HFHSD and HFHSD+M groups had significantly higher leptin plasma levels relative to the STD group, whereas the plasma leptin levels in the HFHSD+L animals did not differ when compared to those in the STD and HFHSD groups. Observing the insulin serum levels, the HFHSD+L females had significantly higher fasting insulinemia compared to the STD females, but the same trend was not statistically significant in the male groups. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) score was also calculated. The highest and statistically significant score was achieved by HFHSD+L females. Furthermore, phosphorylated tyrosine moieties of the insulin receptor substrate 1 (IRS-1) increased significantly in the skeletal muscle of all treated groups, but especially in HFHSD+L groups. Plasma corticosterone levels were not informative, while females in general exhibited higher adiponectin levels than males.

Improved insulin sensitivity was the expected secondary outcome of antidiabetic treatment; hence, the insulin tolerance test (ITT) was performed, and mathematically modeled ITT function was calculated in the experimental week 18 (Figure 2D; Extended Data Figures 3D, E) when we assumed insulin resistance could be developed. All the groups fed the HFHSD had significantly higher AUCs of glucose blood levels than the STD group. Mathematically modeled ITT functions (Figure 2D) revealed that the response to hyperinsulinemic challenge was highest in STD and lowest in HFHSD+L (minimal glucose concentration (H_{min})), indicating low insulin sensitivity under liraglutide treatment. STD group exhibited prolonged hypoglycemic levels lasting longer than 2 h. Animals fed the HFHSD had exaggerated glycemic compensatory responses in the ITT postacute recovery period, but both metformin-treated groups and males on liraglutide regained normoglycemia (H_0). However, females in HFHSD+L that resisted acute hypoglycemia the best also remained in reactive hyperglycemia for the longest time, which can be explained by their highest tendency to develop insulin resistance relative to other animal groups.

Corticosterone, a potent insulin-antagonizing hormone, is commonly negatively associated with insulin sensitivity. Measuring its levels could provide a possible explanation for the dysglycemia observed in HFHSD+L females. Because a one-point measurement of corticosterone level is a low presentation for overall daily corticosterone fluctuations, we used Hans Selye's historical finding of an association between adrenal gland size and cumulative corticosterone levels (28). The surface areas of mid-sections of

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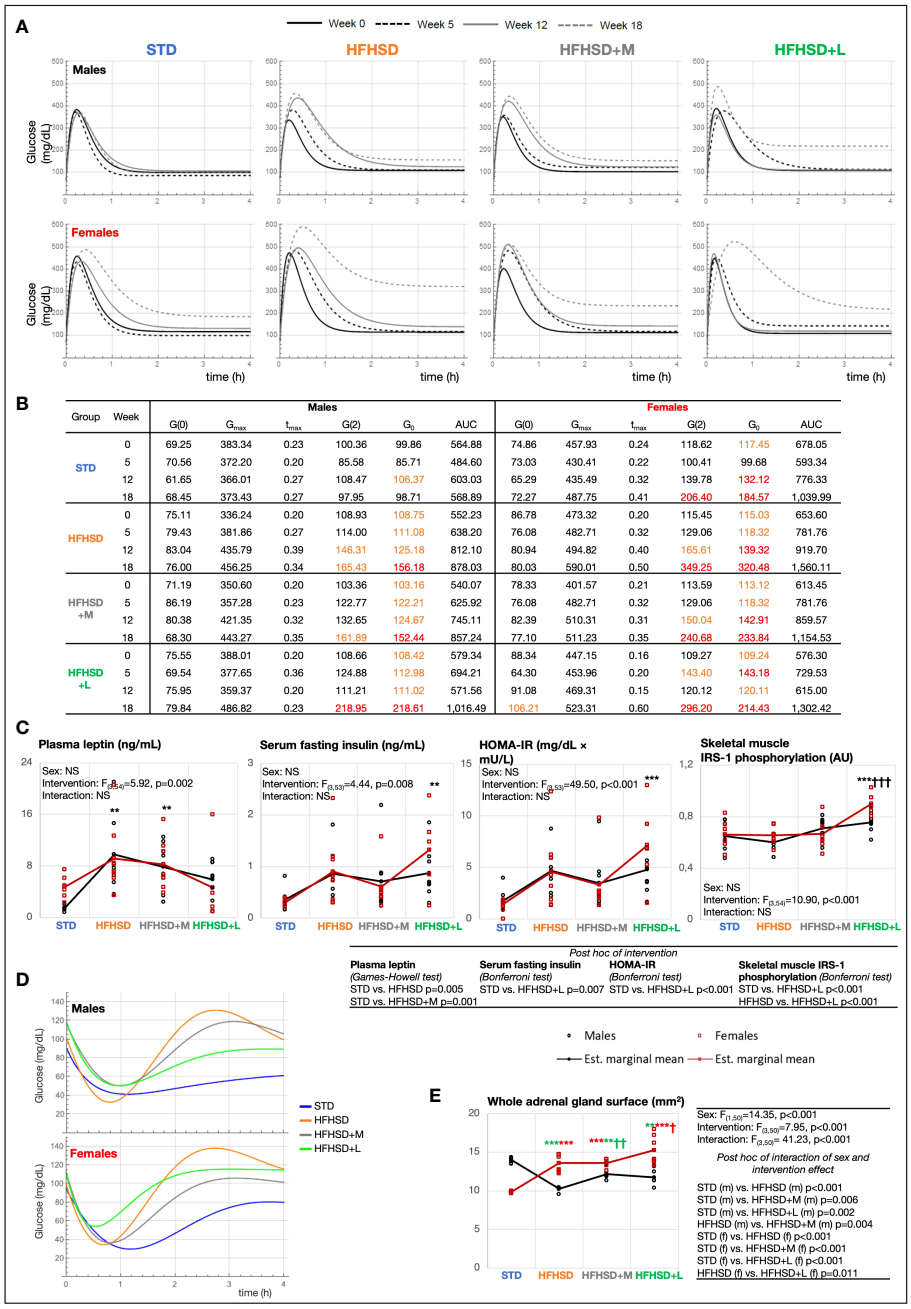

Group	Week	Males G(0)	Males G_{max}	Males t_{max}	Males G(2)	Males G_0	Males AUC	Females G(0)	Females G_{max}	Females t_{max}	Females G(2)	Females G_0	Females AUC
STD	0	69.25	383.34	0.23	100.36	99.86	564.88	74.86	457.93	0.24	118.62	117.45	678.05
	5	70.56	372.20	0.20	85.58	85.71	484.60	73.03	430.41	0.22	100.41	99.68	593.34
	12	61.65	366.01	0.27	108.47	106.37	603.03	65.29	435.49	0.32	139.78	132.12	776.33
	18	68.45	373.43	0.27	97.95	98.71	568.89	72.27	487.75	0.41	206.40	184.57	1,039.99
HFHSD	0	75.11	336.24	0.20	108.93	108.75	552.23	86.78	473.32	0.20	115.45	115.03	653.60
	5	79.43	381.86	0.27	114.00	111.08	638.20	76.08	482.71	0.32	129.06	118.32	781.76
	12	83.04	435.79	0.39	146.31	125.18	812.10	80.94	494.82	0.40	165.61	139.32	919.70
	18	76.00	456.25	0.34	165.43	156.18	878.03	80.03	590.01	0.50	349.25	320.48	1,560.11
HFHSD +M	0	71.19	350.60	0.20	103.36	103.16	540.07	78.33	401.57	0.21	113.59	113.12	613.45
	5	86.19	357.28	0.23	122.77	122.21	625.92	76.08	482.71	0.32	129.06	118.32	781.76
	12	80.38	421.35	0.32	132.65	124.67	745.11	82.39	510.31	0.31	150.04	142.91	859.57
	18	68.30	443.27	0.35	161.89	152.44	857.24	77.10	511.23	0.35	240.68	233.84	1,154.53
HFHSD +L	0	75.55	388.01	0.20	108.66	108.42	579.34	88.34	447.15	0.16	109.27	109.24	576.30
	5	69.54	377.65	0.36	124.88	112.98	694.21	64.30	453.96	0.20	143.40	143.18	729.53
	12	75.95	359.37	0.20	111.21	111.02	571.56	91.08	469.31	0.15	120.12	120.11	615.00
	18	79.84	486.82	0.23	218.95	218.61	1,016.49	106.21	523.31	0.60	296.20	214.43	1,302.42

FIGURE 2
Aging in women impaired glucose metabolism more than in men, while long-term treatment with liraglutide exacerbated hyperinsulinemia and insulin resistance. **(A)** Model function [$G(t)$] of blood glucose concentration (mg/dL) based on measurements from glucose tolerance test (GTT) in experimental weeks 0, 5, 12, and 18. **(B)** Characteristics of model function: fasting blood glucose concentration [mg/dL) ($G(0)$], maximal glucose concentration (mg/dL) (G_{max}), the moment at which G_{max} is reached (h) (t_{max}), 2-h blood glucose at GTT [mg/dL) (G(2)], blood glucose setpoint (asymptote) (mg/dL) (G_0), and area under the curve (AUC). Values in orange, prediabetes (100 mg/dL < $G(0)$ < 125 mg/dL, 140 mg/dL < $G(2)$ < 199 mg/dL); values in red, diabetes ($G(0) \geq 126$ mg/dL, $G(2) \geq 200$ mg/dL). **(C)** Interaction plots of intervention and sex effects on plasma leptin (ng/mL), serum fasting insulin (ng/mL), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (mg/dL × mU/L), insulin receptor substrate 1 (IRS-1) phosphorylation in the skeletal muscle [arbitrary units (AU)]. **(D)** Model function [$H(t)$] of blood glucose (mg/dL) based on measurements from insulin tolerance test (ITT) in experimental week 18. **(E)** The adrenal gland surface. Two-way ANOVA; black symbol, experimental groups including both sexes; green symbol, male groups; and red symbol, female groups; */†p < 0.05, **/††p < 0.01, ***/†††p < 0.001 (*compared to STD, †compared to HFHSD). NS, not significant; f, female; m, male; STD, standard diet group; HFHSD, high-fat and high-sucrose diet group; HFHSD + M, HFHSD treated with metformin; HFHSD + L, HFHSD treated with liraglutide.

adrenal glands were analyzed (Figure 2E). The male HFHSD animals and male groups receiving medication had significantly smaller adrenal glands than the STD animals, whereas exactly the opposite finding was present in females. The biggest adrenal gland surface in HFHSD+L females indicates the highest cumulative corticosterone levels in these animals, which may be related to metabolic disbalance and a shift in normoglycemia set point.

Both antidiabetic treatments increased leptin sensitivity in hypothalamic satiety nuclei, but only liraglutide had a peripheral anti-inflammatory effect

Improved insulin and leptin sensitivity in hypothalamic satiety nuclei, related to reduced food intake, was the expected tertiary outcome of antidiabetic treatment. Insulin serves as an acute satiety signal, leptin as a chronic one, and insulin-like growth factor (IGF) as a sub-acute signal that adjusts body temperature to energy resources. Their receptors initiate the signal responses in the brain and subsequently reduce feeding. The expression of insulin receptor α (IR-α), leptin receptor (ObR), and insulin-like growth factor 1 receptor β (IGF-1Rβ) was measured as markers of energy-sensing signaling pathways in the following brain nuclei: the arcuate (ARC), lateral hypothalamic (LH), paraventricular (PVN), and ventromedial hypothalamic (VMH) nuclei (Figure 3A; Extended Data Figures 4.1A, 4.2, 4.3, 4.4). Selected hypothalamic nuclei are part of the neural network that controls feeding, and previous studies have shown that they are not equally prone to developing insulin/leptin resistance (29).

The high IR expression in the brain nuclei was associated with low serum fasting insulin levels in STD animals (in females in particular) and their potentially better central insulin response. Long-lasting HFHSD decreased the expression of IR in the LH nucleus, potentially due to increased serum insulin levels. The antidiabetic treatment reversed the receptor decrease in LH nuclei of males but not in females. The animal group with the lowest expression of IR relative to all other groups in all satiety nuclei (HFHSD+L females) also had the highest serum fasting insulin levels. This potential central insulin resistance indicated a small potential of insulin on the feeding switching function. This was also in agreement with the low whole-body sensitivity of insulin receptors (as measured by the ITT response) in HFHSD+L females.

HFHSD did not significantly affect ObR expression in individual nuclei, regardless of the increased plasma leptin levels. On the other hand, antidiabetic treatment was associated with a profound central effect: increased ObR expression was observed in all satiety nuclei in both males and females—more in the case of metformin, than liraglutide. It explained the major metformin beneficial effect: quick reaching satiety and no gain of weight despite an increase in adipocyte surface area. Contrary to HFHSD +M animals, the medication effect was lower in HFHSD+L groups, in particular females, probably due to cross-downregulation of ObR with increased insulin levels. HFHSD+L males, but not females, had increased expression of IGF-1R, which could provide them with additional relief from high-caloric HFHSD (Extended Data Figures 4.1A, 4.4) by its ability to increase body temperature.

The fourth expected outcome of antidiabetic treatment was a reduction in low-grade inflammation. Neuro-inflammation was investigated in the same brain nuclei with the help of two markers: ionized calcium-binding adaptor molecule 1 (Iba1), a microglia marker (Figure 3A; Extended Data Figure 4.5), and the glial fibrillary acidic protein (GFAP), an astrocyte marker (Extended Data Figures 4.1A, 4.6). Although some significant changes, mostly provoked by medication rather than HFHSD itself were shown, there was no clear correlation between hormonal changes and neuro-inflammatory status.

To investigate peripheral aspects of low-grade inflammation, proinflammatory (M1) and anti-inflammatory (M2) macrophages were analyzed in visceral adipose tissue using CD68 and CD163 markers, respectively (Figure 3B; Extended Data Figure 4.7). In addition to macrophages, the expression of the tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6) was analyzed in the visceral and subcutaneous adipose tissue samples (Figure 3C; Extended Data Figure 4.1B). Visceral adipose tissue was chosen for additional research because it appeared to be more related to inflammatory response.

Sex and intervention did not affect the M1 phenotype but did affect M2 in the adipose tissue. STD females had a significantly higher number of M2 macrophages compared to males (beneficial inflammatory response). However, when fed the HFHSD or treated with antidiabetics, both aspects of inflammatory responses were comparable between sexes. A large adipocyte size is a challenge for classical phagocytosis, whose inefficiency is reflected in the secretion of proinflammatory cytokines. In support of this, the TNF-α and IL-6 increase was interconnected with the downregulation of M2 lineages observed in groups with the highest adipocyte sizes, HFHSD and HFHSD+M. Liraglutide treatment reduced adipocyte size more in females than in males, and this resulted in consistently reduced secretion of inflammatory cytokines. A marked decrease in adipocyte size in liraglutide-treated females ultimately resulted in the proinflammatory response and highest M1/M2 ratio.

These results indicated that metformin was less able to alter the peripheral inflammatory response of animals exposed to HFHSD, whereas liraglutide had anti-inflammatory consequences only in males, but in females, liraglutide treatment led to an excessive reduction in adipocyte size and a reversal of the favorable treatment effect.

Sex-specific metabolic difference in liver and skeletal muscle was enhanced by a high-fat and high-sucrose diet and antidiabetic drugs

The fifth expected outcome of antidiabetic treatment was a slower progression of metabolic-dysfunction-associated fatty liver disease (MAFLD) caused by HFHSD. In normal-weight subjects, the presence of hepatic steatosis accompanied by at least two metabolic risk abnormalities is required for MAFLD diagnosis.

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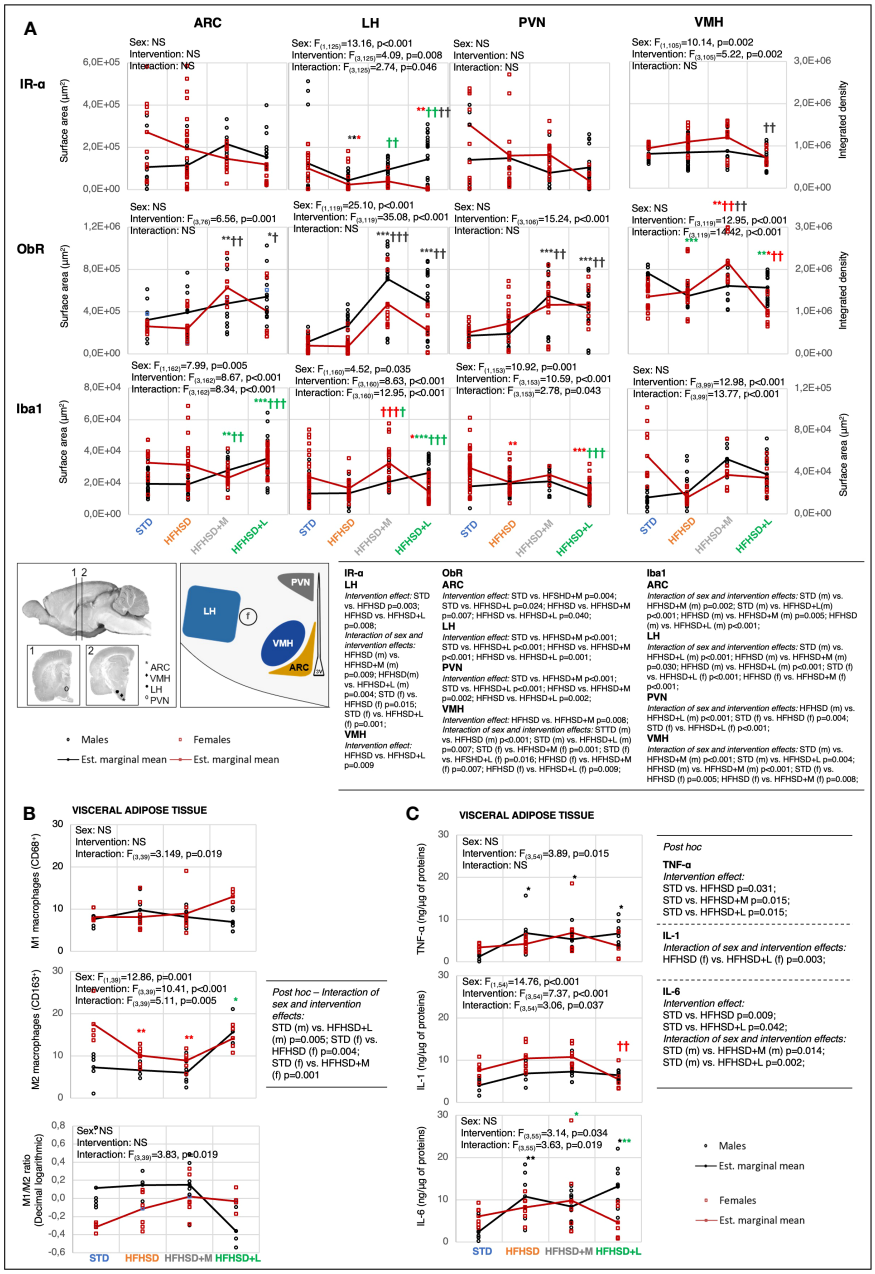

FIGURE 3
Metformin and liraglutide influenced high-fat high-sucrose diet-associated microinflammation in the hypothalamus and visceral adipose tissue in a sex-specific manner. **(A)** Interaction plots of intervention and sex effects on the expression level of the insulin receptor α-subunit (IR-α), leptin receptor (ObR), and ionized calcium-binding adapter molecule 1 (Iba1) in the following hypothalamic nuclei: arcuate nucleus (ARC), lateral nucleus of hypothalamus (LH), paraventricular nucleus (PVN), and ventromedial nucleus (VMH). The location of nuclei within the hypothalamus is indicated on the scheme below the interaction plots (f, fornix; 3V, third ventricle). Two-way ANOVA and Games–Howell *post-hoc* test for between-group comparisons. **(B)** Interaction plots of intervention and sex effects on the number of M1 macrophages (CD68) and M2 macrophages (CD163) in visceral adipose tissue per field of view at 100× magnification, and decimal logarithmic representation of M1 to M2 macrophage ratio in visceral adipose tissue. Two-way ANOVA and Games–Howell *post-hoc* test for between-group comparisons. **(C)** Interaction plots of intervention and sex effects on the TNF-α, IL-1, and IL-6 in the visceral adipose tissue. Two-way ANOVA and Games–Howell *post-hoc* test for between-group comparisons. Black symbol, experimental groups including both sexes; green symbol, male groups; red symbol, female groups. */†p < 0.05, **/††p < 0.01, ***/†††p < 0.001 (*compared to STD, †compared to HFHSD). NS, not significant; f, female; m, male; STD, standard diet group; HFHSD, high-fat and high-sucrose diet group; HFHSD+M, HFHSD treated with metformin; HFHSD+L, HFHSD treated with liraglutide.

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With the exception of STD males, all animal groups fulfilled metabolic criteria for MAFLD and the presence of prediabetes or diabetes (Figures 2B, C). Nevertheless, pronounced liver steatosis was recorded just in the HFHSD and HFHSD+M groups (Figure 4A), as visualized by Sudan black staining. Due to their hydrophobicity, fat droplets were compact, and we used Oil Red staining to calculate their surface (Figure 4B; Extended Data Figure 5.2). The extent of lipid accumulation varied in subcapsular (SPL) and deeper parenchymal portions [central part (CPL)] of the liver (Figures 4A, B). Therefore, these regions were analyzed separately. In all groups, the subcapsular part accumulated more fat droplets, and steatosis was more pronounced in males than in females due to central part involvement. Male groups with the highest steatosis also had the highest levels of serum cholesterol and triglycerides (Extended Data Figure 5.1A), but without an increase of liver damage markers (Extended Data Figure 5.1B)—aspartate transaminase (AST) and alanine transaminase (ALT). Fat droplet accumulation in HFHSD+L groups was comparable to that in STD groups; that is, liraglutide successfully resolved hepatic steatosis. Also, HFHSD+L females significantly increased the liver mass to body mass ratio, probably due to both the loss of body mass and the loss of hepatic lipids (Extended data Figure 5.1C).

To determine whether lipid accumulation led to decreased glycogen storage, liver sections were stained with metachromatic toluidine (Figure 4B; Extended Data Figure 5.3). Surprisingly, the male groups with the highest steatosis, HFHSD and HFHSD+M, also had the highest glycogen content, both subcapsular and within the parenchyma. Contrary to that, liraglutide treatment depleted glycogen stores, especially in subcapsular hepatocytes. In conclusion, liraglutide treatment led to the depletion of glycogen stores.

The sixth expected outcome of antidiabetic treatment was a positive effect on HFHSD-induced skeletal muscle mitochondrial dynamics and antioxidant capacity. The content of mitochondria by

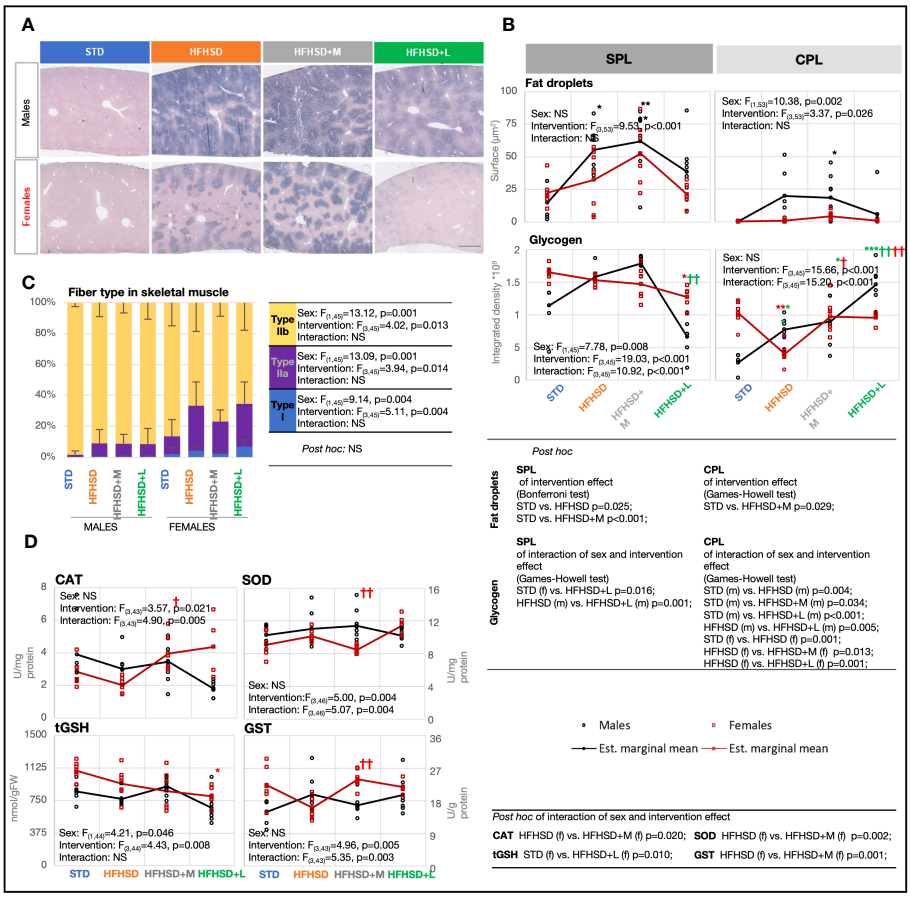

FIGURE 4
Male rats fed the high-fat and high-sucrose diet are more prone to excessive hepatic and skeletal muscle lipid accumulation. **(A)** Cross-section of liver stained using Sudan black, with annotated central (CPL) and subcapsular (SPL) parts. Scale, 500 μm. **(B)** Fat droplet measurement based on Oil red O staining and glycogen measurement based on metachromatic toluidin stain. Two-way ANOVA and Bonferroni or Games–Howell *post-hoc* test for between-group comparisons. **(C)** Percentage of fiber types I, IIa, and IIb in skeletal muscle based on staining with succinate dehydrogenase. Two-way ANOVA and Games–Howell *post-hoc* test for between-group comparisons. **(D)** Interaction plots of intervention and sex effects on the following parameters for determination of the oxidative-antioxidative status of skeletal muscle: catalase (CAT) (U/mg protein); superoxide dismutase (SOD) (U/mg protein); total glutathione (tGSH) (nmol/g of fresh tissue weight (FW)); glutathione *S*-transferase (GST) (U/g protein). Two-way ANOVA and Games–Howell *post-hoc* test for between-group comparisons; black symbol, experimental groups including both sexes; green symbol, male groups; red symbol, female groups; */†*p* < 0.05, **/††*p* < 0.01, ***/†††*p* < 0.001 (*compared to STD, †compared to HFHSD). NS, not significant; f, female; m, male; STD, standard diet group; HFHSD, high-fat and high-sucrose diet group; HFHSD+M, HFHSD treated with metformin; HFHSD+L, HFHSD treated with liraglutide.

skeletal muscle fiber types varies from high (type I), through intermediate (type IIa), to low (type IIb), while oxidative capacity correlates with the number of mitochondria in physiological conditions. Mitochondrial mass per fiber was determined by succinate dehydrogenase (Complex II) histological staining of skeletal muscles from the nuchal region (Figure 4C; Extended Data Figure 5.4). A large sex difference was already visible in animals on STD; females showed a higher percentage of red fibers (I and IIb) than males. Consumption of HFHSD led to a significant increase in mitochondrial mass in both sexes, but the ratio between red and white fibers (IIb) increased to a greater extent in females. Contrary to the diet, both antidiabetic treatments were unremarkable in the skeletal muscles of males. Nevertheless, metformin, known to affect mitochondrial efficiency by inhibiting Complex I, decreased the proportion of red fibers in females. Interestingly, metformin treatment in females had also the greatest effect on the antioxidant capacity of skeletal muscle (Figure 4D), leading to a significant increase in enzyme catalase (CAT) and glutathione *S*-transferase (GST). Medication with liraglutide did not affect mitochondrial mass and was associated with lower total glutathione (tGSH), but higher SOD in females.

The lipid droplet content of skeletal muscle was an indirect indicator of blunted inhibition of adipose tissue lipolysis in the development of insulin resistance, so we measured the average size of fat droplets using Oil Red staining (Extended Data Figures 5.1D, 5.5). As expected, female groups had a higher average size of fat droplets than males, with the exception of metformin-treated males. Nevertheless, the accumulation of lipids was not accompanied by an increased risk for lipid peroxidation (Extended Data Figure 5.1E).

When these results are considered together, increased mitochondrial volume in HFHSD is a sign of serious changes in mitochondrial dynamic that are not matched with antioxidant capacity and pose a challenge to the quality control of mitochondria. The observed changes in skeletal muscle tissue deserved a more careful analysis.

Diet and antidiabetic drugs have a significant effect on the metabolic profile of skeletal muscles in males but in less regard in females

Skeletal muscle tissue is an insulin-dependent organ, and its insulin resistance triggers diabetes (30). The expected outcome of antidiabetic treatment was a beneficial metabolic response opposing skeletal muscle insulin resistance. Therefore, the fresh-frozen samples of muscles from the nuchal region were subjected to the MALDI-TOF imaging mass spectrometry (IMS) that generated big data with the least loss of relevant molecules. Mass spectra were recorded in the range 300–700 Da (Extended Data Figure 6.1) in order to analyze metabolites and in the range 700–1,000 Da (Extended Data Figure 6.2) in order to analyze lipids.

To identify patterns and trends or extract the most important features, principal component analysis (PCA) was used for big data visualization. Overlapping metabolic profiles were observed using the assumption that the sets of metabolic profiles may be represented by the chemical fingerprint containing strong signals (signal intensity > 5% of the strongest signal, $N = 74$) coming from the average TIC-normalized mass spectra in the range 300–1,000 Da (Figure 5A). However, out of 74 strongest m/z signals, 21 were significantly altered in at least one treatment pair selected by the omnibus false discovery rate (FDR) Kruskal–Wallis (KW) ANOVA followed by pairwise Dunn–Bonferroni test (Figure 5B). STD males were the most different (12–15 compounds) in relation to all other animal groups of both sexes. Nevertheless, liraglutide-treated males (HFHSD+L) were closest to STD males in muscle metabolic profile. In all-female groups, the muscle metabolic profile was similar (especially between the STD and HFHSD groups), and metformin treatment had a slightly larger effect than the liraglutide treatment. This result speaks in favor of pre-existing gender-specific differences in muscle metabolic profiles, and their different response to antidiabetic drugs.

To putatively identify significant m/z signals, METASPACE and HMDB databases were used (Table 1; Extended Data Figure 6.3). They included xanthurenic acid 8-*O*-sulfate, inosine monophosphate (IMP), phosphatidic acids (PA), and different types of phospholipids. Xanthurenic acide 8-*O*-sulfate (m/z 307.97), considered to serve as a natriuretic hormone that enhances glycosuria, was lowest in STD groups (Figure 5C). IMP (m/z 387.01), recently introduced as a beneficial nutriceutical affecting the energetic and antioxidant status of the liver and muscles (31) and previously connected with high physical activity in untrained animals, was highest in STD males. Levels of PA (m/z 737.45/761.45/763.46), precursors of phospholipids with positive effects on mitochondrial dynamics, were highest in STD males, liraglutide-treated males, and metformin-treated females.

In order to graphically represent major clusters, a heatmap was constructed using Euclidean distance, and Ward's method was applied to the scaled significant m/z signals (Extended Data Figure 6.4). In concordance with PCA, all treatment groups were clustered together, which implied a large inter-individual variability. According to the samples' dendrogram, liraglutide treatment achieved the expected effects in the muscles of most males (populations of STD and HFHSD+L males were grouped together). Metformin effects were shared between sexes (males and females on metformin were grouped together). The most dispersed classes were HFHSD and HFHSD+L females. m/z's dendrogram showed clustering of phospholipids and their partial overlap with the PA. Conspicuously, IMP was between phospholipids and PA clusters. It was interesting to notice that m/z = 329.95 and 439.03 Da, which we were not able to uniquely identify, formed a cluster with the xanthurenic acid 8-*O*-sulfate.

Taken together, the skeletal muscle metabolic profile of STD males was different from all other groups, and the closest to it was HFHSD+L males. Also, HFHSD had a significant effect on males but not on females, which spoke in favor of developing muscle insulin resistance caused by menopause itself. Aging was a probable basis for large inter-individual differences (since biological and chronological age may mismatch) (32, 33), so it was not unexpected that the overall effect of antidiabetic drugs in female groups was negligible.

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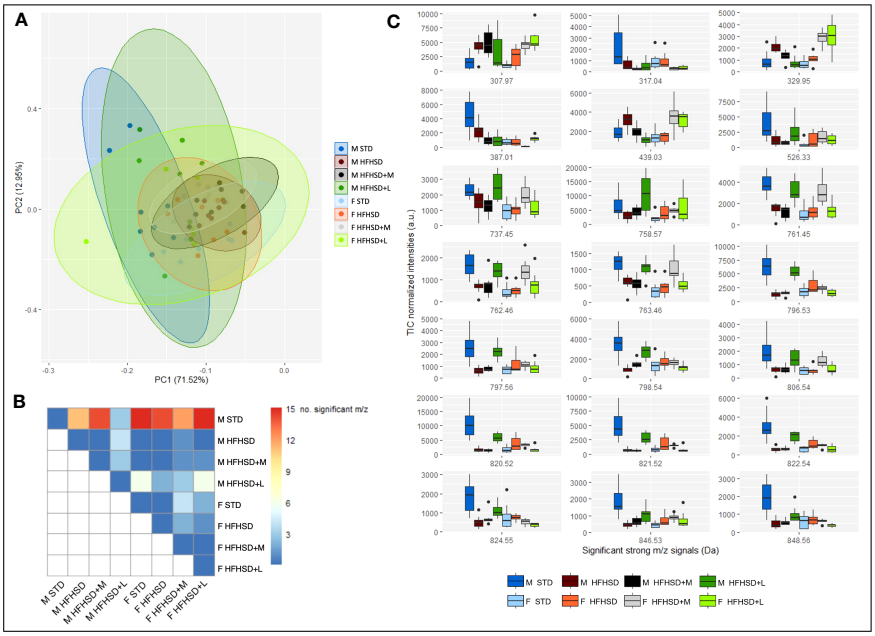

FIGURE 5
MALDI-TOF IMS analysis of Sprague–Dawley rats' nuchal skeletal muscle shows sex-specific metabolic responses to HFHSD, liraglutide, and metformin treatments. **(A)** Principal component analysis (PCA) using strong m/z signals (signal intensity > 5% of the strongest signal) coming from the average total-ion-current (TIC)-normalized mass spectra recorded in the range 300–1,000 Da explains 84.47% variance. **(B)** Distribution of the significantly altered m/z signals by treatment pairs. **(C)** Box and whisker plots of significantly altered m/z signals. Each group sample size was eight (4 biological replicates × 2 technical replicates). F, female; M, male; STD, standard diet group; HFHSD, high-fat and high-sugar diet group; HFHSD+M, rats on HFHSD treated with metformin; HFHSD+L, rats on HFHSD treated with liraglutide; m/z, mass-to-charge ratio.

Discussion

Glucotoxicity and lipotoxicity are the major two drivers of hyperinsulinemia and the concomitant development of multiorgan insulin resistance, culminating with the loss of β-cells as an ultimate deficit in DM2, independent of sex difference and aging (34). In this study, we developed the preclinical rat model to address the long-term effects of diet, aging, and sex in development of the DM2, which proved to be successful in recapitulating the whole sequence of its pathogenesis, from metabolic disorder to prediabetes and finally diabetes. As our motive was to increase the translational relevance, animals of both sexes were used, and the HFHSD was introduced in reproductive senescence (at 45 weeks). HFSHD indeed quickly leads to a metabolic disorder; 5 weeks after its introduction, the animals had early signs of prediabetes (i.e., elevated G_0 parameter derived from the mathematical model), 7 weeks later, they reached clinical prediabetes, and in the next 6 weeks, clinical diabetes. The following mathematical parameters derived from the glucose tolerance test (GTT) were informative in monitoring the progression of metabolic disorder of the studied rats: G_{max}, G_0, $G(2)$, and $G(0)$. The length of the study (18 weeks) provided insight into the transition from prediabetes to diabetes. Subsequently, this allowed evaluation of the effects of the therapeutic interventions by metformin and liraglutide introduced at week 5. The long-term monitoring of the medications (for 13 weeks) distinguished early and late effects of therapy and revealed sex differences due to aging, diet, and antidiabetic treatment.

Aging was the primary metabolic culprit in the DM2 pathology of females

Thanks to the fact that the study was initiated in middle-aged animals, it demonstrated the importance of aging as the primary metabolic culprit in DM2 pathology, which was more prominent in females. The females developed already on STD the indicators of metabolic disorder; an increase in parameters describing GTT-provoked glucose disposal [G_{max}, G_0, and $G(2)$], and higher skeletal muscle mitochondrial mass. Similarly, in our previous study (35) on young (3.5 months) and mature (12 months) rats, STD-fed perimenopausal females had a higher G_{max} than males. Therefore, an increase in G_{max} can be considered a prodromal sign of metabolic risk associated with aging, at least in females.

The response to HFHSD was sex-specific implying that females may develop skeletal muscle insulin resistance, while males may develop hepatic insulin resistance

HFHSD exacerbates the female tendency toward glucose intolerance, dramatically increases skeletal muscle mitochondrial mass, and increases the associated potential development of insulin resistance. Glucose tolerance reflects the β-cell ability to offset insulin resistance by increased insulin secretion, and as long as this balance holds, glucose tolerance remains the same (36). At the

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TABLE 1 Statistically significant alterations in the strong m/z signal intensities of male and female rat nuchal skeletal muscle with tentative annotations.

m/z (Da)[a]	Adduct	Treatment pairs	Tentative endogenous metabolite annotation[b]	Metabolic and physiological role	Comments
307.97	M+Na	F STD/F HFHSD+L	Xanthurenic acid 8-*O*-sulfate	Trp/Kynurenine metabolism Natriuresis	*N*-Acetyl-L-glutamyl 5-phosphate is a result of database searches that is less likely to be present in muscles (1–3).
317.04	–	M STD/F HFHSD M STD/F HFHSD+M M STD/M HFHSD+M	–	–	Quinolinic acid is a result of the HMDB search but not of the METASPACE search. Database searches provide two more hits that are less likely to be present in muscles: methoxybrassenin B/ wasalexin A, B, and homocarnosine.
329.95	–	F HFHSD+L/F HFHSD F HFHSD+M/F HFHSD F STD/F HFHSD+L M HFHSD+L/F HFHSD+L M HFHSD+M/ F HFHSD+L M STD/F HFHSD+L F STD/F HFHSD+M M HFHSD+L/F HFHSD+M M STD/F HFHSD+M	–	–	3-Iodotyrosine is a result of HMDB and METASPACE search, but this compound is not likely to be present in muscles: instead, xanthurenic acid 8-*O*-sulfate adduct of type M+2Na-H is a more likely annotation.
387.01	M+K	M STD/F HFHSD M STD/F HFHSD+L M HFHSD/F HFHSD+M M STD/F HFHSD+M M STD/F STD M STD/M HFHSD+L M STD/M HFHSD+M	IMP	Impaired ATP biosynthesis during physical activity	– (4, 5)
439.03	–	F HFHSD+M/F HFHSD M HFHSD+L/F HFHSD+L F STD/F HFHSD+M M HFHSD+L/F HFHSD+M M HFHSD+L/ M HFHSD	–		Glucocheirolin is a result of HDMB and METASPACE search, but this nutrient cannot be present due to controlled diets. Maleylacetoacetic/4-fumarylacetoacetic acids are results of HMDB but not of the METASPACE search. This m/z signal also corresponds to the matrix adduct 2CHCA+Na+K-H and may reflect the variable cellular K content.
526.33	–	M STD/F SD M STD/M HFHSD+M	–	–	LysoPC C20:4 adduct of type M-H_2O+H is a result of HMDB search, but it is not likely to be produced in MALDI source.
737.45/761.45/ 763.46[c]	M+K	M HFHSD+L/F HFHSD M STD/F HFHSD M HFHSD+L/F HFHSD+L	PA C36:3/PA C38:5/PA C38:4	Triglyceride/ phospholipid biosynthesis PIP_2/DAG signaling Insulin sensitivity	DG C38:4;O, DG C40:5;O, PG C31;O2, and PG C36:6;O2 are results of HMDB search, which are not present in the METASPACE database due to adduct types not likely to be produced in MALDI source (6).

(Continued)

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TABLE 1 Continued

m/z (Da)[a]	Adduct	Treatment pairs	Tentative endogenous metabolite annotation[b]	Metabolic and physio-logical role	Comments
		M STD/F HFHSD+L F STD/F HFHSD+M M HFHSD+M/ F HFHSD+M M HFHSD+L/F SD M STD/F STD M SD/M HFHSD M HFHSD+M/ M HFHSD+L M STD/M HFHSD+M			
762.46	–	M HFHSD+L/F HFHSD M HFHSD+L/F STD	–	–	PS C29:0 adduct of type M+H+HCOONa is a result of HMDB search only, but it is not likely to be produced in MALDI source. PG(PGJ2/i-12:0) adduct of type M+NH4 is also a result of HMDB search only, but it is not likely to be present in muscles. This m/z may correspond to PA C38:5 containing a (13)C atom.
758.57; 796.53, 797.56, 798.54, 806.54, 820.52, 821.52, 822.54, 824.55, 846.53, 848.56[c]	Different types	M STD/F HFHSD M HFHSD+L/F HFHSD+L M STD/F HFHSD+L M HFHSD+L/F HFHSD+M M STD/F HFHSD+M M HFHSD+L/F STD M STD/F STD M HFHSD+L/ M HFHSD M STD/M HFHSD M HFHSD+M/ M HFHSD+L M STD/M HFHSD+M	Different phospholipids	Insulin sensitivity	– (6, 7)

[a]FDR corrected pairwise Dunn–Bonferroni test applied on strong m/z signals ($p < 0.05$). [b]METASPACE (8) (https://metaspace2020.eu) and HMDB (9) search using ±10 ppm acceptance limit. [c]Some of the listed m/z signals are not significantly altered in all treatment pairs. F, female; HFHSD, high-fat and high-sucrose diet group; HFHSD+M, HFHSD treated with metformin; HFHSD +L, HFHSD treated with liraglutide; STD, standard diet group; M, male; m/z, mass-to-charge ratio. Each group sample size was eight (4 biological replicates × 2 technical replicates), and the m/z range was set to 300–1,000 Da.

end of the experiment, HFHSD females had glucose tolerance almost twice lower than males (elevated AUC results in GTT obtained with and without mathematical modeling). Relatively low AUC values in weeks 5 and 12 in SD and HFHSD indicate a prediabetes period. Our result speaks in favor of the hypothesis that prediabetes is a reversible condition, so we see a small difference between STD and HFHSD. Decompensation in glucose tolerance occurs somewhere between 12 and 18 weeks after prolonged feeding with HFHSD (20). Diminished glucose tolerance as a sign of β-cell loss was considerably higher in females than in males, and it supported earlier onset of diabetes in females compared to males. The increased number of mitochondria by itself posed a female-specific risk for the development of skeletal muscle insulin resistance due to increased reactive oxygen species (ROS) production (37, 38). Nevertheless, the increased mitochondria mitogenesis and their decreased efficacy represent potentially a female-specific protective response to HFHSD oriented toward the dissipation of excess energy.

On the other hand, HFHSD males developed liver steatosis accompanied by elevated levels of cholesterol and triglycerides in the blood, while the pathological changes of skeletal muscles included fat droplets and a significant decrease in IMP. The hepatic findings were consistent with research results for nonalcoholic fatty liver disease, recently renamed metabolic-associated fatty liver disease in order to reflect its association with metabolic syndrome morbidity, where men were more often affected with the disease than women (39–43). The skeletal muscle findings could be interpreted as a sign of reduced physical

activity accompanied by a change in diet (44) and diminished supplies for ATP biosynthesis (31).

Potential HFHSD-induced leptin and insulin resistance of satiety centers contributed to multiorgan disorder in prediabetes

The disturbance of the satiety nuclei in the brain (reflected in the G_0 parameter) was obligatory in the HFHSD-induced pathophysiology of DM2 in both sexes. Moreover, this aspect of diabetes development was documented through increased caloric intake. Satiety centers are influenced by a variety of nutritional factors (glucose, fatty acids, amino acids) and different hormones—insulin and leptin being the most important. In fact, the opposing action of insulin and leptin can explain why animals with HFHSD did not develop polyphagia despite the potential development of central insulin resistance (45). However, the satiety nuclei are not only involved in feeding control but also in various autonomous reactions, such as sympathetic activity, cardiovascular output, stress response, etc. What is important for this study is that the lateral hypothalamic satiety nucleus (LH) inhibits the activity of the paraventricular (PVN) (46) stress response-mediating nucleus and thus exerts insulin-mediated negative feedback regulation of the stress response. Judging by the size of the adrenal gland, which was significantly larger in females than in males on the HFHSD, the central stress homeostasis regulation was more efficient in males than in females. Nevertheless, the development of insulin and leptin resistance in the satiety centers explained how malfunction of the hypothalamic neuronal network contributed to a multiorgan disorder (Figure 6). Clinically relevant, glucose set point (as a functional output of satiety nuclei) which was reflected in mathematical parameter G_0, proved to be reversible in STD groups, while caloric intake returned to the starting level over time in HFHSD groups.

HFHSD increased the production of proinflammatory metabolites of the kynurenine pathway

Another sign of the onset of diabetes in both sexes due to HFHSD was increased production of xanthurenic acid 8-*O*-sulfate and quinolinic acid (putatively identified) in skeletal muscle tissue. These metabolites were generated in the kynurenine pathway, a major pathway for tryptophan metabolism, which is activated in diabetes and shown to contribute to inflammation, oxidative stress, and beta-cell dysfunction (47).

Can prediabetic therapy slow the development of diabetes despite a diabetogenic diet?

The sex-specific effects induced by HFHSD included diminished glucose tolerance and a higher stress response in females, and they may have several important translational connotations. If aging is the main cause of metabolic deterioration, accelerated by HFHSD, then a dietary lifestyle intervention would be necessary but may not be sufficient for metabolic correction. From the example of STD females, an early intervention is desirable and should aim to correct glucose variability. Metformin and liraglutide are obvious choices for medication due to their anti-senescence or anti-obesogenic effects, respectively.

Metformin had more benefits for females than for males due to the systemic disturbance of lipid metabolism

The two antidiabetic drugs had different sex-specific effects when tested in this long-term study. Metformin proved to be effective in the treatment of diabetes in both sexes. The beneficial effect was observed after the first week of treatment, in which animals reduced their food intake. The reduced food intake remained visible until the end of the study, and subsequent analysis of the hypothalamic satiety nuclei showed in three out of the four observed nuclei an increased level of leptin receptors (what could be interpreted as increased sensitivity to leptin). Changes in ObR expression are associated with changes in feeding behavior (48, 49). Therefore, a more precise assessment of sensitivity to leptin could be obtained by a functional study, i.e., administration of recombinant leptin and subsequent assessment of the amount of food consumed, as well as immunochemical determination of downstream molecules in the ObR signaling pathway (pSTAT3 and cFos) of the satiety nuclei (50).

Although metformin reduced food intake, this was not reflected in the morphology of visceral adipose tissue or plasma leptin levels. Just the opposite, HFHSD+M males had increased the surface area of adipocytes and fat droplets in adipocytes, liver, and skeletal muscle when compared to HFHSD males. Subsequently, the inflammation in adipose tissue increased, and it promoted systemic insulin resistance, which reduced the overall beneficial effects of metformin. As already described, enlarged adipocytes become dysfunctional in diabetes and secrete less protective and more inflammatory adipocytokines. When their fat storage capacity is exceeded, fats are stored in other tissues such as the liver, skeletal muscle, and pancreas, and they contribute to their insulin resistance or insulin secretion, as shown in this study as well (51). Metformin is known as an insulin-sensitizing medication, whose effect is achieved by activation of AMP-activated protein kinase (AMPK) and further inactivation of acetyl–CoA carboxylase (Acc1 and Acc2) by phosphorylation (52). The positive effects of metformin treatment come from the very blocking of lipolysis and lowering of the amount of free fatty acids whose lipotoxicity promotes insulin resistance of the liver and skeletal muscle (53).

Females responded better to metformin treatment than males. The beneficial effects, contrary to males, have been observed as improved glucose tolerance: parameters relevant for diabetes (G_{max}, G_0, and $G(2)$) decreased in comparison to the HFHSD-untreated females. Although serum fasting insulin and HOMA-IR, as signs of peripheral insulin resistance, did not decrease significantly,

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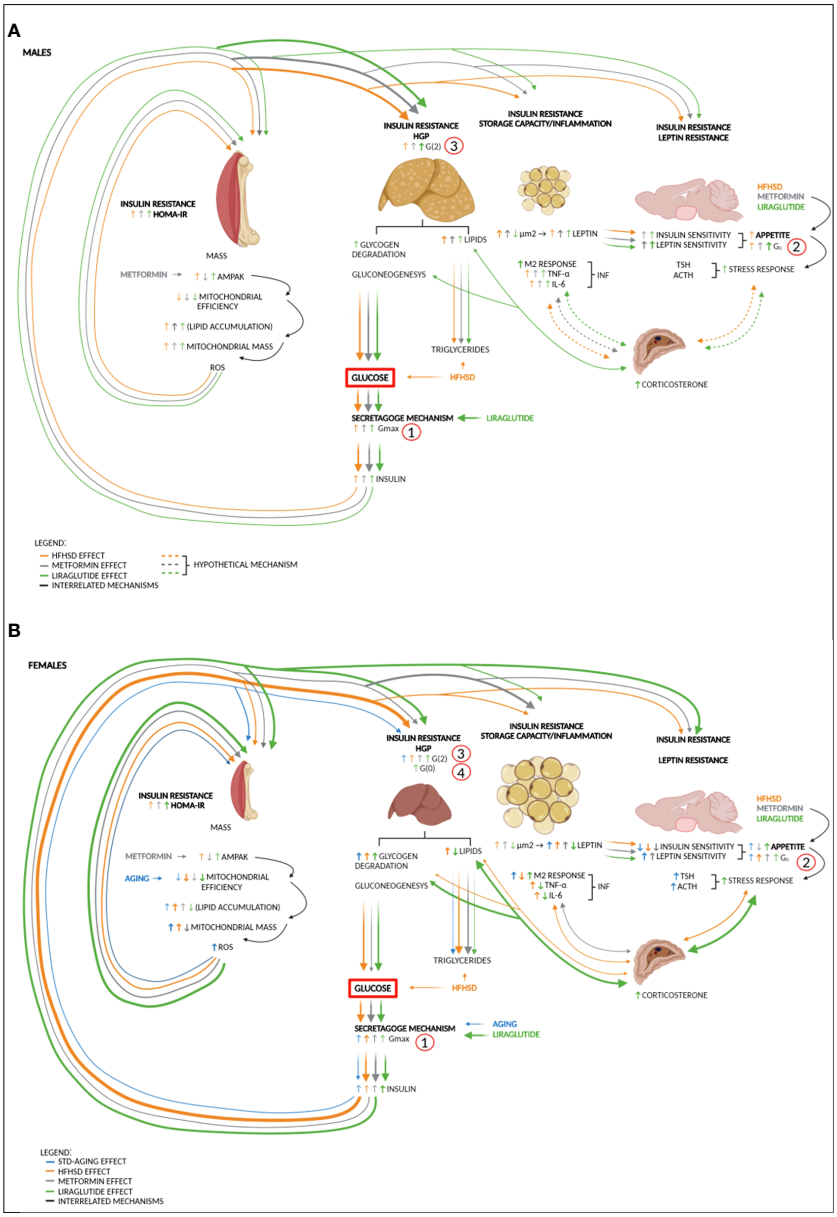

FIGURE 6
Sex-specific impairments in different organs contribute to DM2 to varying degrees—proposed mechanism. **(A)** High-fat and high-sucrose diet (HFHSD) is the major trigger of metabolic changes in males. Diet-induced hyperglycemia and hyperlipidemia burden the secretagogue mechanism, which leads to an increase in glucose variability (G_{max}) and hyperinsulinemia. Hyperglycaemia and hyperinsulinemia increase hunger drive and gradually lead to insulin resistance of skeletal muscle (HOMA index), liver (hepatic glucose production (HGP), $G(2)$), and adipose tissue (fat storage capacity and the appearance of inflammation: M2 macrophages, TNF-α, and IL-6). In males, the liver is more sensitive to hyperinsulinemia than skeletal muscle, so metabolic-dysfunction-associated fatty liver disease (MAFLD) caused by HFHSD occurs more quickly. Under the action of antidiabetic drugs metformin and liraglutide, the sensitivity of the hypothalamic nuclei to insulin and leptin increases. Although both antidiabetics are anorexigenic, there is a gradual increase in the glucose set point (G_0) under the influence of HFHSD. Potentially, further degradation of the satiety mechanism and low-grade inflammation in adipose tissue can lead to an increase in the stress response and entry into a vicious circle in which the stress response increases HGP (reflected in the increase of $G(2)$ mathematical parameter) and contributes to insulin resistance of other organs. **(B)** In females, aging and especially HFHSD increase glucose variability. HFHSD-induced hyperinsulinemia affects skeletal muscle more than in males, which is seen as increased mitogenesis, reduced mitochondrial efficiency, and a less-efficient ROS response, which by itself increases muscle insulin resistance. The introduction of antidiabetic drugs only increases sensitivity to leptin but not to insulin. In liraglutide-treated females, plasma leptin and insulin sensitivity in the satiety centers are so low that both no longer contribute to extinguishing the stress response. The increased stress response further increases HGP, and in females treated with liraglutide, this leads to the full picture of DM2 (increased $G(0)$ (fasting glucose) and hyperphagia). Metformin, as an insulin sensitizer, stops the activation of the stress response and reduces the mass of mitochondria in skeletal muscle, thereby delaying the onset of DM2. AMPAK, AMP-activated protein kinase pathway; DM2, diabetes type 2; G_0, blood glucose set point; $G(0)$, fasting glucose; $G(2)$, plasma glucose 2 h after load; G_{max}, maximal glucose concentration after glucose load; HFHSD, high-fat and high-sucrose diet; HGP, hepatic glucose production; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; IL-6, interleukin 6; M2, macrophage lineage; TNF-α, tumor necrosis factor-alpha. Numbers (1, 2, 3, and 4) indicate the sequence of events in DM2 development. Note that the thickness of the line indicates the strength of the effect. Dashed lines represent hypothetical mechanisms.

mitochondrial mass in skeletal muscle decreased. The reduction in mitochondrial mass corresponded with the finding of elevated levels of PA in the metabolic profile of the skeletal muscle of HFHSD+M females and its positive effect on mitochondrial fusion (54). Moreover, metformin in females was associated with decreased expression of superoxide dismutase (SOD), a central redox enzyme, which was also a bifurcation point between two signaling pathways that were involved in matching the efficacy of mitochondria with metabolic energy needs. Diminished levels of superoxide dismutase (SOD) enable the targeted propagation of superoxide signaling toward aconitase, an enzyme from the citric acid cycle serving as a metabolic switch in mitochondrial uncoupling and safe deciphering of energy (55–57). These two, elevated PA and downregulated SOD, can be related to the eventual counteracting of HFHSD-induced H_2O_2 production and lipid peroxidation.

In conclusion, the group of males treated with metformin maintained their prediabetic status, and the group of females maintained their diabetic status. The numerous beneficial effects justified the use of metformin as a prediabetic drug, especially in females. It would certainly be worth checking its effectiveness in combination with dietary measures and physical activity in future animal studies.

The short-term positive effects of liraglutide are lost in long-term treatment due to hyperinsulinemia

The liraglutide treatment in both sexes was associated with initially reduced food intake, a significant reduction in the surface area of visceral adipocytes, and lower leptin levels. In addition, liraglutide also showed remarkable effects in reversing fatty liver changes and reducing peripheral inflammation. These findings were in accordance with the observed weight-reducing effects of liraglutide, where a significant overall decrease in the percentage of adipose tissue was frequently reported (8, 58). Without any doubt, these effects of liraglutide contributed to the almost complete normalization of glucose tolerance in both sexes after 7 weeks of treatment. Unexpectedly, the effect disappeared during the following 7 weeks of treatment: males outperformed their control HFHSD group and increased G_0 and $G(2)$ (which correspond to blood glucose set points and plasma glucose 2 h after load) up to diabetic values while females developed full clinical picture of DM2 (elevated $G(0)$, fasting glucose).

We tried to identify what led to this unexpected deterioration in both sexes. Liraglutide treatment in males increased insulin and leptin sensitivity in satiety nuclei. Still, the G_0 in this group surpassed that of other male groups. Conspicuously, the glycogen storage in the subcapsular part of the liver was depleted together with the fat droplets, which we interpreted as a sign of hepatic insulin resistance despite its recovery from steatosis. This was supported by the fact that serum fasting insulin and HOMA-IR were at the same level as in the HFHSD group, probably due to the major liraglutide effect of hyperinsulinemia. Despite the mentioned negative effects, IMS analysis of the skeletal muscle showed elevated levels of PA and various phospholipids, which contributed to regaining characteristics of STD males. Namely, in addition to the positive effect of PA on mitochondrial dynamics, phospholipid levels were previously positively associated with mitochondrial efficiency and skeletal muscle sensitivity to insulin (59).

In the case of liraglutide treatment of females, despite short-term positive effects, long-term treatment turned out to be deleterious. Not only was glucose tolerance not significantly improved, but the treated females had the highest serum basal insulin levels, the most pronounced peripheral insulin resistance, and the largest adrenal glands (suggesting the most hyperactive stress response). The sex differences in liraglutide response could be partially explained by human data showing a 24% lower weight-adjusted clearance in women compared to men (60). In accordance with previous literature (48), our results suggested that animals treated with liraglutide had profound central effects of treatment. Glucagon-like peptide 1 receptor (GLP1R) was expressed on satiety nuclei in the brain, and therefore the central action of its agonist liraglutide was expected. Also, polyphagia and high glucose set-point in HFHSD+L females can be explained by the main peripheral action of liraglutide, stimulating insulin secretion and concomitant development of insulin resistance primarily in the satiety nuclei. The argument about insulin resistance of satiety nuclei was based on the reduced expression of IR, a phenomenon associated with hyperphagia (61). For final proof of insulin resistance, it would be necessary to perform a functional study—to apply insulin and determine the values of pAkt and pGSK3 in the tissue 1 h after insulin application (62). In addition, hyperinsulinemia was combined with a loss of protective leptin signaling due to a decrease in adipocyte surface area and a consequent decrease in their ability to excrete leptin and adiponectin. All these results suggested that the females treated with liraglutide have a high tendency to develop adipocyte insulin resistance—an inability to store lipids (or blocked lipolysis) and excrete leptin at sufficient levels to counteract central insulin resistance. Moreover, they concomitantly developed exaggerated stress responses. Sympathetic activation is demonstrated to promote the conversion of stored lipids into energy metabolism pathways (63). Intriguingly, the liraglutide-treated animals exhibited significantly smaller visceral adipocytes compared to other HFHSD-fed animals, which could also be due to the centrally dysregulated sympathetic activity.

Unlike males, liraglutide did not increase PA or phospholipid levels in female skeletal muscle, but it did increase xanthurenic acid 8-*O*-sulfate, which was expected to increase natriuresis (64) and consequent glucouresis. This may explain previously reported liraglutide-induced natriuresis that has not been mediated by natriuretic peptides (65). Given that xanthurenic acid interferes with the synthesis of insulin in β-cells and creates inactive complexes with insulin (47, 66, 67), it could be part of the last protective mechanism that acts against hyperinsulinemia and insulin resistance and reflects the severity of the diabetic disorder. In these circumstances, the development of insulin resistance in female skeletal muscle after liraglutide treatment could be considered a protective mechanism that saves energy-hungry muscle (loaded with mitochondria) from glucose loss in postprandial hypoglycemia that occurred after hyperinsulinemia

following hyperphagia in conditions of gradual exhaustion of all energy stores.

We do not know if longer follow-up would result in a similar effect of liraglutide in males. It is worth noting that HFHSD+L males had the lowest glucose tolerance among all male groups, which meant that they had a significant loss of β-cells and were close to decompensation. Yet, liraglutide has its place in prediabetic therapy, especially in men, under the condition of personalized dosing and strict control of hyperinsulinemia.

The strengths and limitations of the study

The strength of our study is that it included animals of both sexes, the use of a mathematical model that sheds light on the sequence of metabolic deterioration from prediabetes to diabetes, and finally whole-body analysis. In translational studies, the female gender is less represented due to difficulties in achieving synchronization in the estrous cycle (typical for rats) and the expectation of large variations in biochemical parameters influenced by sex hormones. Recent studies show that the variation among females (unstaged for cycle) is not greater than the variation among males (68), so excluding females is one of the obstacles to gender-sensitive personalized medicine. The use of mathematical modeling is still rare in biological studies, although it can reveal parameters that have a higher sensitivity than those used in clinical practice. Finally, whole-body analysis is complex, but it can reveal the mutual connection of pathophysiological mechanisms.

We recognize the following limitations of our study: (1) variability in female animals could be smaller if animals were synchronized for their estrus cycle (which was omitted because of animal age, duration of the study, and low variability among females, independent of cycle stage); (2) due to the high number of animals handled at the same time points, even if experiments were performed at the same time of day, certain parameters (like hormones) could be influenced by the circadian rhythm; (3) handling of animals, even if done with special care and by properly trained technicians, could be a source of stress that could be avoided by using metabolic cages and continuous glucose monitoring (which were unavailable due to limited resources); (4) dosage of treatment medications was calculated based on the current human therapy guidelines; however, other doses should also be tested; (5) murine models are not well tested for metabolic syndrome in aged animals, and female predisposition toward development of diabetes might be strain-dependent; (6) assessment of insulin/leptin resistance at the level of the hypothalamic nuclei or skeletal muscle would be more precise if it was done within the framework of a functional study, i.e., after insulin/leptin administration followed by assessment of GLUT4 and STAT3 expression; (7) in addition to IRS-1 phosphorylation, GLUT4 translocation should be determined 30–60 min after insulin challenge; and (8) the use of additional methods directed at whole-body changes in mitochondrial function (indirect whole-body calorimetry) should be recommended. The finding of liraglutide side effects was unexpected but was reproduced in a replicated study (data not yet published).

Prediabetic interventions should start earlier and become sex-specific

In conclusion, the pathophysiology of DM2 is very complex and requires the monitoring of several clinical parameters instead of focusing solely on insulin insensitivity. A plethora of impairments in many different tissues and organ systems contribute to DM2 in various degrees, including the liver (impaired carbohydrate metabolism, hyperreactive gluconeogenesis), skeletal muscle (impaired glucose uptake and energy metabolism), adrenal glands (impaired stress responses), adipose tissue (impaired secretion of adipocytokines), brain (impaired central regulation of energy homeostasis and stress reaction), and possibly many other organs (69, 70). While this study undoubtedly confirmed the complexity of multiple organ involvement in the development and progression of DM2, it also shed light on the role of sex differences, aging, and the relative contribution of various organs or tissues to disease severity. Moreover, significant sex differences were noted even in response to antidiabetic medication, which was strikingly obvious from the MALDI-TOF skeletal muscle analysis of the treated animals. Although neither of the two antidiabetic drugs used as prediabetes treatment, metformin and liraglutide, were able to reverse HFHSD-induced DM2, metformin was the superior intervention over liraglutide due to improved central leptin sensitivity and peripheral insulin sensitivity in females. The short-term success and long-term failure of liraglutide therapy can be explained by its central effect on satiety nuclei and hyperinsulinemia, which ultimately lead to insulin resistance. Thanks to its positive effects on the metabolism of skeletal muscle, liver, and adipocytes, with good titration, liraglutide can find its place, especially in the treatment of males. If some future prediabetic therapy is to be considered, this study suggests that its success will depend on the correct identification of early biomarkers of prediabetes and, equally early, the application and monitoring of a well-targeted sex-specific approach.

Methods

Animal model and study design

The animal study was approved by the National Scientific Ethical Committee on Animal Experimentation (Hungary, registration number: IV/3084/2016). The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII. tv. 32.§).

The study was carried out on 32 male and 32 female Sprague–Dawley rats (Innovo Ltd, Gödöllő, Hungary). Three-week-old rats were fed *ad libitum* with standard rodent chow (Innovo Ltd, Gödöllő, Hungary) and water. They were kept in cages in a room with

controlled temperature (20°C–23°C), humidity (40%–60%), and light/dark cycle (12 h light/12 h dark). STD consisted of 65% carbohydrate (5% disaccharide, 39% polysaccharide), 11% fat, and 24% protein. When the rats reached 45 weeks of age (week 0 of the experiment), they were randomly separated into four groups, each composed of eight males and eight females: (1) STD, (2) HFHSD, (3) HFHSD+M, and (4) HFHSD+L. The STD group continued consuming standard food until the end of the experiment, while others were transferred to HFHSD (Altromin Spezialfutter GmbH & Co, Lage, Germany, Cat. No. C-1101) consisting of 56% carbohydrate (18% disaccharide, 36% polysaccharide), 28% fat, and 16% protein. From experimental week 6 (when the rats were 51 weeks old), HFHSD+M group was treated subcutaneously with 50 mg/kg/day of metformin [resuspended in sterile/distilled water (50 mg/mL)]; Sigma Aldrich, Budapest, Hungary) and the HFHSD+L group was injected subcutaneously with 0.3 mg/kg/day of liraglutide (resuspended in sterile/distilled water (50 mg/mL)); Creative Peptides Inc., Shirley, NY, USA). STD and HFHSD groups were administered with vehicle (sterile/distilled water) only. The dose was determined according to reports in the literature (71, 72). Antidiabetic treatment lasted for 13 weeks, until the end of the experiment when rats reached 64 weeks of age.

Body mass and food consumption were measured weekly (every morning of the first day of each experimental week) using a digital scale (SPX621, Ohaus Corp., Parsippany, NJ, USA). Food consumption was calculated weekly by the rodent pellet reduction in the feeder rack of cages and expressed as caloric intake (kcal/g of body mass) per animal group. The metabolic energy of the STD was 2.84 kcal/g and of the HFHSD was 3.89 kcal/g.

During the experiment, several animals succumbed, as follows: three males and one female from the STD group died during or after the GTT due to aortic aneurysm; one female from the HFHSD+M group died due to pulmonary edema; and one female from the HFHSD +L group died due to abdominal tumor and vaginal bleeding.

At the end of the experiment, animals were sacrificed during deep isoflurane anesthesia (Forane) (Baxter Healthcare Corp. Deerfield, IL, USA) by cardiac puncture, followed by the collection of whole blood. Prepared serum and plasma samples were stored at −20°C for later analysis. The organs (brain, liver, adrenal glands, adipose tissue) were weighed, snap frozen, or fixed with 4% paraformaldehyde, as previously described (73), and stored at −80°C for further molecular studies. Nuchal region skeletal muscles (*Semispinalis capitis*, *Splenius capitis*, and *Splenius cervicis*) were cryoprotected and embedded in methylcellulose that does not interfere with iMS. Histology was performed on fixed cryoprotected sections, and iMS on fresh frozen. Part of the tissue necessary for histological staining was embedded in paraffin (adipose tissue, adrenal glands, liver). Liver mass to body mass ratio was calculated and shown as a percentage.

Glucose and insulin tolerance tests

The GTT was carried out four times, as follows: at the beginning of the experiment (week 0), immediately before beginning treatments with antidiabetics (metformin, liraglutide; week 5), after 6 weeks of treatments (week 12), and the week before animals were sacrificed (week 18). Animals fasted for 16 h before the GTT. Fasting glucose level was measured first, followed by intraperitoneally injection of glucose solution (25%) at a 2-mg/kg dose. Blood glucose levels were determined 15, 30, 45, 60, 90, 120, and 240 min after the injection. Blood samples were obtained from a tail vein using a needle to collect one drop of blood (5 μL) and place it on a test strip. Blood glucose levels were determined using a glucometer (OneTouch UltraMini, Milpitas, CA, USA), and glucose concentration curves were plotted. The ITT was carried out during the week the animals were sacrificed (week 18). Animals fasted for 4 h before the ITT. The fasting glucose level was measured first, and then each rat was injected intraperitoneally with 0.5 U/kg of Humulin R insulin (Eli Lilly, Indianapolis, IN, USA). Blood glucose levels were determined 15, 30, 45, 60, 90, 120, and 180 min after the injection.

In addition to calculating the AUC, the measurements were also used for plotting the model function of glucose concentration during GTT and ITT. AUC determination and modeling were performed as previously described (35). NonlineraModelFit module of Mathematica (ver. 11.0, Wolfram Research, Inc., Champaign, IL, USA) was used to solve functions describing glucose concentration fluctuation during GTT and ITT. The following obtained parameters revealed alterations in glucose dynamics: fasting blood glucose concentration ($G(0)$ in GTT/$H(0)$ in ITT), maximal/minimal glucose concentration (G_{max}/H_{min}) and corresponding moment (t_{max}/t_{min}), 2-h blood glucose ($G(2)$), coefficient of oscillation amplitude decline (α), basic period of function (T), blood glucose setpoint (G_0/H_0), initial speed of blood glucose increase/decrease ($G'(0)$)/($H'(0)$), blood glucose concentration at which maximal speed of glucose concentration decrease/increase is attained (G_I), maximal speed of glucose concentration decrease (G'_I) and corresponding moment (t_I). To evaluate the goodness-of-fit of the model, a determination coefficient (R^2) was calculated.

Tissue, serum, and plasma measurements

Serum fasting insulin was measured using a Rat Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH, USA). Plasma leptin, adiponectin, corticosterone, and TNF-α, IL-1, and IL-6 from subcutaneous and visceral adipose tissues were measured using appropriate ELISA kits from R&D Systems (Minneapolis, MN, USA). Phosphorylated IRS-1 from the liver, visceral adipose tissue, and muscles were measured using a Phospho-IRS-1 (panTyr) ELISA kit (Cell Signaling Technology, Danvers, MA, USA). The enzymes AST and ALT were measured using standard clinical laboratory methods. HOMA-IR was calculated using insulin and glucose data with equation HOMA-IR = {fasting glucose (mg/dL) × fasting insulin (μU/mL)}/405.

Adipose tissue histomorphometry

Visceral adipocyte surface areas were measured based on hematoxylin and eosin histological staining (HE) of 5-μm-thick sections of paraffin-embedded tissue using following protocol

(xylene for 10 min; 100% ethanol (EtOH), 100% EtOH, 96% EtOH, 70% EtOH for 5 min each; distilled water (dH_2O) for 5 min; Mayer's hematoxylin for 10 min; dH_2O for 1 min; tap water for 10 min; dH_2O for 1 min; eosin Y for 30 s; dH_2O for 5 s; 70% EtOH, 96% EtOH, 100% EtOH at 5 dips in each, and 100% EtOH for 3 min; xylene for 5 min and covesliped). Digital micrographs, collected by an Olympus D70 camera (Olympus, Hamburg, Germany) set up on a Zeiss Axioskop 2 MOT microscope (Carl Zeiss Microscopy, Thornwood, NY, USA), were analyzed in CellProfiler (v. 3.1.9) using a semiautomated protocol consisting of several modules (74). Each micrograph was split into three color channels. The green channel was converted to a grayscale image for subsequent analysis. In the next module, the adipocytes were identified as primary objects based on their typical diameter and intensity range, as determined manually for each micrograph and using a global threshold strategy, which classifies the pixels above the threshold as foreground (i.e., adipocytes) and below as background. Otsu's algorithm was used as a thresholding method because the percentages of areas covered by the foreground varied. Objects touching the borders of the images were discarded from further analysis. When a single adipocyte was identified as two or more objects due to the intensity gradient, the Split or Merge Objects module was applied, using the distance-based merging method. Finally, the surface areas of the identified adipocytes were measured in pixels and converted to square micrometers using the scale bars of the original micrographs. Furthermore, surface areas were divided into four distinct adipocyte size classes, based on the distribution of median values and upper and lower quartiles in STD groups: class 1 (< 2,197.5 μm^2), class 2 (2,197.5–4,395 μm^2), class 3 (4,395–6,592 μm^2), and class 4 (> 6,592 μm^2).

Adrenal gland histopathology

Paraffin-embedded adrenal glands were cut using a microtome (Leica SM2000R; Nussloch, Germany) into 5-µm-thick sections. The HE staining of tissue was followed by a collection of digital micrographs at ×100 magnification.

Analysis of liver fat droplets, glycogen, and ferric ion

Liver tissue was cut using a cryostat (Leica CM3050S; Nussloch, Germany) into 20-µm-thick sections. Liver fat droplets were stained using Sudan Black B to show overall fat distribution and Oil Red O for quantification, as described by Vacca (75). Digital micrographs were collected at 200× magnification. Fat droplet size determination was performed using FIJI software (76). Images were split into red, green, and blue channels and converted into 8-bit images. The red color threshold was set at 0-100, and the surface area of the remaining particles was analyzed. The size of fat droplets was expressed in square micrometers.

Liver glycogen was stained using a metachromatic toluidine stain, as described by Vacca (75). Digital micrographs were collected at 400× magnification. The images were deconvoluted with a Feulgen light green vector in FIJI software, and Color 1 was used to measure integrated density value (IDV). Glycogen values are presented as positively correlated integrated color density (the number obtained from quantification was subtracted from maximal IDV, which corresponds to the total pixel number of an image multiplied by 255).

Skeletal muscle analyses

Paraformaldehyde-fixed skeletal muscle tissue from nuchal region tissue was cut using a cryostat into 35-µm-thick sections. Skeletal muscle fat droplets were stained using Oil Red O, as described by Vacca (75). Digital micrographs were collected at 200× magnification and analyzed using FIJI software. Images were split into red, green, and blue channels. In order to characterize fat droplets, the green channel threshold was set at 0–140, and the remaining particles were segmented by *binary/watershed*. The resulting particles were analyzed with a lower limit of 25 square pixels. The fat droplet size is presented in square pixels. To determine the number of muscle fibers in a section, edges were detected in a green channel, whose threshold was set at 0–35. With interior holes included, the remaining particles were analyzed with a lower limit of 2,000 square pixels. To obtain an average count of fat droplets per fiber, the total number of droplets per section was divided by the number of muscle fibers in a given section.

To determine skeletal muscle fiber type composition, snap-frozen skeletal muscles were cut using a cryostat into 14-µm-thick sections. Slices were stained with succinate dehydrogenase, as described by Vacca (75). Digital micrographs were collected at 200× magnification and analyzed using FIJI software. Images were split into red, green, and blue channels. Only a transversely cut area of the sample was selected for further analysis. The selected region was duplicated, the background was subtracted, and segmentation was performed using the Statistical Region Merging algorithm. Q was set to 4: background and three muscle fiber types (I, IIa, IIb). To quantify areas of the specific muscle types, the image threshold was set at 0–120 for type I fibers, 121–180 for type IIa fibers, 181–240 for type IIb fibers, and 0–240 for total area. The protocol was adjusted to conform to the muscle fiber classification described by Kano et al. (77, 78).

Analysis of snap-frozen skeletal muscle tissue was used to determine lipid peroxidation (LPO), total glutathione (tGSH), and the activities of the following antioxidant enzymes: glutathione reductase (GR), glutathione *S*-transferase (GST), catalase (CAT), and superoxide dismutase (SOD).

LPO was estimated by measuring the thiobarbituric acid reactive substances (TBARS), according to the method described by Ohkawa et al. (79) The TBARS were calculated according to a standard curve prepared from 1,1,3,3-tetraethoxypropane and expressed in nanomoles per milligram of fresh tissue weight (nmol/mg FW).

tGSH content was assayed using a spectrophotometric kinetic method based on a reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid by glutathione (GSH), recorded at 412 nm and expressed in nmol/mg FW (80).

Protein extracts (1:10, w/v) were prepared for the antioxidant enzyme activity assay by homogenizing tissue in 100 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and by centrifugation at 20,000×*g* for 15 min at 4°C. Protein concentration in extracts was estimated using the Bradford assay (81).

GR activity was determined indirectly by measuring the consumption of NADPH during GSSG reduction, demonstrated by a decrease in absorbance at 340 nm. The assay mixture (1 mL) consisted of 1 mM GSSG, 0.1 mM NADPH, and protein extract in 100 mM phosphate buffer (pH 7.5). The GR activity was calculated using a molar extinction coefficient for NADPH (ϵ = 6.220 mM/cm) and expressed in units per gram of (U/g) protein (82).

GST activity was determined by measuring the conjugation of 1-chloro-2,4-dinitro benzene (CDNB) with GSH, demonstrated by an increase in absorbance at 340 nm (83). The GST activity was calculated using a molar extinction coefficient of glutathione-1-chloro-2,4-dinitrobenzene conjugate (ϵ = 9.6 mM/cm) and expressed in U/g protein.

CAT activity was estimated spectrophotometrically using H_2O_2 as a substrate, as described by Aebi (84) and expressed in U/g protein.

SOD activity was determined by measuring the inhibition of cytochrome c reduction with superoxide radicals generated by the xanthine/xanthine oxidase system. The reduction rate was recorded spectrophotometrically at 550 nm (85). Results were expressed in units per milligram of proteins, where one unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of cytochrome c reduction under the assay.

MALDI-TOF skeletal muscle analysis

MALDI-TOF IMS analysis was performed using the Shimadzu IMScope TRIO MALDI-IT-TOF MS instrument (Shimadzu, Kyoto, Japan). Fresh-frozen nuchal skeletal muscle tissue sections (25 µm thick) were mounted on an indium tin oxide (ITO)-coated glass slide, with a surface resistivity of 15–25 Ω/sq (Sigma-Aldrich, St. Louis, MO, USA). After snap washing with 20 mM ammonium acetate buffer, the sections were dried and immediately further processed. Matrix α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich, St. Louis, MO, USA) was applied to samples using an iMLayer sublimation device (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions (10 min sublimation at 180°C). Sublimation was followed by 2 min of recrystallization at 70°C with 0.5% methanol in a vapor chamber.

Imaging in the positive ion mode was performed using m/z ranges 300–700 and 700–1,000 Da with the following setup: approximately 500 pixels with a pitch of 10 × 10 µm, a laser diameter of 10 µm, a laser intensity of 15%, 50 laser shots/pixel, and a 200-Hz laser frequency. Data analysis was performed with R software (86) ver. 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria). Total-ion-current (TIC)-normalized m/z signals were used for the image generation and data analysis. Only the strong, TIC-normalized m/z signals averaged over all pixels were used in the statistical analysis; strong m/z signals were the ones for which the sum of intensities across all images (ΣIm/z) was greater than 5% of the largest ΣIm/z. For the selection of significant m/z signals, a FDR-corrected KW ANOVA followed by pairwise Dunn–Bonferroni test was used. Graphical presentation of the IMS results was performed by ImageReveal ver. 1.1.010128 (Shimadzu, Kyoto, Japan). Human metabolome database (HMDB) (87) and METASPACE database (88) (https://metaspace2020.eu) were used for the tentative metabolite identification. ± 10 ppm m/z accuracy tolerance and a statistical significance cutoff of 0.05 were used in all instances.

Immunohistochemistry

Macrophages of M1 and M2 phenotypes were immunostained on 5-µm-thick sections of visceral adipose tissue and 20-µm-thick cryosections of the liver. IR-α, ObR, IGF-1Rβ, Iba1, and GFAP were immunohistochemically stained on 35-µm-thick coronal brain cryosections.

Slide-mounted sections of visceral adipose tissue were stained using the VENTANA Benchmark Ultra IHC/ISH System (Roche, Basel, Switzerland) and anti-CD68 (clone KP-1; Roche) and anti-CD163 (clone MRQ-26; Roche) antibodies.

Liver and brain tissues were stained by free-floating immunohistochemistry developed with 3,3′-diaminobenzidine (DAB) as previously described by our group at 4°C and without detergents applied. The following antibodies were used: rabbit anti-CD197 diluted 1:1,000 (Abcam, Cambridge, UK) and rabbit anti-CD206 diluted 1:1,000 (Abcam) for liver tissue; rabbit anti-alpha subunit of IR-α diluted 1:250 (IR-α; Santa Cruz Biotechnology, Dallas, TX, USA; SC-710), rabbit anti-beta subunit of IGF-1Rβ diluted 1:250 (IGF-1Rβ; Santa Cruz Biotechnology; SC-713), rabbit anti-ObR diluted 1:50 (ObR; Santa Cruz Biotechnology; SC-8325), and biotinylated goat anti-rabbit IgG diluted 1:1,000 (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) for brain tissue.

GFAP and Iba1 expressions were analyzed by free-floating fluorescent immunohistochemistry using the same protocol described above. After the incubation with secondary antibodies, the sections were incubated for 10 min at room temperature with 0.1% Sudan Black B prepared in 70% ethanol to suppress autofluorescence. Afterward, the sections were shortly rinsed with distilled water, slide-mounted, and coverslipped with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Rabbit anti-GFAP diluted 1:4,000 (Dako, Agilent Technologies, Santa Clara, CA, USA), rabbit anti-Iba1 diluted 1:1,000 (Wako Chemicals, Neuss, Germany), and goat anti-rabbit IgG conjugated with Cy3 diluted 1:300 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used.

The digital micrographs of DAB-developed staining of visceral adipose tissue and liver tissue were collected at ×200 magnification. Brain tissue digital micrographs were collected at ×400 magnification from hypothalamic areas associated with energy maintenance: ARC, LH, PVN, and VMH. Immunopositive macrophages were counted per field of view. IR-α, IGF-1Rβ, and ObR immunopositive reactions within the areas of 0.02 mm^2 were

analyzed using FIJI software by the following steps: images were converted to 8-bit, the threshold was set to omit background (0–127 till 142), and the corresponding color area of immunopositive reaction or IDV was measured. ROIs on fluorescent micrographs (areas of five glial cells per image) were analyzed using the Color Pixel Counter plugin for FIJI with a minimum intensity value set to 30 after enhancing local contrast (block size: 127, histogram bins: 256, maximum slope: 3.00, mask: none) and adjusting the gamma value to 1.80.

Statistical analysis

Statistical analysis was performed using Statistica 12 software (TIBCO, Palo Alto, CA, USA). The statistical significance level was set at $p < 0.05$. For significance analysis of between-group comparisons to determine the influence of sex, intervention, or their interaction, two-way ANOVA was applied followed by Bonferroni or Games–Howell test depending on the equality of variances assessed by Levene's test. To assess the influence of sex, intervention, and duration of intervention on the ratio of the whole-group caloric intake to the whole-group body mass, repeated measures of three-way ANOVA were applied, and the Bonferroni *post-hoc* test was performed. The statistical analysis of MALDI-TOF was performed as mentioned above.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by National Scientific Ethical Committee on Animal Experimentation (Hungary, registration number: IV/3084/2016). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

The authors confirm contribution to the paper as follows: Study conception and design: SV, RG, MH, TT, and SG. Data collection: VI, MZ, MF, KS, FB, SB, RV, AR, DM, ZD, MBer, MBal, AI, MBak, AS-B, and SM. Analysis and interpretation of results: VI, MZ, IL, MH, MF, RS, SB, AR, DM, ZD, MBer, MBal, and SM. Draft manuscript preparation: VI, MF, MH, SB, AR, SG, RG, SV, and MZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1181064/full#supplementary-material

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Long-term green-Mediterranean diet may favor fasting morning cortisol stress hormone; the DIRECT-PLUS clinical trial

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Background: Fasting morning cortisol (FMC) stress hormone levels, are suggested to reflect increased cardiometabolic risk. Acute response to weight loss diet could elevate FMC. Richer Polyphenols and lower carbohydrates diets could favor FMC levels. We aimed to explore the effect of long-term high polyphenol Mediterranean diet (green-MED) on FMC and its relation to metabolic health.

Methods: We randomized 294 participants into one of three dietary interventions for 18-months: healthy dietary guidelines (HDG), Mediterranean (MED) diet, and Green-MED diet. Both MED diets were similarly hypocaloric and lower in carbohydrates and included walnuts (28 g/day). The high-polyphenols/low-meat Green-MED group further included green tea (3-4 cups/day) and a Wolffia-globosa Mankai plant 1-cup green shakeFMC was obtained between 07:00-07:30AM at baseline, six, and eighteen-months.

Results: Participants (age=51.1years, 88% men) had a mean BMI of 31.3kg/m^2, FMC=304.07nmol\L, and glycated-hemoglobin-A1c (HbA1c)=5.5%; 11% had type 2 diabetes and 38% were prediabetes. Baseline FMC was higher among men (308.6 $\pm$ 90.05nmol\L) than women (269.6$\pm$ 83.9nmol\L;p=0.02). Higher baseline FMC was directly associated with age, dysglycemia, MRI-assessed visceral adiposity, fasting plasma glucose (FPG), high-sensitivity C-reactive-protein (hsCRP), testosterone, Progesterone and TSH levels ($p \leq 0.05$ for all). The 18-month retention was 89%. After 6 months, there were no significant changes in FMC among all intervention groups. However, after 18-months, both MED groups significantly reduced FMC (MED=-1.6%[-21.45 nmol/L]; Green-MED=-1.8%[-26.67 nmol/L]; p<0.05 vs. baseline), as opposed to HDG dieters (+4%[-12 nmol/L], p=0.28 vs. baseline), whereas Green-MED diet FMC change was significant as compared to HDG diet group (p=0.048 multivariable models). Overall, 18-month decrease in FMC levels was associated with favorable changes

in FPG, HbA1c, hsCRP, TSH, testosterone and MRI-assessed hepatosteatosis, and with unfavorable changes of HDLc ($p<0.05$ for all, weight loss adjusted, multivariable models).

Conclusion: Long-term adherence to MED diets, and mainly green-MED/high polyphenols diet, may lower FMC, stress hormone, levels,. Lifestyle-induced FMC decrease may have potential benefits related to cardiometabolic health, irrespective of weight loss.

Clinical trial registration: ClinicalTrials.gov, identifier NCT03020186.

KEYWORDS

fasting plasma cortisol, lifestyle intervention, mediterranean diet, weight loss, insulin resistance, cardiometabolic health

1 Introduction

Cortisol is the main glucocorticosteroid (GC) produced by the adrenal glands, specifically in the zona fasciculata of the adrenal cortex and is commonly known as a "stress hormone". It is a key component of the body's stress response system, which is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. In addition, cortisol is involved in many physiological processes including metabolism, glycemic control, immune response, growth, cardiovascular function, mood, cognitive functions, reproduction, and development (1). Cortisol levels can exhibit three distinct patterns of fluctuation: ultradian, characterized by pulses of circulating hormone levels occurring approximately every 60 minutes; circadian, representing diurnal fluctuations; and stimulus-induced, triggered by external components that activate the HPA axis. The basal activity often encompasses both the circadian and ultradian rhythms. In humans, cortisol levels reach their peak in the morning and are lowest at night, primarily regulated by the central pacemaker located in the suprachiasmatic nucleus (2).

Dysregulation of cortisol can lead to significant health implications, primarily by inducing insulin resistance and diabetes through its effects on key organs involved in the pathogenesis of insulin resistance, including the liver, skeletal muscle, adipose tissue, and pancreas (3–6). Several studies have reported an association between elevated GCs levels and the development of multiple adverse metabolic complications, including type 2 diabetes, cardiovascular disease, dyslipidemia, and ectopic fat accumulation (3, 4, 7–10). In addition, prolonged exposure to GC's can lead to the accumulation of central fat, accompanied by low-grade inflammation. Garciía-Eguren et al, identified that, transcriptional and epigenetic signatures induced by elevated cortisol levels in visceral adipose tissue (VAT) may explain the adverse consequences of long-term VAT impairment (11). While research has highlighted the adverse effects of excessive cortisol exposure, it has certain limitations. Much of the research has focused on animal models, while in human studies, the primary emphasis has been on prolonged glucocorticoid therapy and Cushing's syndrome. the determinants and metabolic consequences of elevated cortisol levels within the physiological ranges in humans are yet to be determined.

Studies of lifestyle interventions on changes in GCs levels have yielded mixed results. In a systematic review conducted by Chawla et al., it was observed that the timing of food consumption, particularly time-restricted eating (TRE), can impact the circadian cortisol fluctuations. Notably, during Ramadan practice of TRE, a reduced waking cortisol response was observed, and a statistically significant increase in cortisol levels in the evening compared to non-Ramadan periods. In non-Ramadan TRE studies, skipping dinner was associated with a decrease in evening cortisol levels and potentially an elevated morning level, although not all cases accounted for waking time (12). Additionally, diet content can. Also alter cortisol fluctuations. Shively et al. demonstrated in non-human primates that adherence to the Mediterranean (MED) diet resulted in reduced cortisol responses to acute stress and ACTH challenges, accompanied by increased stress resilience. Furthermore, Carvalho et al. revealed that the MED diet countered the link between various cortisol biomarkers and inflammation (13, 14). Additionally, specific products rich in polyphenols such as Hibiscus sabdariffa, tea, dark chocolate, as well as specific compounds such as resveratrol and epigallocatechin gallate, have shown associations with cortisol dynamics. This suggests a potential role for these compounds in modulating the body's response to stress (15–18). Stimson et al., showed that high-fat low-carbohydrate diet enhance cortisol regeneration and reduce cortisol in activation, independent of changes in energy consumption and weight-loss (19).The relationship between caloric restriction and changes in cortisol levels is unclear. Although some studies suggest that acute short-term caloric restriction can increase in cortisol levels, it appears that cortisol elevation is primarily attributable to fasting or a short duration of caloric restriction rather than a long-term caloric restriction (20–22). The limited number of studies and the variability in nutritional interventions make it difficult to draw clear conclusions on the

impact of lifestyle interventions on cortisol levels, highlighting the need for further research in this area.

In this study, we aimed to investigate the impact of long-term dietary interventions on fasting morning cortisol (FMC) and its association with metabolic health, beyond the effects of weight loss.

2 Methods

2.1 Study design

The DIRECT-PLUS trial (ClinicalTrials.gov ID: NCT03020186) was designed as an 18-month study of the effectiveness of a lifestyle intervention program on weight loss and cardiovascular risk reduction. The trial was conducted in an isolated workplace, the Nuclear Research Center Negev (NRCN) in Dimona, Israel, where the participants had access to a monitored lunch programs. Of the 378 volunteers who initially enrolled, 294 met the inclusion criteria of age greater than 30 years with abdominal obesity (waist circumference of over 102cm for men and over 88cm for women) or dyslipidemia (triglycerides greater than 150mg/dL and high-density lipoprotein cholesterol less than or equal to 40mg/dL for men or less than or equal to 50mg/dL for women). The exclusion criteria were an inability to participate in physical activity, abnormal liver function, a serum creatinine level of 2 mg/dL or higher, major illnesses that might require hospitalization, active malignancy or undergoing chemotherapy within the prior 3 years, participation in another trial, treatment with warfarin (due to its interaction with vitamin K), or having an implant that would preclude magnetic resonance imaging (MRI). Furthermore, none of the participants were on continuous steroid or anti-inflammatory treatment. There were no new diagnoses requiring the initiation of anti-inflammatory or steroid therapies during the study. The study protocol was approved by the Soroka University Medical Centre medical ethics board and institutional review board, and all participants provided written informed consent. The participants did not receive any financial compensation or gifts for their participation in the study. Clinical and medical measurements were taken at baseline, 6-months, and 18-months.

2.2 Randomization and intervention

Participants were assigned to one of three treatment groups: healthy dietary guidelines (HDG), MED diet, or green MED diet. The randomization process is described in detail in Supplementary Data 1. The participants were aware of their assigned intervention (open-label protocol). All participants were provided with a complimentary monitored gym membership and received physical activity instructions. It is crucial to emphasize that this study did not involve a physical activity intervention but rather equal accommodations were provided across all intervention groups. HDG participants were given basic health-promoting dietary recommendations. MED participants were instructed to adhere to a calorie-restricted traditional MED diet, low in simple carbohydrates, as in prior trials (23, 24). The assigned MED diet was high in vegetables, with poultry and fish replacing beef and lamb. MED participants were given 28g of walnuts per day [containing 440 mg polyphenols/day; gallic acid equivalents (GAE), according to United States Department of Agriculture (USDA) Phenol-Explorer: http://phenol-explorer.eu/food-processing/foods, including, mostly, ellagitannins, ellagic acid and its derivatives (25)]. *Green-MED* participants were also given 28g/day walnuts and instructed to avoid red/processed meat. The green MED diet was richer in plants and polyphenols, as participants were specifically provided with 3-4 cups of green tea per day and 400ml green shake of Wolffia globosa (Mankai strain; a newly developed duckweed grown under highly supervised conditions) as a green plant-based protein, replacing animal protein at dinner. Both green tea and Mankai together provided an additional daily intake of 800 mg polyphenols [(GAE), according to Phenol-Explorer and Eurofins lab analysis, including catechins (flavanols)] beyond the polyphenol content in the prescribed MED diet. The calorie count for the day included the green tea and Mankai shake. The MED and green MED diets had the same calorie restriction (1500-1800 kcal/day for men and 1200-1400 kcal/day for women). Further details of the dietary interventions and physical activity protocols are provided in Supplementry Data 2.

2.3 Nutritional adherence assessment

Self-reported food frequency questionnaires (FFQ) were administered via computer at baseline, after 6 months, and at the trial's conclusion (26, 27), which encompassed an assessment of provided item intake. We tracked overall changes in specific food group consumption, as previously detailed (28) alongside the intake of Mankai and green tea. The outcomes of these changes have been discussed in our prior work (29).

2.4 Lifestyle changes guidance

Regarding lifestyle interventions, participants received 90-minute sessions at the workplace, combining nutritional and physical activity guidance from a multidisciplinary team, including physicians, clinical dietitians, and fitness instructors. All lifestyle educational programs were provided at the same intensity to all three groups, These sessions occurred weekly during the first month, monthly over the next five months, and every other month until the 18th month. Simultaneously, the exercise program involved a gradual increase in aerobic training, starting at 20 minutes and 65% of the maximum heart rate, progressing to 45-60 minutes at 80% of the maximum heart rate, performed 3-4 times per week. Resistance training began with a single set of weights at 60% of the maximum weight and eventually advanced to two sets at 80% of the maximum weight. These resistance exercises included leg extensions, leg curls, squats, lateral pull-downs, push-ups, shoulder presses, elbow flexions, triceps extensions, and bent leg sit-ups. These adjustments aimed to enhance both aerobic and strength components of the workout program. To maintain motivation, participants received timely text messages with

relevant information based on their intervention group, and a website provided access to specific nutritional and physical activity information for each assigned intervention group.

2.5 Clinical parameters and fasting blood biomarkers

A standard wall-mounted stadiometer was used to measure height to the nearest millimeter. Without shoes, body weight was measured to the nearest 0.1 kg. WC was measured to the nearest millimeter halfway between the last rib and the iliac crest using standard procedures and an anthropometric measuring tape. After resting, two blood pressure (BP) measurements and a pulse rate were recorded using an automatic BP monitor (Accutorr-4, Datascope, New Jersey, USA). The blood pressure was computed as the mean of the 2 measurements. FMC and all other laboratory markers were measured in blood samples drawn between 07:00-07:30 AM after a 12-hour fast. Additionally, participants were instructed to avoid physical activity in the 12 hours preceding their blood tests. We also verified that on the day of each examination, all patients were not currently suffering from acute illnesses and were not receiving acute or subacute medical treatments. All FMC measurements were analyzed by LC-MS/MS in serum, as previously described (30). samples were centrifuged and stored at -80°C. Visceral adipose tissue (VAT) and intrahepatic fat (IHF) were assessed at baseline and after 18-months of intervention by MRI. Further details are provided in Supplementry Data.

2.6 Statistical analysis

The primary aim of the DIRECT-PLUS randomized controlled trial was to explore the effects of the interventions on weight and adiposity (29, 31).This secondary analysis report explores the dynamics of FMC during weight loss interventions and its associations with metabolic health, beyond weight loss.

Continuous variables are presented as mean ± standard deviation and categorical variables are presented as percentages. Variables were tested for normal distribution using the Shapiro-Wilk test. Baseline characteristics of the study population were analyzed across sex-specific tertiles of baseline FMC; we tested for trend using the kendall tau test. Correlations were evaluated using Pearson's or Spearman's correlation tests based on the distribution of the variables (normal vs. non-normal). 5 participants were lacking FMC at baseline and were not included in further analysis (n=289). VAT were expressed as proportions out of abdominal adipose tissue depots assessed (I.e., Deep-subcutaneous fat, superficial-subcutaneous fat and visceral adipose fat); this was to genuinely reflect the abundancy of each fat layer, irrespective of total adipose tissue. IHF was assessed using H-MRS as elaborated in Supplement 3. To assess the relationship between changes in FMC by intervention and sex-based groups, we first examined the differences within each group, compare to baseline, using a paired-sample t-test for variables that were normally distributed or the Wilcoxon signed-rank test for variables that were not normally distributed. For assessing between groups differences at baseline across intervention group we used analysis of variance (ANOVA) model. Subsequently, we evaluated between groups differences in FMC dynamics, separately comparing intervention groups and sex-based groups using a multivariable model (Analysis of covariance[ANCOVA]), adjusting for weight loss since baseline, age, and sex for the intervention group comparisons, and weight loss since baseline and age for the sex-based group comparisons. In addition, the change in FMC was presented as mean percentage of change from baseline, absolute change from baseline and median with IQR, due to its high variability. To examine the association between changes in FMC and markers of metabolic health, we conducted partial correlation analyses. Specifically, we examined the relationship beyond the effect associated with weight loss at time 18 using three types of models: a crude (univariate) model, model 1 adjusted for age, sex, and intervention group, and model 2 adjusted for age, sex, intervention group, and weight loss change. The same analyses were performed for changes over 6 months and reported in Supplementary Figure 1. All associations in the partial correlation analyses were adjusted for multiple comparisons using FDR-BH (with a q value of 5%(. We examined the differences within glycemic-groups and glycemic-status shifting groups classified by their transition from three glycemic states (Normoglycemic, Pre-diabetic, Diabetic). Glycemic status was defined following the American Diabetes Association (ADA) guidelines (32, 33). Static glycemic migration (SGM) classified as participants who started (Baseline) and finished (18-months) in the same glycemic state (i.e., from normoglycemic to normoglycemic, Pre-diabetic to Pre-diabetic and Diabetic to Diabetic). Negative glycemic migration (NGM) classified as participants who had a worsened state change from baseline to 18-months(i.e., from normoglycemic to pre-diabetic and from pre-diabetic to diabetic). Positive glycemic migration (PGM) classified as participants who improved their glycemic state from baseline to 18 months. No participant migrated from diabetic to normoglycemic nor from normoglycemic to diabetic. These analyses used paired-sample t-tests for variables that were normally distributed or the Wilcoxon signed-rank test for variables that were not normally distributed. To detect differences between these groups, first we used an ANOVA model and then implemented an independent t-test for the baseline comparison. For the comparison of the percentages change we used a multivariable model (ANCOVA) adjusted for weight loss from baseline, intervention group, age, and sex. Analyses were performed Python Software Foundation, version 3.9.13, available at https://www.python.org/. Significance was set at $P < 0.05$.

3 Results

3.1 Baseline characteristics

Among the 294 study participants (88% men, mean age 51.1) the mean BMI was 31.3kg/m^2 and mean FMC=304.07nmol\L); 51% were normoglycemic, 38% prediabetic, and 11% had type 2 diabetes. Baseline FMC was higher among men (308.6 ± 90.05 nmol\L) than

women 269.6± 83.9 nmol\L, p=0.02). Baseline FMC levels were similar across intervention groups, (HDG= 307.1± 91.5 nmol\L, MED=304± 81.4 nmol\L, Green-MED= 301.1± 97.3 nmol\L; p= 0.87). Baseline FMC was positively associated with age (r=0.11, p=0.05), dysglycemia (p of trend = 0.02), visceral adiposity (r=0.15, p=0.01), glucose (r=0.18, p<0.01), hsCRP (r=0.10, p=0.08), testosterone (r=0.12, p=0.04), progesterone (r=0.22, p<0.01) and TSH (r=0.11, p=0.05). It is important to highlight that fasting glucose, hsCRP, and progesterone exhibited a trend across the second and third tertiles of baseline FMC, aligning with visceral adiposity and dysglycemia, while this was not evident with changes in age, weight, or BMI. Baseline characteristics across sex-specific tertiles of FMC are further detailed in Table 1.

3.2 Adherence to the intervention

As reported previously (29, 31), the retention rate of the study was 98% after 6-months and 89% after 18-months. Dropouts rates were primarily due to a lack of motivation and unrelated medical issues. Moderate weight loss was observed in both calorie-restricted MED groups (MED: -2.81% ± 5.6% [-2.71 ± 5.6 kg]; Green-MED: -3.9% ± 6.3% [-3.7 ± 6.26 kg]), which was significantly greater than that in the HDG group (-0.4% ± 5% [-0.4 ± 4.7 kg]) (p < 0.05 for both MED groups vs. HDG group). The Green-MED diet group exhibited a significant increase in the consumption of fish, Mankai, and green tea, and a decrease in the consumption of red meat and poultry compared to the other two groups (p < 0.01 for all). Furthermore, both the Green-MED and MED diet groups showed a decrease in carbohydrate consumption during both the 18-month and 6-month periods of the trial (p<0.05 for all). Additional information regarding changes in macronutrient intake across intervention groups can be found in Supplementary Table 2.

3.3 FMC dynamics

At baseline, FMC levels were not statistically different across intervention groups (HDG =307 ± 91.5 nmol/L, MED=304.05 ± 81.4 nmol/L, Green-MED=301.1 ± 97.3 nmol/L; p=0.89). Following a 6-month intervention period (HDG=317.3 ± 126 nmol/L, MED=328.1 ± 107.5 nmol/L, Green-MED=313.5 ± 106.27 nmol/L) (Figure 1A), there

TABLE 1 Baseline characteristics of DIRECT PLUS participants across tertiles of fasting morning coristol, n=289.

	Low tertile	Median tertile	top tertile	P of trend	r-correlation
Cortisol, nmol/L	207.69 (± 41.64)	300.62 (± 27.57)	402.86 (± 52.18)	–	–
count	96	96	97	-	-
Diabetes and Pre-diabetes, %	41.67	44.79	61.05	0.02*	–
Age, years	49.2 (± 9.12)	52.02 (± 11.22)	52.19 (± 10.91)	0.05*	0.11*
Weight, kg	95.08 (± 15.95)	93.51 (± 12.91)	92.84 (± 13.81)	0.32	0.01
BMI, kg/m2	31.26 (± 3.78)	31.48 (± 3.86)	31.23 (± 4.27)	0.65	-0.03
Waist circumference, cm	109.29 (± 9.45)	109.94 (± 7.65)	110.07 (± 10.88)	0.6	0.06
Diastolic-BP, mm Hg	81.1 (± 10.03)	80.92 (± 9.74)	80.97 (± 10.96)	0.85	0.05
Systolic-BP, mm Hg	127.82 (± 11.59)	130.8 (± 13.34)	130.46 (± 14.55)	0.27	0.08
Blood Biomarkers					
Fasting glucose, mg/dL	100.99 (± 16.77)	99.74 (± 14.69)	109.69 (± 33.36)	0.02*	0.18*
Insulin, µU/mL	15.36 (± 8.1)	14.57 (± 7.17)	14.09 (± 8.16)	0.15	-0.06
HOMA IR	3.94 (± 2.48)	3.67 (± 2.1)	3.69 (± 2.35)	0.34	-0.02
HbA1c, %	5.44 (± 0.58)	5.45 (± 0.45)	5.55 (± 0.83)	0.8	0.04
Cholesterol, mg/dL	189.55 (± 30.85)	191.55 (± 35.87)	190.14 (± 32.59)	0.89	0.08
Triglycerides, mg/dL[&]	4.93 (± 0.41)	4.84 (± 0.51)	4.93 (± 0.41)	0.8	*0.10*
LDLc, mg/dL	126.46 (± 29.47)	124.11 (± 30.09)	124.83 (± 29.83)	0.55	0.04
HDLc, mg/dL	43.42 (± 9.94)	48.36 (± 13.08)	46.22 (± 11.46)	0.11	0.04
FFA mmol/L	0.49 (± 0.19)	0.5 (± 0.16)	0.49 (± 0.16)	0.49	0.01
ALT, U/L	36.19 (± 17.76)	33.29 (± 15.18)	35.29 (± 17.49)	0.69	0.03
AST, U/L	26.57 (± 8.64)	25.01 (± 7.38)	25.43 (± 7.25)	0.43	0.01
ALKP, mg/dL	74.99 (± 18.59)	71.53 (± 18.72)	76.15 (± 20.39)	0.67	-0.02

(Continued)

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TABLE 1 Continued

	Low tertile	Median tertile	top tertile	P of trend	r-correlation
hsCRP[&], mg/L	0.92 (± 0.95)	0.98 (± 0.72)	1.11 (± 0.9)	0.04*	*0.10*
IL6 pg/m	3.69 (± 1.71)	4.23 (± 4.97)	3.89 (± 2.31)	0.88	-0.01
Leptin, ng/mL	13.01 (± 8.99)	14.2 (± 11.26)	15.22 (± 13.79)	0.92	-0.07
Fetuin A, µg/mL	334.49 (± 95.03)	347.73 (± 94.21)	350.24 (± 98.92)	0.27	0.09
Chemerin, ng/mL	206.78 (± 46.18)	202.26 (± 36.4)	215.11 (± 47.1)	*0.09*	0.08
FGF 21, pg/mL	193.65 (± 119.25)	175.68 (± 109.21)	225.49 (± 148.95)	0.15	0.09
TSH mIU/liter	2.28 (± 1.47)	2.33 (± 1.05)	2.44 (± 1.14)	0.14	0.11*
Folic Acid, ng/dl	7.65 (± 3.12)	8.65 (± 4.12)	7.85 (± 3.4)	0.82	0.04
Ghrelin, pg/mL	527.76 (± 235.15)	520.11 (± 227.42)	531.45 (± 283.47)	0.65	*-0.11*
Aldosterone, nmol/L	231.69 (± 182.18)	208.21 (± 118.48)	225.7 (± 114.07)	0.84	0.04
Progesterone, nmol/L					
Entire	2.17 (± 8.74)	0.87 (± 3.81)	0.36 (± 0.16)	>0.01*	0.22*
Women	17.6(± 20.64)	2.70(± 8.04)	0.38(± 0.27)	0.11	-0.31
Men	0.27(± 0.07)	0.32(± 0.19)	0.35(± 0.13)	>0.01*	0.31*
Testosterone, nmol/L					
Entire	13.15 (± 6.18)	14.12 (± 7.49)	13.92 (± 6.7)	0.39	0.12*
Women	0.77(± 0.41)	0.93(± 0.41)	0.95(± 0.53)	0.32	0.15
Men	14.74(± 4.54)	15.82(± 6.12)	15.75(± 4.88)	0.26	0.05
Estradiol, pmol/L					
Entire	123.37 (± 107.29)	113.84 (± 131.85)	107.02 (± 96.75)	*0.07*	*-0.11*
Women	309.25(± 213.66)	237.66(± 356.19)	226.94(± 239.67)	0.27	-0.18
Men	98.90(± 42.05)	96.53(± 44.40)	90.82(± 40.85)	0.17	-0.06
MRI derived fat deposits					
VAT proportion, %	28.32 (± 7.78)	27.36 (± 8.46)	30.57 (± 9.97)	0.04*	0.15*
IHF content, %	10.46 (± 8.84)	10.38 (± 8.63)	9.91 (± 8.75)	0.52	-0.04

1. Values are reported as mean ± SD.
2. P of trend was analyzed using Kendall's Tau test.
3. Sex-specific Tertiles: Q1: men <=265 nmol/L; women: <=232 nmol/L; Q2: men: 266 nmol/L to 351 nmol/L women: 233 nmol/L to 304 nmol/L; Q3: men: =>352 nmol/L, women: =>305 nmol/L.
4. FMC, fasting morning cortisol; BMI, body mass index; BP, blood pressure; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HbA1c, glycated-hemoglobin-A1c; LDL,Low-density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; FFA, free -fatty acids; ALT, Alanine Transaminase; AST, Aspartate aminotransferase; ALKP, alkaline phosphatase; hsCRP, High Sensitivity C-Reactive Protein; IL6, Interleukin-6; FGF-21, Fibroblast Growth Factor 21; TSH, Thyroid Stimulating Hormone; VAT, visceral adipose tissue; DSC, deep subcutaneous adipose tissue; SSC, superficial subcutaneous adipose tissue; IHF, intrahepatic fat.
5. *p<0.05.
6. italic font style = p<0.1.
7. [&]Lan transformed.
8. The gonadal hormone levels were calculated for the same tests as presented in the entire table. However, the analysis further stratified these hormone levels by gender, including women and men. Subsequent testing was conducted within each of these strata to examine potential variations.

were no significant changes in FMC among all intervention groups (mean FMC percentages and absolute change: HDG= 7.6%± 38.8% [10.72 ± 114.10 nmol/L], MED=12.4%± 41.5% [22.37 ± 108.7 nmol/L], Green-MED=11.6% ± 44.6% [13.1 ± 111.2 nmol/L]; Median percentages change and IQR: HDG= 3.6%[-20.61%,32.09%], MED=3.1%[-15.49%, 34.34%], Green-MED=3.08%[-17.5%, 29.96%]) as compared to baseline. Similarly, after a 6-month intervention, the levels of FMC in men (323.42 ± 112.22 nmol/L) and women (290.7 ± 119.54 nmol/L) did not show any significant changes within their respective groups (men=10.3% ± 41.6% [14.6 ± 112 nmol/L], women=12.4% ± 42.4% [21.26 ± 106 nmol/L]) (Figure 1B).

However, after 18-months(HDG=297.51 ± 85.81 nmol/L, MED=281.82 ± 76.6 nmol/L, Green-MED=273 ± 98.6 nmol/L), both the Med and Green-MED diets achieved significant reductions in FMC levels (mean FMC percentages and absolute change: MED=-1.6% ± 39% [-21.45 ± 92.7 nmol/L], Green-MED=-1.8% ± 45% [-26.67 ± 117.1 nmol/L]; p<0.05 vs. baseline for both; Median percentages change and IQR: MED=-7%[-25.86%, 12.8%], Green-MED=-10.47%[-29.12,

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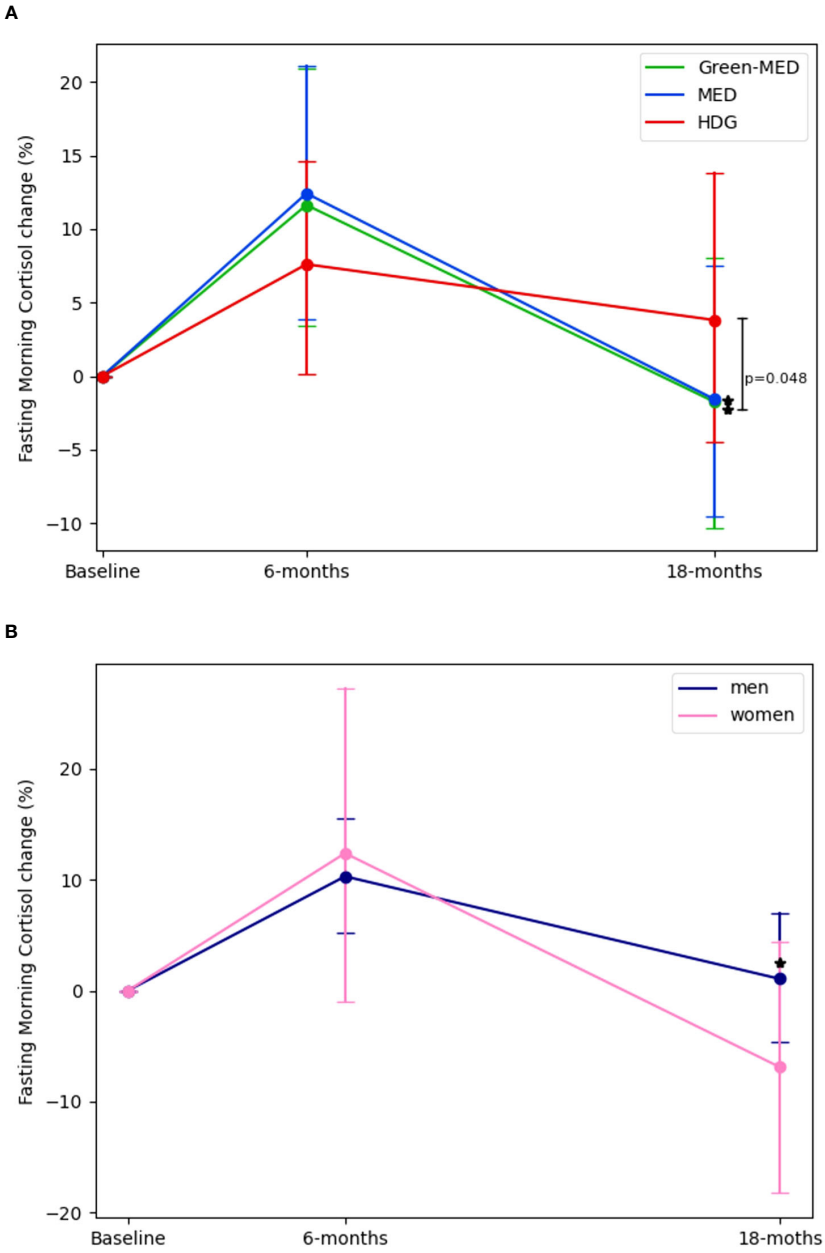

FIGURE 1
Fasting morning cortisol changes across intervention and sex groups. 1. **(A)**:FMC changes across intervention by time, Baseline, 6-months and 18-months (end of intervention). **(B)**: FMC changes across sex by time, Baseline, 6-months and 18-months (end of intervention) 2. For panel **(A)**. p between groups was analyzed in an ANCOVA model adjusted for Δweight, age, and gender. For panel **(B)**. p between groups was analyzed in an ANCOVA model adjusted for Δweight and age. 3. * significant within change vs. baseline at 0.05; FMC levels in both MED and Green-MED diets reduced significantly vs. baseline. There were no significant differences between sex groups. 4. Abbreviations: HDG, healthy dietary guidelines; Med, Mediterranean diet; Green-Med, green-Mediterranean diet.

17.83]), while the HDG group showed a non-significant change in FMC (mean FMC percentages and absolute change:+4% ± 46.54% [-12 ± 104 nmol/L], p=0.28; Median percentages change and IQR: HDG=-3.9%[-27.46%,18.9%]). Additionally, after 18-months men (289 ± 86 nmol/L) experienced a significant change in FMC levels (1% ± 44.9% [-19.2 ± 107.9 nmol/L], p<0.01) whereas the women's group (237.45 ± 83.9 nmol/L) displayed non-significant reduction (-6.9 ± 31.2% [-27.2 ± 80.93 nmol/L], p=0.08). The difference in FMC percentage change between the Green-MED and HDG groups at 18-months, was statistically significant (p=0.048, multivariable model adjusted for age, sex, and weight-loss from baseline). There were no significant differences between sex-based groups at each time point (multivariable model adjusted for age and weight-loss from baseline).

To further examine the association between the 18-month change in FMC and various metabolic biomarkers, we performed three correlation models. In a univariate correlation model, 18-months FMC decrease was positively correlated with 18-month changes of glucose (r=0.19), ALT (r=0.17), Fetuin A (r=0.20), TSH

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(r=0.28), and testosterone (r=0.18) and HDLc (r=0.18), p<0.05 for all. After adjusting for age, sex, and intervention group, the same markers remained significantly correlated with the 18-month change in FMC (glucose=0.17, HDLc=0.19, ALT=0.16, Fetuin A= 0.2, TSH=0.28, testosterone=0.2). Additionally, after further adjustment for 18-month weight loss, the 18-month decrease in FMC levels was positively correlated with 18-month changes of glucose (r=0.18), HbA1c (r=0.15), ALT (r=0.2), hsCRP (r=0.16), Fetuin A (r=0.2), TSH (r=0.28), MRI-assessed IHF (r=0.19), testosterone (r=0.18) and HDLc (r=0.19), p<0.05 for all. Further associations are illustrated in Figure 2. Associations of 6-months change FMC and 6-months change of metabolic markers is illustrated in Supplementary Figure 1.

3.4 Cortisol dynamic and glycemic status

We further examined FMC changes from three glycemic states at baseline: Normoglycemic(Baseline FMC=285.44 ± 85.44 nmol/L), Pre-diabetic (Baseline FMC=320.65 ± 90.52 nmol/L), Diabetic (Baseline FMC=325.65 ± 92.5 nmol/L). At baseline diabetic and pre-diabetic groups had higher FMC levels than the normoglycemic group(p<0.05 for both). After 6-months (Normoglycemic=308.28 ± 106.44 nmol/L, pre-diabetic=319.28 ± 112.04 nmol/L, diabetic=370.8 ± 134.3 nmol/L), the group of probands with type 2 diabetes showed the greatest elevation (19% ± 42.9% [50.37 ± 114.40 nmol/L], p=0.01 vs. baseline), as compared to the pre-diabetic (4.8% ± 42% [-0.70 ± 112.7 nmol/L], p=0.94 vs. baseline) and normoglycemic (13.7%± 40.6% [22.56 ± 105.15 nmol/L], p=0.02 vs. baseline) groups (p<0.05 for difference between groups; in multivariable models adjusted for age, sex, and 6-month weight loss). After 18-months (Normoglycemic=276.32 ± 88.38, pre-diabetic=291.8 ± 86.56 nmol/L, diabetic=299.42 ± 88.41 nmol/L), only the pre-diabetic group exhibited a statistically significant reduction in FMC levels vs. baseline (-3.9% ± 33.6% [-28.9 ± 100 nmol/L]; p<0.01), whereas the diabetic and normoglycemic groups showed a non-significant change vs. baseline (-4.7% ± 29%[-30.12 ± 117.6 nmol/L]; p=0.17; (5.1%± 51.7% [-8.13 ± 102.77 nmol/L]; p=0.36; respectively). Further details are illustrated in Figure 3.

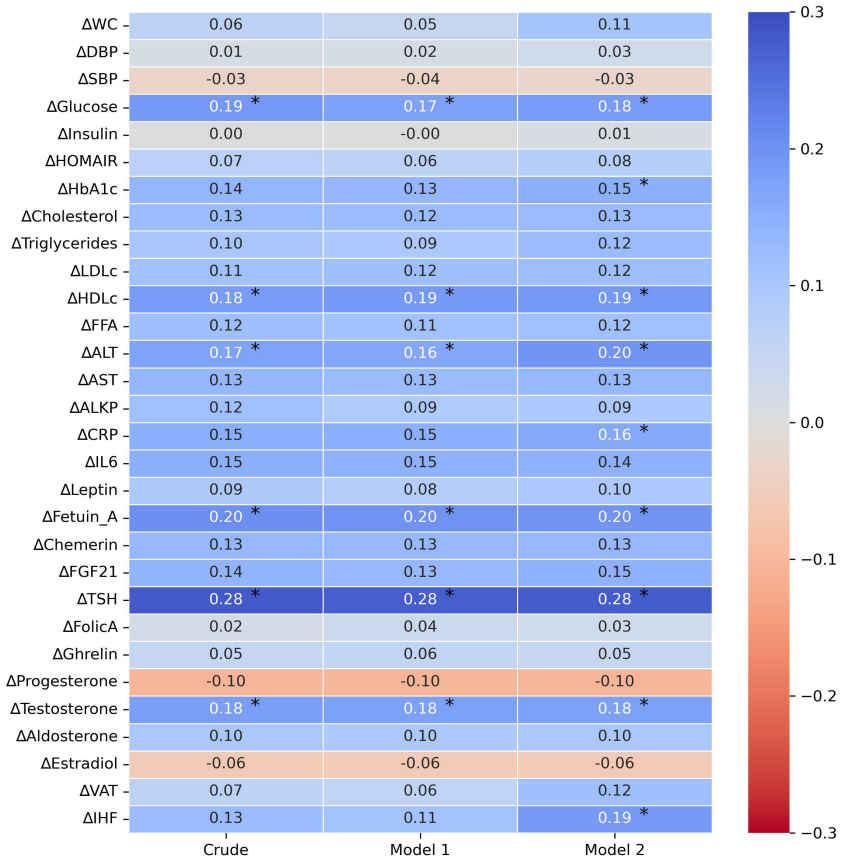

FIGURE 2
Crude and adjusted correlations between 18-month biomarkers changes and 18-month fasting morning cortisol change. 1. Partial correlation analysis of $\Delta variable_{18months}$ with $\Delta FMC_{18months}$ 2. Crude – univariate correlation of $\Delta variable_{18months}$ with $\Delta FMC_{18months}$ 3. Model 1 – adjusted for: age, gender, and intervention group. 4. Model 2 – adjusted for: age, gender, intervention group and $\Delta weight_{18months}$. 5. Abbreviations: FMC, fasting morning cortisol; BMI, body mass index; BP, blood pressure; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HbA1c , glycated-hemoglobin-A1c; LDL ,Low-density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; FFA, free -fatty acids; ALT, Alanine Transaminase; AST, Aspartate aminotransferase; ALKP, alkaline phosphatase; hsCRP, High Sensitivity C-Reactive Protein; IL6, Interleukin-6; FGF-21, Fibroblast Growth Factor 21; TSH, Thyroid Stimulating Hormone; VAT, visceral adipose tissue; IHF, intra-hepatic fat. 6. * p≤0.05. 7. All pvalues are corrected for multiple comparisons (FDR-BH, Q=5%).

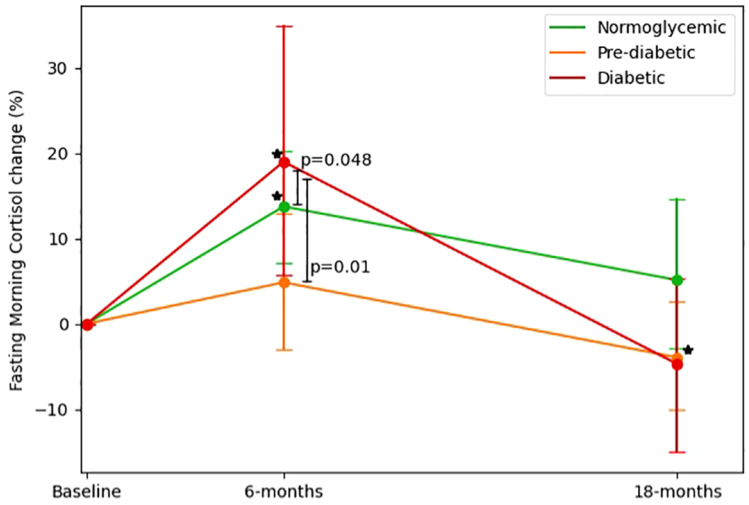

FIGURE 3
Fasting morning cortisol changes across glycemic-status groups. 1. FMC changes across glycemic status groups (defined at baseline) by time, Baseline, 6-months and 18 months (end of intervention). 2. Diabetic defined as fasting glucose >=126 or HbA1c >=6.5 or receiving medical treatment for diabetes. 3. Pre-diabetic defined as 126>fasting glucose >=100 or 6.5>HbA1c>=5.7. 4. P between groups was analyzed in an ANCOVA model adjusted for Δweight, age, and gender. 5. * Significant ($p<0.05$) within change vs. baseline at 0.05; Diabetic and normoglycemic groups had significantly elevated FMC levels in 6-months and pre-diabetic groups had significantly reduced FMC levels.

Participants were classified into three groups by their transition from three glycemic status groups [Normoglycemic[n=148, mean weight=91.11 ± 13.01 kg, fasting glucose=91.94 ± 5.55 mg/dL, HbA1c=5.19% ± 0.31%], Pre-diabetic[n=110, mean weight=96.13 ± 16.14 kg, fasting glucose=106.51 ± 7.34 mg/dL, HbA1c=5.51% ± 0.34%], Diabetic[n=32, mean weight=96.36 ± 12.41 kg, fasting glucose=147.16 ± 46.73 mg/dL, HbA1c=6.75% ± 0.97%]; glycemic status groups were defend using ADA definition (32, 33)]. We identified 190 participants who had a static glycemic migration (SGM; started [Baseline] and finished [18-months] at the same group), 35 participants who had negative glycemic migration (NGM; started [Baseline] at one group and finished [18-months] at worse diagnosed group) and 33 participants who had a positive glycemic migration (PGM; started [Baseline] at one group and completed [18-month] at a better diagnosed group). At baseline, NGM group (274.26 ± 70 nmol/L) had lower FMC levels than the SGM (305.14 ± 87.36 nmol/L, p=0.05) and PGM (318.8 ± 110.77 nmol/L, p=0.05) groups. After 6-months(SGM=317.5 ± 107.61, PGM=316 ± 127.85, NGM=326.11 ± 134.26 nmol/L), only the NGM group showed a significant increase in FMC levels (22.71± 49.89% [50.4 ± 128 nmol/L]; p=0.03 vs. baseline) while SGM and PGM showed non-significant elevation in 6-months FMC (9.28% ± 40.87% [12.31 ± 107.62 nmol/L], p=0.12 vs baseline; 3.30%± 35.05% [-2.80 ± 104.88 nmol/L], p=0.87 vs baseline; respectively), 6-months FMC change levels of the NGM group was significantly higher than that of the PGM group, after controlling for age, sex, weight loss at 6-months, and type of intervention (p=0.04). Further details are illustrated in Figure 4.

4 Discussion

In this trial, we examined the effect of MED diets on fasting morning cortisol levels, and their associations with cardiometabolic markers. Our findings suggest that long-term lifestyle-induced FMC decrease may play a role in metabolic health, independent of weight loss. Additionally, our study highlights the potential of MED diets and green-MED in particular, to effectively lower the stress hormone FMC over the long term.

This study has several limitations. First, the study population was mainly men, reflecting the sex profile of the workplace. While this was taken into account in various models, it may limit the generalizability of the results to women. In addition, caution is needed in interpreting the results related to gender differences. Second, we could not study effects on peak cortisol response, since the blood samples from our participants were collected between 7:00-7:30 AM making it hard to confirm the precise timing of the peak cortisol response. Instead, we relied on a standardized fasting morning cortisol level. Additionally, we were unable to factor in potential sleep alterations, a potentially significant influence on HPA axis regulation (34). Nonetheless, we did analyze the proportion of shift-workers within each diet intervention group, a factor that can potentially affect cortisol secretion. Notably, The proportion of shift-workers did not differ across diet groups neither in the baseline nor at the end of the trial. Future research should consider the potential impact of sleep alterations on FMC. Third, adherence to the interventions was mainly assessed through self-reporting, which may introduce bias and limit the accuracy of the results. However, objective measures such as serum folate analysis, reflecting green leafy vegetable consumption, or urine polyphenols, demonstrated the accuracy of the reporting (26, 29, 35). In addition, given the subtle differences in FMC and the almost borderline statistical significance of the findings, further research is needed to validate our results. Lastly, it is important to note that we relied solely on self-reported data and did not employ objective measures for physical activity assessment. While physical activity might influence FMC levels, it is essential to underscore that

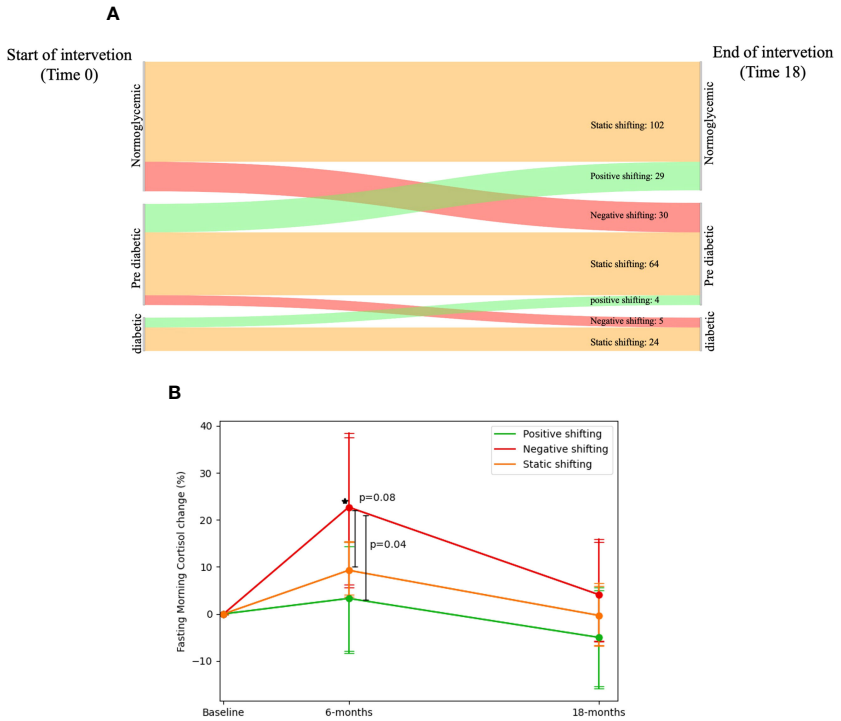

FIGURE 4
Fasting morning cortisol and shifting of glycemic-status. 1. **(A)** Sankey diagram showing the glycemic-status shifting between three groups: Normoglycemic, Pre-diabetic and Diabetic; 190 participants had a static glycemic-status shift (started and finished in the same group: Normoglycemic → Normoglycemic = 102, Pre-diabetic → Pre-diabetic = 64 and Diabetic → Diabetic = 24), 35 participants who had negative glycemicstatus shifting (started in one group and finished in a worse-diagnosed group: Normoglycemic → Pre-diabetic = 30 and Pre-diabetic → Diabetic = 5), and 33 participants who had a positive glycemic-status shifting (started at one group and finished at a better-diagnosed group: Pre-diabetic → Normoglycemic = 29 and Diabetic → Pre-diabetic = 4). 2. **(B)** FMC dynamics in percent across glycemic-status shifting groups. Between P values analyzed in ANCOVA model adjusted for age, gender, Intervention group and Dweight. 3. Diabetic defined as fasting glucose >=126 or HbA1c >=6.5 or receiving medical treatment for diabetes. 4. Pre-diabetic defined as 126>fasting glucose >=100 or 6.5>HbA1c>=5.7. 5. * significant within change vs. baseline at 0.05; Negative shifting group had elevated FMC levels in 6-months vs. Baseline.

that our study did not involve a physical activity intervention; instead, we provided equivalent accommodations across each intervention group.

This trial has several noteworthy strengths. One key strength is that the study was conducted in a closed workplace setting, providing participants with access to an on-site clinic and a monitored provided lunch. This facilitated intensive dietary guidance and group meetings with multidisciplinary teams of healthcare professionals including physicians, dietitians, and physical trainers, ensuring high adherence. The relatively large sample size and high retention rate enhanced the statistical power and strengthened the reliability of the results.

Cortisol levels may be influenced by various lifestyle interventions such as timing of food intake (12), dietary restriction (20–22), and macronutrient content (19). Despite the potential influence of diet on cortisol levels, the association between the MED diet and cortisol levels has not been extensively studied. While existing research in this area has produced inconclusive results, some evidence suggests that the MED diet may actually help to reduce cortisol levels (13, 14). This study confirms and considerably extends those findings by demonstrating a reduction in FMC by MED diet in a randomized trial and indicating that the reduction in FMC was associated with a reduction in metabolic health biomarkers including glucose, HbA1c, ALT, hsCRP, Fetuin A, TSH, testosterone, and intra-hepatic fat content, suggesting potential improvement in specific metabolic biomarkers. Interestingly, these biomarkers showed an improvement after accounting for weight loss, suggesting the possibility that FMC reduction may improve metabolic health.

Another notable lifestyle factor we observed was calorie restriction. All participants across the study arms were motivated to achieve weight loss by adhering to their respective diets and engaging in physical activity. As previously reported by our research group (31), the 6-month timepoint was marked by rapid weight loss. This rapid weight loss could potentially trigger a significant stress response from the body. This, in turn, leads us to consider that this highly stressed state of rapid weight loss might be associated with an elevated FMC response as witnessed in our study. However, it is important to highlight that the 6-months changes in FMC across the intervention group, were highly variable. Thus, we also reported a median and IQR for better interpreting the results.

Several studies have investigated the association between elevated cortisol levels and dyslipidemia (36, 37), but the relationship between cortisol and HDL remains inconclusive. The precise contribution of cortisol to HDL levels remains unclear (38, 39). To the best of our knowledge, this is the first study to

demonstrate a distinct pattern of FMC reduction resulting from specific dietary interventions, with the observed changes in FMC being independently associated with improved metabolic health, irrespective of changes in weight.

Observational studies have established the relationship of elevated cortisol levels and diabetes risk (4, 10). This is thought to occur through the dysregulation of glucose metabolism by cortisol, leading to insulin resistance and impaired glucose tolerance (8). In this study, We found that participants with diabetes had an elevated cortisol response at 6-months, and those who experienced a worsening shift in glycemic state over the 18-month study period also had an elevated cortisol response at 6-months. These findings suggest that acute changes in cortisol levels may be associated with long-term shifts in glycemic status. Further research is needed to fully understand the underlying mechanisms linking cortisol acute response and diabetes risk.

In conclusion, our study adds to the growing body of evidence on the potential benefits of the MED diet for metabolic health beyond weight loss. The results of our trial suggests that long term adherence to a Green-MED/high polyphenols diet may primarily reduce FMC in obese individuals, potentially resulting in improvements in metabolic health biomarkers. Further research is essential to confirm and establish these preliminary findings, as well as to investigate the long-term effects of these dietary interventions on cortisol levels and metabolic health. This future research should specifically explore the associations between changes in FMC and distinct metabolic biomarkers, nonetheless, our study highlights the importance of considering the role of cortisol in metabolic health.

Data availability statement

Data may be accessible upon request, subject to approval by the study's principal investigator, IS.

Ethics statement

The studies involving humans were approved by Soroka University Medical Centre medical ethics board and institutional review board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. This study was conducted in accordance with the CONSORT statement.

Author contributions

IS, GT, AM, HZ, AK, and ER designed the research and conducted the study. IS, LA, and GT analyzed the data, preformed the statistical analysis, wrote the manuscript, and are responsible for final content. IS, GT, and LA conceived and designed the research. MB, UC, BI and MStu reviewed and edited the manuscript. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis as well as for the decision to submit the manuscript for publication. IS, GT, and LA are the guarantors of this work and take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

IS advises the nutritional committee of Hinoman, Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1243910/full#supplementary-material

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Excessive weight gain onset-age and risk of developing diabetes mellitus: a large, prospective Chinese cohort study

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Background: Excessive weight gain and obesity are widely accepted as risk factors for diabetes mellitus, and the age at which obesity onsets may be related to the development of cardiovascular diseases and certain cancers. Here, we aimed to investigate associations between the onset-age of overweight/obesity and risk of developing diabetes mellitus in China.

Methods: 42,144 people with the normal weight range and without diabetes at baseline, were enrolled from the Kailuan cohort which began on the 1st June 2006. All participants were followed-up, biennially, until 31st December 2017. During follow-up, 11,220 participants had become overweight/obese. For each case, one normal-weight control was matched according to age (± 1 year) and sex. Our final analysis included 10,858 case-control pairs. An age-scaled Cox model was implemented to estimate hazard ratios (HR) with corresponding 95% confidence intervals (CI) for diabetes mellitus incidence across age-groups.

Results: At a median follow-up of 5.46 years, 1,403 cases of diabetes mellitus were identified. After multivariate adjustments, age-scaled Cox modelling suggested that risk gradually attenuated with every 10 year increase in age of onset of overweight/obesity. Diabetes mellitus adjusted HRs (aHRs) for new-onset overweight/obesity at <45years, 45-54 years, and 55-64 years were 1.47 (95%CI, 1.12-1.93), 1.38 (95%CI, 1.13-1.68), 1.32 (95%CI, 1.09-1.59), respectively. However, new-onset of overweight/obesity at ≥65 years did not relate to diabetes mellitus (aHR, 1.20; 95%CI, 0.92-1.57). This trend was not observed in women or the new-onset obesity subgroup but was evident in men and the new overweight onset subgroup.

Conclusion: Participants with early onset of excessive weight gain issues are at considerably higher risk of developing diabetes mellitus compared to those who maintain a normal weight.

KEYWORDS

overweight, obesity, onset age, diabetes mellitus, prospective cohort

1 Introduction

Lifestyles change substantially with increased economic prosperity although these changes are not always positive. We are witnessing a type 2 diabetes mellitus pandemic which is closely linked to sedentary lifestyles and weight-gain; however, the prevalence of type 1 is also rising. Diabetes has therefore become a major public health concern in both India and China, where there has been substantial economic development and almost one 5th of the world's population reside. In China alone, the prevalence of overweight/obese adults is approximately 34.3% and 16.4%, respectively (1, 2). Perhaps even more alarming is the prevalence of obesity in children which is rapidly rising (3, 4).

Coincidentally, the prevalence of diabetes has rapidly increased from 9.7% in 2007 to 11.2% in 2017 among Chinese adults (5). Therefore, special attention must be paid to excessive weight gain and obese individuals in China because diabetes mellitus creates a huge economic burden for governments and for individuals, who not only encounter well-known macro and micro-vascular complications, but also encounter depression, anxiety, and all too frequently, die early (6, 7). In more developed societies, excessive weight gain and obesity are widely accepted as risk factors for diabetes mellitus. However, there are genetic differences and lifestyle factors which contribute to insulin resistance and therefore the prevalence of diabetes varies between nationalities and within ethnicities (8). Chinese researchers have postulated that multisectoral efforts are required to address the diabetes epidemic in China; however, these efforts must not be entirely reactive. We need to develop evidence-based preventive strategies to tackle this growing problem.

Demarcation between pre-symptomatic diabetic cases and those who encounter symptoms remains unclear, especially for the public. For example, people may attribute fatigue and macro and micro-vascular issues to ageing rather than being signs of diabetes which should initiate health seeking behaviors. Given the magnitude of the clinical iceberg in China, this is not always the case and so we, as a global community must learn about the differences between and within nationalities in order to identify (and intervene) pre-symptomatic cases. Two studies from Kailuan cohort found that hypertension and diabetes mellitus are risk factors for developing cardiovascular diseases (CVD), which were different across different onset ages in China (9, 10). Further research has suggested that the age at which obesity onsets may be related to the development of cardiovascular diseases and certain cancers (11, 12). However, little is known about correlations between the age of onset of overweight/obesity and risk of developing diabetes mellitus, especially across the mainland Chinese population. It is also hoped that by studying a large, Chinese cohort, we will add to the comparative evidence-base to ensure public health interventions are more highly specified. Therefore, we aimed to investigate associations between the onset-age of overweight/obesity and risk of developing diabetes mellitus in China.

2 Methods

2.1 Study design and participants

This is an exposure-control matched cohort study based on Kailuan cohort in Tangshan, Hebei province. From June 2006 to October 2007, participants from the Kailuan community completed questionnaires and a first survey in Kailuan General Hospital and 10 affiliated hospitals. Subsequent surveys including questionnaires and blood tests were provided every two years, in 2008 to 2009, 2010 to 2011, 2012 to 2013, 2014 to 2015, and 2016 to 2017. All questionnaires were completed by trained nurses and blood tests were taken by laboratory technicians. Data entry was performed by double entry using Epidata, and those with more than half the required data missing were excluded.

A total of 101,510 participants aged between 18-98 years were recruited between 2006 to 2007. After excluding 59,366 participants who did not have baseline body mass index (BMI) or fasting plasma glucose (FBG) information (n = 313), overweight/obese participants (n = 42,586), 3,144 with low-weight, had been diagnosed with diabetes mellitus (n = 9,489) at baseline, or 3,834 lost to follow-up and there were 42,144 participants available for matching (Figure S1A). From 2008 to 2015, 11,220 were considered new-onset excessive weight gain, which included overweight cases and those considered obese. After randomly matching participants with overweight or obese participants with those who maintained a normal BMI across the follow-up period according to age (+/- 1 year), sex and visitations, a total of 21,716 participants (overweight/obese, n=10,858; normal-weight, n=10,858) were finally included. The study was based on The Kailuan Study (trial identification: ChiCTR-TNC-11001489), approved by ethics committee of Kailuan General Hospital. Informed consent was required before individuals were granted access to participate.

2.2 Exposure: new-onset weight gain, considered overweight/obesity

During each examination, weight and height were recorded using calibrated RGZ-120 scales with participants removed footwear and over-clothes. Measures were rounded to the nearest 0.1 kg for weight and 0.1 cm for height. Weight status was ascertained according to BMI, which was calculated by dividing body weight (kg) by height squared (m^2). Cut-off points were predetermined according to the World Health Organization's standard ranges (i.e. underweight, BMI < 18.5 kg/m^2; normal-weight, 18.5 ≤ BMI <25.0 kg/m^2; overweight, 25.0 ≤ BMI < 30.0 kg/m^2; and obesity, BMI ≥ 30.0 kg/m^2) (13).

New-onset weight gain was defined according to BMI changes from normal-weight at baseline to overweight or obese recorded prior to 31st December 2015 or the point of diabetes mellitus diagnosis. Every case was matched with a control who maintained normal-weight across the follow-up period, according age (+/-1 year), sex and visit time (Figure S1B).

2.3 Outcomes: diabetes mellitus incidence during follow-up

Diabetes mellitus incidence was determined according to FBG ≥7.00 mmol/L (126 mg/dL) and/or treatment with anti-hyperglycemic drugs, and/or self-reported physician-diagnosed diabetes mellitus during the follow-up period according to American Diabetes Association guidelines (14). Blood samples from each participant were collected in the morning of each survey after at least a 12-hour fast. FBG was tested using hexokinase method by automatic biochemical analyzer (Hitachi 747; Hitachi, Tokyo, Japan). Diabetes histories and related treatments were collected by trained nurses through structured questionnaires (details are provided in the Supplementary Materials). We did not further distinguish type 1 or type 2 diabetes mellitus. The baseline for this study was defined according to the onset time of excessive weight gain or the time that participants with normal-weight were matched. All participants were followed-up until the date of diabetes mellitus diagnosis or until the final visit on the 31st December 2017, whichever came first.

2.4 Assessment of covariates

Data around other related variables were also collected and updated through questionnaires and blood tests every two year. Covariates were derived from the examination year at which each matched pair was confirmed. Family history of diabetes, education level, physical activity, cigarette smoking and alcohol drinking status were obtained through self-reported questionnaires. Education level was defined as "less than high school", "high school", or "university degree or higher". Active physical activity was defined as aerobic exercise ≥ 3 times per week. Smoking and alcohol drinking status were stratified into three levels: "current", "former" and "never". Total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) were also tested using an automatic biochemical analyzer (Hitachi 747; Hitachi, Tokyo, Japan). Blood pressure was measured three times, and mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) was used.

2.5 Statistical analysis

New-onset weight gain including participants with overweight or obesity and matched controls were stratified into four groups according to onset age: <45 years, 45-54 years, 55-64 years and ≥65 years. Continuous variables were expressed as means with corresponding standard deviations (SD) and compared using Student's t test or one-way analysis of variance (ANOVA) analysis. Categorical variables were shown as proportions and compared using a standard Chi-Square test.

An age-scaled Cox regression model, which took age rather than follow-up time as the time scale, was used to calculate the hazard ratios (HR) and 95% confidence intervals (CI) for risk of developing diabetes mellitus at new-onset overweight/obesity, compared with normal-weight participants across age-groups (15). Multivariate adjusted models were implemented for systolic blood pressure (SBP), FBG, TG, HDL-C, LDL-C, cigarette smoking status, alcohol drinking status, physical activity, family history of diabetes and education level, considering a high collinearity between SBP and DBP as well as TC, HDL-C and LDL-C. A further form of subgroup analysis compared by sex and among overweight and obese participants, separately. To evaluate fluctuations in body weight, we conducted subgroup analysis of those who reduced their weight to within the normal BMI range and those who maintained their overweight or obese status.

To test robustness and address the potential for reverse causation, we further performed sensitivity analyses by excluding participants who were diagnosed with malignant tumors during study, and those who encountered diabetes mellitus within the first year of follow-up.

All analyses were performed using SAS (Version 9.4). A fully conditional specification method was used to impute missing values for covariates using multivariate imputation by chained equation (MICE) method (16, 17). Details of missing covariates were presented in Table S1. All statistical tests were two-sided, and p <0.05 set as the threshold for statistical significance.

3 Results

3.1 Baseline characteristics

A total of 21,716 participants (overweight/obese, n=10,858; normal-weight, n=10,858) were finally included. Aggregated participant characteristics are provided in Tables 1, 2. Compared with participants categorized as normal-weight, new-onset overweight/obese subjects had higher FBG, SBP, DBP, TC, TG

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TABLE 1 Baseline characteristics of new-onset overweight/obesity and normal-weight controls†.

Variables	New-onset overweight/obesity	Normal-weight	*P*
Participants, n	10,858	10,858	
Age, years	52.71 ± 12.21	52.71 ± 12.21	-
Male sex, n(%)	8018(73.8)	8018(73.8)	-
BMI, kg/m^2	26.52 ± 2.36	22.24 ± 1.55	<0.01
FBG, mmol/L	5.26 ± 0.62	5.17 ± 0.61	<0.01
SBP, mmHg	131.01 ± 19.13	126.37 ± 18.95	<0.01
DBP, mmHg	84.04 ± 10.70	81.19 ± 10.40	<0.01
TG, mmol/L	1.63 ± 1.27	1.33 ± 1.14	<0.01
TC, mmol/L	5.05 ± 0.99	4.97 ± 1.00	<0.01
HDL-C, mmol/L	1.46 ± 0.46	1.56 ± 0.47	<0.01
LDL-C, mmol/L	2.65 ± 0.88	2.56 ± 0.85	<0.01
Cigarette smoking, n(%)			
Current	3174(29.2)	3486(32.1)	<0.01
Former	840(7.8)	790(7.3)	
Never	6844(63.0)	6582(60.6)	
Alcohol drinking, n(%)			
Current	2235(20.5)	2287(21.1)	0.55
Former	1983(18.3)	1935(17.8)	
Never	6640(61.2)	6636(61.1)	
Physical exercise, n (%)	1841(17.0)	1851(17.0)	0.86
Family history of diabetes, n(%)	934(8.6)	926(8.5)	0.85
Education, n(%)			0.05
Less than high school	860(7.9)	824(7.6)	
High school degree	9165(84.4)	9108(83.9)	
University degree or higher	833(7.7)	926(8.5)	

BMI, body mass index; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.
† Baseline refers to the examination cycle when new-onset overweight/obesity was first identified.

and LDL-C level, but lower HDL-C levels and a lower prevalence of smokers (Table 1). Among people with new-onset overweight/obesity a younger onset age correlated with lower FBG, SBP, DBP, TC, HDL-C, and LDL-C levels (Table 2). Additionally, these people were more likely to be smokers, alcohol drinkers, and physically inactive. They also had higher levels of TG and education overall but an increased prevalence in the family history of diabetes compared to those with an older onset age.

3.2 Diabetes mellitus incidence

During a median of 5.46 years (118,381 person-years) follow-up, we identified 1,403 previously undiagnosed diabetes cases. Compared to those who maintained a normal-weight during follow-up, people with new-onset overweight/obesity showed a higher risk of developing diabetes (adjusted HR [aHR], 1.29; 95% CI 1.02-1.63). However, the risk was different across onset ages (*P* for interaction < 0.05). As shown in Figure 1, the incidence and rate of diabetes were higher in people with new-onset overweight/obesity across all age-groups. In conjunction with increasing age, the number and rate appeared to consistently increase.

Compared with those considered to have maintained a normal weight, people with overweight/obesity were at a higher risk of developing diabetes, after adjusting for education level, cigarette smoking and alcohol drinking status, physical activity, family history of diabetes, SBP, TG, LDL-C and HDL-C level. The risk of developing diabetes gradually attenuated with every decade increase in age at onset of overweight/obesity, with a aHR of 1.47 (95% CI, 1.12-1.93) in those onset age <45 years, 1.38 (95% CI, 1.13-1.68) in those onset age from 45 to 54 years and 1.32 (95% CI, 1.09-1.59) in those onset age from 55 to 64 years. Although, those whose onset age was 65 years or older did not appear at higher risk (aHR,1.22; 95% CI, 0.94-1.59).

3.3 Subgroup analysis

Stratified by sex, results did not change substantially in males, with an aHR of 1.56 (95% CI, 1.16-2.10) in those onset age<45 years, 1.31 (95% CI, 1.04-1.65) in those onset age from 45 to 54 years and 1.30 (95% CI, 1.05-1.62) in those onset age from 55 to 64 years (Table 3). However, a positive correlation was observed in the relationship between women with overweight/obesity whose onset age was between 45 to 54 years (aHR, 1.58; 95% CI, 1.09-2.28). Although this was not considered a significant interaction (*P* = 0.743).

We further divided the overweight/obesity group into participants with overweight and participants with obesity in order to compare risks of developing diabetes (Table 4). The overweight group had a significant association with DM occurrence, with an aHR of 1.44 (95% CI, 1.09-1.91) in those onset age<45 years, 1.37 (95% CI, 1.12-1.67) in those onset age from 45 to 54 years and 1.32 (95% CI, 1.09-1.59) in those onset age from 55 to 64 years. However, there was no significant finding in those considered obesity (All *P* > 0.05).

To assess the influence of weight fluctuations, we further divided the overweight/obese group into participants who were initially categorized as overweight or obese but who subsequently achieved normal BMI, and those who remained in the overweight or obese category (Table 5). Results showed that regardless of the onset age reducing weight to within a normal BMI range significantly reduced the risk of developing diabetes. The HR was 0.35 (95% CI, 0.24-0.49) in those with an onset age <45 years, and 0.31 (95% CI, 0.24-0.41) in those onset age from 45 to 54 years, 0.35 (95% CI,

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TABLE 2 Baseline characteristics of new-onset overweight/obesity across age groups†.

Variables	New-onset overweight/obesity according to age				*P*
	<45 years	45-54 years	55-64 years	≥65 years	
Participants, n	2,739	3,284	3,258	1,577	
Age, years	36.8 ± 5.9	50.2 ± 2.9	59.4 ± 2.8	71.7 ± 5.3	–
Male sex, n(%)	2049(74.8)	2215(67.4)	2439(74.9)	1315(83.4)	–
BMI, kg/m^2	26.5 ± 2.3	26.5 ± 2.5	26.5 ± 2.2	26.5 ± 2.4	<0.01
FBG, mmol/L	5.1 ± 0.6	5.2 ± 0.6	5.3 ± 06	5.4 ± 0.6	<0.01
SBP, mmHg	121.7 ± 14.7	128.8 ± 17.3	136.0 ± 19.7	141.6 ± 20.0	<0.01
DBP, mmHg	81.2 ± 9.9	84.5 ± 10.8	85.8 ± 10.9	84.5 ± 10.6	<0.01
TG, mmol/L	1.8 ± 1.4	1.7 ± 1.4	1.6 ± 1.1	1.4 ± 0.9	<0.01
TC, mmol/L	4.9 ± 1.0	5.1 ± 1.0	5.2 ± 1.0	5.1 ± 1.0	<0.01
HDL-C, mmol/L	1.4 ± 0.4	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	<0.01
LDL-C, mmol/L	2.6 ± 0.8	2.6 ± 0.9	2.7 ± 0.9	2.7 ± 1.0	<0.01
Cigarette smoking, n(%)					<0.01
Current	1001(36.6)	1101(33.5)	803(24.7)	269(17.1)	
Former	184(6.7)	226(6.9)	294(9.0)	136(8.6)	
Never	1554(56.7)	1957(59.6)	2161(66.3)	1172(74.3)	
Alcohol drinking, n(%)					<0.01
Current	744(27.2)	746(22.7)	522(16.0)	223(14.1)	
Former	624(22.8)	662(20.2)	500(15.4)	197(12.5)	
Never	1371(50.0)	1876(57.1)	2236(68.6)	1157(73.4)	
Physical exercise, n(%)	257(9.4)	467(14.2)	719(22.1)	398(25.2)	<0.01
Family history of diabetes, n(%)	373(13.6)	345(10.5)	189(5.8)	27(1.7)	<0.01
Education, n(%)					<0.01
Less than high school	39(1.4)	115(3.5)	333(10.2)	364(23.1)	
High school degree	2163(79.0)	3014(91.8)	2840(87.2)	1155(73.2)	
University degree or higher	537(19.6)	155(4.7)	85(2.6)	58(3.7)	

BMI, body mass index; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SBP, systolic blood pressure.

† Baseline refers to the examination cycle when new-onset overweight/obesity was first identified.

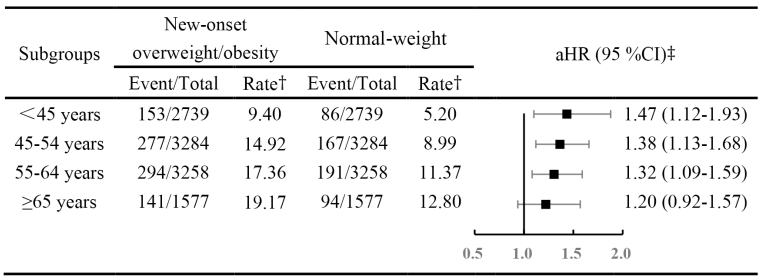

FIGURE 1
Hazard rations for diabetes mellitus across age-based onset groups, among new onset overweight and obesity versus normal-weight participants. † The rate was per 1,000 person years. ‡ Adjusted for systolic blood pressure, fasting blood glucose, triglyceride, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, cigarette smoking status, alcohol drinking status, physical exercise, family history of diabetes, education degree. Abbreviations: aHR, adjusted hazard ratio; CIs, confidence intervals.

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TABLE 3 Hazard ratios for diabetes mellitus across age-based onset groups, among new-onset overweight and obesity versus normal-weight participants by sex.

Subgroup	New-onset overweight/obesity		Normal-weight		aHR (95% CI) †	*P* for interaction
	Event/Total	Rate†	Event/Total	Rate†		
Male (n=16,036)						0.74
<45 years	130/2049	10.63	73/2049	5.80	1.56 (1.16-2.10)	
45-54 years	185/2215	14.89	122/2215	9.80	1.31 (1.04-1.65)	
55-64 years	221/2439	17.49	145/2439	11.70	1.30 (1.05-1.62)	
≥65 years	116/1315	19.14	76/1315	12.59	1.21 (0.90-1.63)	
Female (n=5,680)						
<45 years	23/690	5.69	13/690	3.31	0.98 (0.47-2.04)	
45-54 years	92/1069	14.99	45/1069	7.32	1.58 (1.09-2.28)	
55-64 years	73/819	16.98	46/819	10.44	1.38 (0.94-2.03)	
≥65 years	25/262	19.35	18/262	13.77	1.27 (0.67-2.42)	

aHR, adjusted hazard ratio; CI, confidence interval.
† Adjusted for systolic blood pressure, fasting blood glucose, triglyceride, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, cigarette smoking status, alcohol drinking status, physical exercise, family history of diabetes, education degree.

0.27-0.45) in those onset age from 55 to 64 years and 0.30 (95% CI, 0.21-0.44) in those onset age ≥65 years.

3.4 Sensitivity analysis

Results remained consistent after excluding participants who were diagnosed with malignant tumors during study and after excluding those with outcome events within the first year of follow-up (Table 6).

4 Discussion

Our findings suggest that new-onset overweight/obesity is associated with a higher risk developing diabetes mellitus among Chinese mainlanders, although the magnitude of this effect varied across the lifespan. Participants considered (using BMI) as overweight or obese at age <45 years were at the highest risk of developing diabetes mellitus, compared with age- and sex- matched controls. The aforementioned risk gradually attenuated with each decade increase in excessive weight gain onset age. The results also

TABLE 4 Hazard ratios for incident diabetes mellitus among patients with new-onset overweight and obesity versus normal-weight participants, across separate age-groups.

Subgroup	New-onset overweight or obesity		Normal-weight		aHR (95% CI)‡
	Event/Total	Rate†	Event/Total	Rate†	
Overweight (n=20,692)					
<45 years	146/2623	9.35	84/2623	5.29	1.44 (1.09-1.91)
45-54 years	263/3113	14.90	161/3113	9.13	1.37 (1.12-1.67)
55-64 years	282/3125	17.30	184/3125	11.38	1.32 (1.09-1.59)
≥65 years	131/1485	18.76	88/1485	12.61	1.17 (0.89-1.54)
Obesity (n=1,024)					
<45 years	7/116	10.53	2/116	3.02	3.08 (0.25-37.89)
45-54 years	14/171	15.21	6/171	6.39	1.41 (0.53-3.78)
55-64 years	12/133	18.74	7/133	11.18	1.38 (0.53-3.58)
≥65 years	10/92	26.80	6/92	16.40	2.42 (0.58-9.98)

aHR, adjusted hazard ratio; CI, confidence interval.
† Rate was per 1,000 person years.
‡ Adjusted for systolic blood pressure, fasting blood glucose, triglyceride, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, cigarette smoking status, alcohol drinking status, physical exercise, family history of diabetes, education degree.

TABLE 5 Hazard ratios for incident diabetes mellitus of changed to a normal BMI among patients with new-onset overweight and obesity, across separate age-groups.

Subgroup	Changed to a normal BMI		Stable overweight/obesity		aHR (95% CI)‡
	Event/Total	Rate†	Event/Total	Rate†	
<45 years	45/1264	5.45	108/1475	13.47	0.35 (0.25-0.50)
45-54 years	76/1659	7.51	201/1625	23.82	0.32 (0.25-0.42)
55-64 years	84/1533	9.33	210/1725	26.47	0.35 (0.27-0.46)
≥65 years	44/850	9.81	97/727	33.78	0.30 (0.21-0.43)

aHR, adjusted hazard ratio; CI, confidence interval.
† Rate was per 1,000 person years.
‡ Adjusted for systolic blood pressure, fasting blood glucose, triglyceride, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, cigarette smoking status, alcohol drinking status, physical exercise, family history of diabetes, education degree.

remained stable in men and when analysis was constricted to overweight participants, but not for those considered obese.

To date, few studies have investigated the relationship between excessive weight gain onset age and risk of developing diabetes. In a related, British birth cohort study, which compared those who had never been obese, childhood obesity and younger-adulthood obesity have a 4.38-fold (95% CI, 1.86–10.31) and 3.96-fold (95%CI, 2.10-7.43) risk of hemoglobin A1c (HbA1c) ≥7%, respectively (18). HbA1c is an indicator for glucose metabolism, where a threshold of 6.5% could also be used to diagnose diabetes mellitus (19). An HbA1c reading of between 6.0–6.9% corresponds with 60% developing diabetes within a 10 years follow-up period (20). In this study, similar trends were found for onset age of overweight/obesity which associations were non-significant at a younger-adulthood and mid-adulthood onset age (18), which is similar with our findings. These studies suggest the impact of excessive weight gain in terms of diabetes across the life course, although single comprehensive studies which investigate these changes across a specific nation are few in number.

Excessive weight gain is associated with an increased risk of developing insulin resistance and type 2 diabetes. In obese individuals, adipose tissue releases increased amounts of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines and other factors that are involved in the development of insulin resistance (21). When insulin resistance is accompanied by dysfunction of pancreatic islet beta-cells, failure to control blood glucose levels results in type 2 diabetes. A previous study reported that in addition to initial BMI, obesity onset at younger ages also means cumulative exposure, which is associated with an increased risk of developing type 2 diabetes (22). This supports our finding that an early onset age of excessive weight gain also means a longer cumulative exposure to detrimental factors. Secondly, evidence suggests that earlier excessive weight gain is closely related to genetic predispositions (23, 24). Previous studies have also identified numerous genetic loci and gene variants such as FTO, MC4R, ADAMTS9 and GRB14/COBLL1, which have been found to be associated with overweight/obesity and diabetes (23, 25, 26). Early-onset overweight/obesity participants are likely to be

TABLE 6 Sensitivity analysis.

Subgroup	New-onset overweight/obesity		Normal-weight		aHR (95% CI) ‡
	Event/Total	Rate†	Event/Total	Rate†	
Excluding participants with malignant tumors (n=21,436)					
<45 years	153/2725	9.45	86/2725	5.37	1.37 (1.05-1.80)
45-54 years	273/3240	14.91	167/3240	9.41	1.31 (1.08-1.60)
55-64 years	291/3207	17.49	191/3207	11.54	1.26 (1.05-1.52)
≥65 years	138/1546	19.14	86/1546	11.90	1.27 (0.96-1.67)
Excluding participants with outcome events within the first year of follow-up (n=21,682)					
<45 years	153/2736	9.40	86/2736	5.2	1.45 (1.11-1.91)
45-54 years	275/3278	14.82	167/3278	8.99	1.35 (1.11-1.64)
55-64 years	294/3253	17.36	190/3253	11.31	1.29 (1.07-1.55)
≥65 years	140/1574	19.04	94/1574	12.8	1.21 (0.93-1.59)

aHR, adjusted hazard ratio; CI, confidence interval.
† Rate was per 1,000 person years.
‡ Adjusted for systolic blood pressure, fasting blood glucose, triglyceride, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, cigarette smoking status, alcohol drinking status, physical exercise, family history of diabetes, education degree.

genetically susceptible and carry the above genes which create a higher risk of diabetes. Thirdly, being overweight or obese could interfere with age induced epigenetic changes including DNA methylation, non-coding RNA (ncRNA) and histone modifications. Altered DNA methylation in different tissues (human pancreatic islets, skeletal muscle and adipose tissue) could reduce insulin secretion and increases insulin resistance via differs patterns. Additionally, early onset overweight/obesity may initiate DNA methylation and gene expression in early adulthood and lead to high risk of diabetes (27–32). It is also reported that changes in BMI are accompanied by widespread metabolic changes in early adult, resulting in chronically increased levels of circulating free fatty acids and adipokines, which is again closely associated with diabetes (33–36). Finally, individuals with young-onset overweight/obesity tended to have an unhealthy lifestyle, which likely contributes to the development of diabetes.

In this study, participants with overweight/obesity whose onset age ≥65 years were not observed to be statistically correlate with a higher risk of incident diabetes, compared with normal-weight participants. We supposed that there were some competing risks in older adults who may develop diabetes and obesity simultaneously, reducing the HR of the association. This phenomenon was also observed in other studies (37, 38).

We conducted gender-specific stratification analysis, only observing a positive correlation among women who were overweight or obese and had an onset age of between 45 to 54 years. In overweight or obese women with an onset age <45 years, there was no correlation (aHR, 0.98; 95% CI, 0.47-2.02), which was different from men who had an aHR = 1.52 (95% CI, 1.13-2.04). In a related longitudinal Australian study of women's health researchers found that obesity onset age negatively correlates with an increased risk of developing diabetes (aHR, 0.87; 95% CI, 0.79-0.96, per 1 year increment) (22). This may have a biological basis because premenopausal women may have a degree of protection from circulating estrogen (39). Another reason could be differences in fat distribution between men and women, where women have a greater proportion of subcutaneous fat and adipose tissue whereas men tend to harbour visceral fat on the abdomin which is a known driver in the progression of disease (40). Therefore, even though these women who became overweight/obese at an onset age <45 years, the risk of developing diabetes mellitus does not necessarily increase. This assertion is supported by sex differences in the prevalences because there are more men with pre-pubescent diabetes, whereas there are more women with postmenapausal diabetes (41). The development of diabetes mellitus after menopause is thought to occur through alterations in insulin secretion, insulin sensitivity, and glucose effectiveness (42).

We observed that the risk of women at onset age between 45 to 54 years was higher than men at same age (aHR, 1.57 *vs* aHR, 1.29), which suggests that the risk of developing diabetes mellitus by weight gain may be much higher in women after menopause. Though a positive correlation was observed in women at onset age higher than 55, such association was not statistically significant. This may be due to the comparatively small sample size of these groups (1,638 at an onset age of between 55 to 64 years and 524 at an onset age higher than 65 years). On the basis of the incidence of 7.26% and 8.20%, the power to find a relationship in these two groups was 0.60 and 0.26 (expected HR = 1.5).

According to previous reports, the risk of obesity may be higher than being overweight in a similar age group (18), but we did not observe that in this study. Hypothetically, this may be due to the comparatively low prevalence of obesity in China when we defined obesity according to World Health Organization (WHO) cutoff (BMI ≥ 30 kg/m^2). On the other hand, because of the incidence of 6.25% and sample size of 1024, the power to find a relationship in women was only 0.37 (expected HR = 1.5). Therefore, a larger sample is required to explore this further.

Considering the influence of weight loss, we conducted a separate subgroup analysis of overweight or obese category between those who changed to a normal BMI and those maintained in the overweight or obese category. We observed a steady risk reduction in those who changed to a normal BMI, which was consistent with a retrospective cohort study of U.S. adults (HR, 0.33; 95% CI, 0.14-0.76) (43). Surprisingly, the risk reduction effect we observed was steady in all age group. That is to say, weight loss at any age to reduce the risk of developing diabetes.

Apart from diabetes mellitus, researchers have also found weight gain positively correlates to mortality and some cancers, and the younger the onset age of weight gain, the higher the risk (37, 44, 45). A typical study from the United States reported that weight gain at all ages positively relate to mortality, but with stronger associations for weight gain between ages 18 and 35 years and ages 35 and 50 years than between ages 50 and 69 years (44). Another study from the United States showed such relationship between onset age of weight gain and pancreatic cancer (37). The risk change from 1.50 (95% 1.26-1.77) from age 14 to 19 to 1.16 (95% 1.02-1.32) from age 50 to 59, and non-significant from age 60 to 69 (37). Also, age of onset of obesity may, at least in part, affect the prevalence of cardiovascular risk factors in severe obesity (46). Therefore, weight gain over different stages of life from early childhood is implicated in the development of diabetes, specific cancer, cardiovascular diseases and even mortality. Uncertainty remains about whether some life stages are more influential than others.

This was a large prospective study investigating the association between onset age of overweight/obesity and the risk of developing diabetes which use age, rather than study time, as the time scale to optimally account for the observational study design. However, this study also has several limitations. The distribution of gender in our study was imbalanced (men 73.8%) and the characteristics and mechanisms of overweight/obesity and diabetes may be different between men and women. Secondly, our definition of overweight/obesity is based on BMI, which is only an indicator of total body adiposity and could not accurately represent the distribution of adipose tissue or bone density. However, BMI is the most commonly used and practical measure of obesity both in children and adults. There are studies which have proved that BMI is generally consistent with indicators of central adiposity such as waist circumference and waist-to-height ratio (47, 48). Thirdly, the diagnosis of diabetes was based on a single measurement of FBG rather than oral glucose tolerance testing or the measurement of HbA1c, and therefore, the incidence of diabetes might be underestimated. Finally, our regression model included

adjustments only for a small number of covariates, which means residual confounders may still exist.

5 Conclusion

Our study found that new-onset overweight/obesity correlates with an increased risk of developing diabetes mellitus among Chinese population <65 years. Participants with early onset excessive weight gain or obesity were at a higher risk of having diabetes. The results highlight the importance of preventing the onset of excessive weight gain as we age. Although, further research is needed to link lifestyles, developmental stages, and physiological changes to understand these interactions more clearly.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the ethics committee in Kailuan General Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

WF: Formal Analysis, Software, Writing – original draft. XY: Formal Analysis, Software, Writing – original draft. WL: Data curation, Writing – review & editing. SS: Writing – review & editing. GC: Data curation, Writing – review & editing. ZFC: Data curation, Writing – review & editing. ZH: Data curation, Writing – review & editing. XW: Data curation, Writing – review & editing. WW: Data curation, Writing – review & editing. ZCC: Data curation, Writing – review & editing. YL: Funding acquisition, Methodology, Writing – review & editing. SW: Conceptualization, Project administration, Writing – review & editing. YC: Conceptualization, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1281203/full#supplementary-material

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Association between the weight-adjusted waist index and the odds of type 2 diabetes mellitus in United States adults: a cross-sectional study

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Objective: To examine the association between the weight-adjusted waist index (WWI) and the odds of type 2 diabetes mellitus(T2DM)among U.S. adults.

Methods: Data from the National Health and Nutrition Examination Survey (NHANES) spanning six years (2007–2018) were utilized, encompassing 31001 eligible participants. Weighted multivariate logistic regression models and smoothed fit curves were employed to assess the association between WWI and the odds of T2DM, as well as dose-response relationships in the overall population and the odds of T2DM in various subgroups.

Results: In the fully adjusted continuous model, each one-unit increase in WWI was associated with a 1.14-fold increase in the odds of T2DM within the entire study population (2.14 [1.98,2.31], P < 0.0001). In the fully adjusted categorical model, when using the lowest tertile of WWI (T1) as the reference group, the second tertile (T2) and the third tertile (T3) were associated with a 0.88-fold (1.88 [1.64,2.17], P < 0.0001) and a 2.63-fold (3.63 [3.11,4.23], P < 0.0001) increase in the odds of T2DM. These findings indicated a positive correlation between WWI values and the odds of T2DM, aligning with the results of the smoothed-fitted curves. In the analysis of subgroups, in addition to maintaining consistency with the overall population results, we found interactions between age and hypertension subgroups.

Conclusion: In conclusion, WWI was found to be positively associated with the odds of T2DM in U.S. adults.

KEYWORDS

weight-adjusted waist index, type 2 diabetes mellitus, NHANES, United States adults, cross-sectional study

1 Introduction

Type 2 diabetes mellitus (T2DM) stands as a chronic metabolic disorder known for its hallmark features of insulin resistance and elevated blood glucose levels. Once diagnosed, it typically proves challenging to reverse, and it frequently leads to complications affecting the kidneys, retinal health, cardiovascular system, neural function, and liver. Regrettably, effective treatments for this condition have been lacking (1). Recent statistics indicate a concerning trend, with the prevalence of diabetes projected to surge to 578 million individuals by 2030 (2). Therefore, we must take effective measures to identify people at risk of developing diabetes and to give relevant preventive guidance and advice promptly, to achieve the goal of slowing down the onset and development of diabetes.

Globally, the rates of obesity have reached epidemiological levels and are impacting an increasing population of individuals (3–5). Studies have demonstrated a strong association between obesity and the development of type 2 diabetes (6). Although Body Mass Index (BMI) is the most commonly used method to assess obesity, an important limitation of applying BMI is that it does not reflect the true body fat distribution (7). Waist circumference (WC) is a simple and reliable method for assessing abdominal obesity and has been used to measure total body fat (8), but fails to distinguish between subcutaneous and visceral fat (9). It has been shown that visceral fat produces more free fatty acids than subcutaneous fat, which increases insulin resistance and the risk of diabetes (10, 11). Quantifying visceral fat and muscle mass by applying Computer Tomography (CT) or Magnetic Resonance Imaging (MRI) lacks feasibility in clinical work. Therefore, Park Y et al. proposed a novel index of obesity called the "weight-adjusted waist circumference index (WWI)" (12). The WWI is calculated as waist circumference (WC) divided by the square root of body weight, which not only weakens the relationship with BMI but also combines the advantages of WC to provide a better reflection of body fat distribution and muscle mass, as well as reflecting the problem of central obesity, which is not related to body weight (13). Therefore, WWI has potential advantages as a predictor of obesity.

Previous studies have shown a positive association between WWI and the prevalence of type 2 diabetes (14, 15), but these studies have been conducted in Asian countries, and there is a lack of research on the association between WWI and the prevalence of type 2 diabetes in the US population. Therefore, we applied a large sample of data obtained from the NHANES database from 2007 to 2018 to further validate the potential relationship between WWI and the odds of T2DM.

2 Methods

2.1 Study population

NHANES, a nationally representative cross-sectional study, was purposefully designed to evaluate the health and nutritional status of an ambulatory population within the United States. The U.S. Centers for Disease Control and Prevention secured approval from the Research Ethics Review Board for this study, and all participants provided written informed consent, obviating the need for further ethical review. The present study adhered to the guidelines outlined in the Epidemiologic Statement for Enhanced Reporting of Observational Studies (16).

For the investigation, a comprehensive screening of the entire 2007-2018 NHANES database was carried out, encompassing a total of 59842 participants. To ensure the relevance and accuracy of the study, specific exclusion criteria were applied. Participants who were younger than 20 years old were excluded, resulting in the exclusion of 25072 participants. Additionally, participants with missing data on the waist circumference were excluded, leading to the exclusion of 3385 participants. Participants with missing data on weight were excluded, leading to the exclusion of 40 participants. Moreover, those with incomplete records regarding a diagnosis of T2DM were also excluded, accounting for an additional 344 participants. Consequently, the final dataset analyzed for the study consisted of 31001 subjects. Further details are illustrated in Figure 1.

2.2 Diagnosis of T2DM

Under the 2013 U.S. Diabetes Guidelines (17), T2DM was categorized as meeting any of the following criteria: 1) self-reported physician-diagnosed T2DM; 2)Currently taking hypoglycemic drugs or injecting insulin;3) random blood glucose level equal to or exceeding 11.1 mmol/L; 4) glycosylated hemoglobin (HbA1c) level equal to or exceeding 6.5%; 5) fasting blood glucose level equal to or exceeding 7.0 mmol/L; 6) 2-h blood glucose level (determined by oral glucose tolerance test (OGTT)) equal to or exceeding 11.1 mmol/L. In the analysis, the incidence of T2DM was regarded as the outcome of interest.

2.3 Calculation of WWI

WWI (cm/√kg) was measured by calculating WC (cm) divided by the square root of the weight (kg). Anthropometric

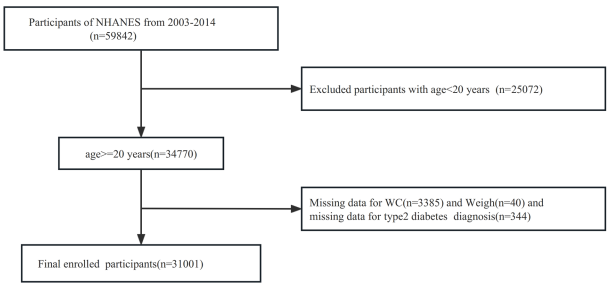

FIGURE 1
Flowchart of participants' selection.

measurements were recorded by trained medical personnel and specialized recorders to ensure the exact accuracy of the data. Body weight was determined using a digital scale. Participants were asked to wear their examination clothing, and then stand barefoot on the digital scale with arms held close to their bodies and gaze fixed straight ahead, as outlined previously (18). WC was calculated using a tape measure, which was positioned at the intersection of the midaxillary line and the horizontal line just above the outermost upper point of the right kneecap (19). The WWI value was used as an exposure variable in this study.

2.4 Covariates

Covariates, such as age, gender, race/ethnicity (Non-Hispanic white, Non-Hispanic black, Mexican-American, etc.), educational level (less than high school, high school or equivalent, college or above), marital status (married/living with partner, never married, widowed/divorced/separated), family poverty-to-income ratio (PIR), smoking status (never, now, former), alcohol user (never, former, mild, moderate, heavy), Physical activity(PA, Mets), Alanine aminotransferase (ALT, U/L), Aspartate aminotransferase (AST, U/L), creatinine(umol/L), uric acid(UA,umol/L), triglycerides (TG, mmol/L), high-density lipoprotein-cholesterol (HDL-C, mmol/L), total cholesterol (TC, mmol/L). Hypertension was defined as meeting any of the following criteria: 1) self-reported physician diagnosis of hypertension; 2) current use of antihypertensive medication; 3) an average of three measurements of systolic blood pressure (SBP) exceeding 140 mmHg and/or diastolic blood pressure (DBP) exceeding 90 mmHg. Hyperlipidemia can be diagnosed by meeting any of the following conditions: 1) Diagnosed as hypertriglyceridemia (TG>=150mg/dL or>=1.70mmol/L); 2) Diagnosis of hypercholesterolemia can be made by meeting any of the following conditions: 1. TC>=200mg/dL (>=5.18mmol/L) 2. Low-density lipoprotein-cholesterol(LDL-C)>=130mg/dL (>=3.37mmol/L) 3. Male HDL<40mg/dL (<1.04mmol/L), female HDL<50mg/dL (<1.30mmol/L); 3) Currently taking lipid-lowering medication. The diagnosis of coronary heart disease (CHD) was determined based on self-reported responses to the NHANES questionnaire. The calculation of physical activity is based on multiplying the number of days (in days/week) of specific activities per week by the duration of specific activities per day (in minutes/day) to calculate the resulting exercise metabolic equivalents (METs). Stroke diagnoses were derived from self-reported stroke diagnoses from the questionnaire. Some covariates had missing values, although the extent of missing data for each variable was less than 20%. These missing values were addressed through the utilization of the "mice" R package, employing multiple interpolations to impute the missing data for continuous variables.

2.5 Statistical analysis

The statistical analysis was conducted using R 4.3.1 software. The analysis incorporated the variables for weights, sdmvstra, and sdmvpsu. Continuous variables were expressed as mean ± standard deviation, while categorical variables were presented as numbers and percentages. To make the comparison between the two groups, weighted Student's t-test, Mann-Whitney U test, and the Chi-square test were utilized.

To explore the relationship between WWI level and the odds of developing T2DM, the multivariate logistic regression model was employed. Odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were computed using the "survey" R package. Model 1 was unadjusted for any covariates. Model 2 was adjusted for age, gender, and race/ethnicity. Model 3 further included adjustments for educational level, marital status, PIR, smoking status, alcohol user, ALT, AST, creatinine, UA, TC, TG, HDL, hypertension, CHD, stroke, and Hyperlipidemia. The multivariate logistic regression model was divided into categorical and continuous models. In the categorical model, WWI values were categorized into thirds, and nonlinear trends were assessed by treating the median value of each trichotomy as a continuous variable.

In addition, subgroup analysis was conducted to indicate whether age, gender, race/ethnicity, hypertension, CHD, stroke, and Hyperlipidemia were associated with the odds of T2DM in different population subgroups. The level of statistical significance was set at $P<0.05$.

3 Results

3.1 Characteristics of the study population

A total of 31001 participants were included, and Table 1 shows the demographic and clinical features of participants by tertiles of baseline WWI values. All variables were statistically significant among the three WWI subgroups. Compared with participants in the T1 group, participants in the T3 group were mostly female, older, non-Hispanic white, less educated, Divorced/widowed/separated, exhibited a higher prevalence of former smokers and previous drinkers, had lower PIR, creatinine, PA and HDL, had higher values of WC, Weight, ALT, AST, UA, TC, TG, and BMI. Besides, they had higher incidence rates of hypertension, CHD, Hyperlipidemia, T2DM, and stroke.

3.2 Association between the WWI and the odds of T2DM

As indicated in Table 2, in the completely adjusted continuous model, with each 1-unit increase in WWI, the total odds of T2DM increased by 1.14-fold [2.14 [1.98,2.31], $P<0.0001$]. In the completely adjusted categorical model, compared to the lowest tertile of WWI (T1) serving as the reference group, T2 and T3 were associated with a 0.88-fold [1.88 [1.64,2.17], $P<0.0001$] and 2.63-fold [3.63 [3.11,4.23], $P<0.0001$] increase in the odds of developing T2DM, respectively. These findings underscore a significant positive correlation between WWI and the odds of T2DM. Furthermore, the results from the fitted curves indicated a non-linear trend (P non-linear=0.0017), as illustrated in Figure 2.

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TABLE 1 Basic characteristics of participants by weight-adjusted-waist index tertile.

variable	total	T1(=<10.70)	T2(11.70-11.44)	T3(>11.44)	Pvalue
Age (years old)	47.40 ± 0.22	38.93 ± 0.27	49.03 ± 0.25	56.81 ± 0.28	< 0.0001
Sex, (%)					< 0.0001
Female	15719(50.7)	4206(42.33)	5030(49.81)	6483(63.96)	
Male	15282(49.3)	6125(57.67)	5301(50.19)	3856(36.04)	
Race/ethnicity, (%)					< 0.0001
Mexican American	4668(15.06)	970(5.98)	1709(9.84)	1989(10.50)	
Non-Hispanic Black	6612(21.33)	2765(13.40)	2106(10.09)	1741(9.32)	
Non-Hispanic White	12617(40.7)	4184(66.47)	4013(65.04)	4420(67.50)	
Others	7104(22.92)	2412(14.15)	2503(15.03)	2189(12.69)	
Educational level, (%)					< 0.0001
College or above	5128(16.54)	2145(22.55)	1736(17.86)	1247(12.04)	
High school or equivalent	18289(58.99)	6476(66.21)	6104(66.29)	5709(66.34)	
Less than high school	7584(24.46)	1710(11.23)	2491(15.85)	3383(21.63)	
Marital status, (%)					< 0.0001
Divorced/widowed/separated	6830(22.03)	1488(11.82)	2148(17.77)	3194(27.32)	
Married/living with partner	18439(59.48)	5805(60.28)	6687(68.69)	5947(60.98)	
Never married	5732(18.49)	3038(27.90)	1496(13.53)	1198(11.70)	
PIR	2.99 ± 0.03	3.16 ± 0.04	3.03 ± 0.04	2.70 ± 0.04	< 0.0001
Smoking status, (%)					< 0.0001
Former	7335(23.66)	1743(18.20)	2571(26.51)	3021(30.66)	
Never	17282(55.75)	6046(60.11)	5715(54.18)	5521(51.17)	
Now	6384(20.59)	2542(21.70)	2045(19.32)	1797(18.17)	
Alcohol user, (%)					< 0.0001
Former	4311(13.91)	883(6.83)	1440(12.11)	1988(16.74)	
Heavy	5694(18.37)	2399(23.99)	1904(19.25)	1391(14.69)	
Mild	9456(30.5)	3387(34.99)	3199(33.61)	2870(32.39)	
Moderate	4315(13.92)	1674(17.57)	1481(16.68)	1160(13.35)	
Never	7225(23.31)	1988(16.62)	2307(18.34)	2930(22.84)	
Waist circumference (cm)	99.26 ± 0.23	87.87 ± 0.18	100.60 ± 0.18	112.86 ± 0.26	< 0.0001
Weight (kg)	82.66 ± 0.24	75.86 ± 0.25	83.89 ± 0.29	90.28 ± 0.42	< 0.0001
Alt(U/L)	25.30 ± 0.16	23.57 ± 0.19	26.86 ± 0.34	25.79 ± 0.27	< 0.0001
Ast(U/L)	25.22 ± 0.12	24.64 ± 0.15	25.66 ± 0.23	25.48 ± 0.23	< 0.001
UA(umol/L)	322.38 ± 0.79	310.26 ± 1.17	324.03 ± 1.22	336.62 ± 1.13	< 0.0001
Creatinine(umol/L)	77.87 ± 0.23	78.59 ± 0.31	77.26 ± 0.35	77.63 ± 0.36	0.01
Total cholesterol (mmol/L)	5.01 ± 0.01	4.89 ± 0.02	5.11 ± 0.02	5.04 ± 0.02	< 0.0001
Triglyceride (mmol/L)	1.72 ± 0.02	1.41 ± 0.02	1.84 ± 0.02	2.00 ± 0.02	< 0.0001
HDL-C (mmol/L)	1.38 ± 0.01	1.46 ± 0.01	1.35 ± 0.01	1.31 ± 0.01	< 0.0001
BMI (kg/m^2)	28.99 ± 0.09	25.46 ± 0.07	29.31 ± 0.08	33.33 ± 0.13	< 0.0001
Physical activity(Mets)	3837.49 ± 65.86	4995.10 ± 105.55	3689.74± 96.11	2465.93± 68.28	< 0.0001

(Continued)

TABLE 1 Continued

variable	total	T1(=<10.70)	T2(11.70-11.44)	T3(>11.44)	Pvalue
Hypertension, (%)					< 0.0001
No	17799(57.41)	7942(79.43)	5894(60.67)	3963(41.53)	
Yes	13202(42.59)	2389(20.57)	4437(39.33)	6376(58.47)	
Coronary heart disease, (%)					< 0.0001
No	29774(96.04)	10199(98.90)	9971(96.88)	9604(93.30)	
Yes	1227(3.96)	132(1.10)	360(3.12)	735(6.70)	
Stroke, (%)					< 0.0001
No	29819(96.19)	10172(98.86)	9975(97.41)	9672(94.58)	
Yes	1182(3.81)	159(1.14)	356(2.59)	667(5.42)	
Hyperlipidemia, (%)					< 0.0001
No	9579(30.9)	4989(47.59)	2728(25.12)	1862(18.26)	
Yes	21422(69.1)	5342(52.41)	7603(74.88)	8477(81.74)	
T2DM, (%)					< 0.0001
No	25110 (81)	9749(96.03)	8566(87.03)	6795(70.67)	
Yes	5891 (19)	582(3.97)	1765(12.97)	3544(29.33)	

PIR, poverty-to-income ratio; BMI, body mass index; HDL-c, high-density lipoprotein-cholesterol; T2DM, type 2 diabetes mellitus; UA, uric acid; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.
Continuous variables were presented as mean with standard deviation (mean ± SD), and categorical variables were expressed as proportion.
Continuous variables were analyzed via one-way ANOVA; categorical variables were analyzed using the Chi-square test or the Fisher's exact test, and P-value less than 0.05 was considered statistically significant.

3.3 Subgroup analysis

The subgroup analysis was performed to assess the association between WWI and the odds of T2DM in different populations. As shown in Table 3, the relationship between WWI and the odds of T2DM was not markedly affected by age, gender, race/ethnicity, hypertension, CHD, stroke, and Hyperlipidemia(P<0.05). Significant interaction (p for interaction < 0.0001) was identified within subgroups based on age(<60、>=60). Among individuals younger than 60 years, each 1-unit increase in WWI was associated with a 1.79-fold elevated odds of T2DM. In contrast, for those aged 60 years or older, each 1-unit increase in WWI was linked with a 0.84-fold increased odds of T2DM. We also found a certain degree of interaction (p for interaction=0.01) in the hypertension (yes/no) subgroup. In hypertensive populations, each 1-unit increasement in WWI was correlated with a 1.06-fold increased odds of T2DM, and in non-hypertensive populations, each 1-unit increasement in WWI was related to a 1.20-fold increased odds of T2DM.

TABLE 2 The association between weight-adjusted-waist index and T2DM.

WWI	Event(%)	Type 2 diabetes OR (95%CI)					
		Model 1	P	Model 2	P	Model 3	P
Per 1 increment	5891(19.00)	3.20(3.00,3.40)	<0.0001	2.73(2.54,2.93)	<0.0001	2.14(1.98,2.31)	<0.0001
Tertiles							
T1	582(3.97)	1.00(reference)		1.00(reference)		1.00(reference)	
T2	1765(12.97)	3.61(3.14, 4.15)	<0.0001	2.59(2.25,2.97)	<0.0001	1.88(1.64,2.17)	<0.0001
T3	3544(29.33)	10.05(8.75,11.53)	<0.0001	6.11(5.29,7.06)	<0.0001	3.63(3.11,4.23)	<0.0001
p for trend			<0.0001		<0.0001		<0.0001

Model 1 was adjusted for none.
Model 2 was adjusted for age, sex, and race/ethnicity.
Model 3 was adjusted for age, sex, race/ethnicity, educational level, marital status, PIR, smoking status, alcohol user, Alt, Ast, UA, creatinine, TC, TG, HDL-C, physical activity,hypertension, stroke, coronary heart disease, and hyperlipidemia.
PIR, poverty-to-income ratio; HDL-c, high-density lipoprotein-cholesterol; UA, uric acid; TG, triglycerides; TC, total cholesterol;ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.

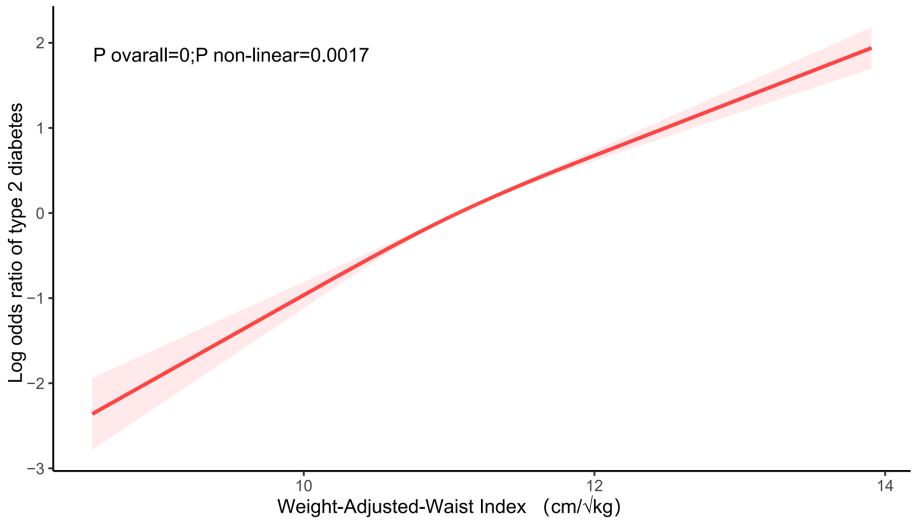

FIGURE 2
The association between WWI and the odds of T2DM. Age, sex, race/ethnicity, educational level, marital status, PIR, smoking status, alcohol user, Alt, Ast, UA, creatinine, TC, TG, HDL-C, physical activity,hypertension, stroke, coronary heart disease, and hyperlipidemia were adjusted.

4 Discussion

In this cross-sectional study involving 31,001 nationally representative participants, we found a strong positive correlation between WWI values and the odds of type 2 diabetes, implying that individuals with a higher WWI are more likely to have type 2 diabetes. Moreover, in the subgroup analyses, we found an interaction between age and hypertension in both subgroups.

A prospective cohort study from Northeast China (n=9205) observed a significant positive correlation between WWI values and the occurrence of type 2 diabetes and suggested that WWI could be a simple and effective predictor for the diagnosis of type 2 diabetes mellitus in a rural population in China (14). Sun H et al. (15) conducted a secondary analysis of a retrospective cohort study in a Japanese population (n=15,464) and found that WWI is a new metabolic index that can be used to predict T2DM in a Japanese population. WWI was found to be a novel metabolic index that can be used to predict T2DM occurrence in the Japanese population. Our findings were generally consistent with the results of these two studies. In addition, subgroup analyses revealed that WWI was more strongly associated with T2DM in people younger than 60 years of age (p for interaction< 0.0001), which may be related to changes in body composition such as significant increases in adiposity and decreases in muscle mass in older adults due to aging (20, 21), and other studies have suggested that it may also be related to differences in adiposity distribution between the younger and the older population (22, 22). differently in younger and older populations (22, 23). Our study also found higher odds of T2DM in the nonhypertensive population than in the hypertensive population.

In addition to being associated with T2DM, WWI is significantly associated with a variety of cardiovascular diseases and poor prognosis. A prospective cohort study from China included 10,338 nonhypertensive subjects with a mean follow-up of 6 years and found that high WWI was significantly linked to an increased risk of hypertension (24).In a cross-sectional study that included 21,040 subjects, Fang H et al. found that high levels of WWI were significantly associated with an elevated risk of development of CVD, especially prominent in those under 50 years of age, suggesting that WWI may be an interventional indicator for reducing the risk of cardiovascular disease in the general adult population (25). In a prospective cohort study (n=26822) with a mean follow-up of 69 months, elevated levels of WWI were found to be independently correlated with an increased risk of cardiovascular mortality and all-cause mortality (26). Zhang D et al. also reported that WWI was associated with heart failure, suggesting that WWI may be a significant marker of heart failure predictability (27). Ye J et al. found in a cross-sectional study of 23,389 cases that larger WWI might be an independently predictive factor for stroke (28). In addition, Cai S et al. have reported that WWI is associated with left heart ventricular fertilization, suggesting that WWI may be an important predictor of cardiometabolic risk (29).

BMI and WC, the traditional measures of obesity, are strongly associated with the development of diabetes mellitus. J M Chan et al. conducted a cross-sectional study that included 51,529 men aged 40-75 years and found that those with a BMI greater than 35 kg/m2 had a substantially increased risk of type 2 diabetes mellitus compared to those with a BMI less than 23 kg/m2 (30). A cohort study from Colombia that included 6,580 subjects with a mean follow-up of 12 years resulted in the definition of waist circumference thresholds of 89 cm in men and 86 cm in women, which were used to identify an elevated risk of developing diabetes (31). A retrospective cohort study from Japan, which included 4754 subjects, found that both BMI and WC were positively associated with the risk of developing diabetes (32). The obesity paradox is still present despite the growing evidence that these traditional obesity markers are correlated with type 2 diabetes mellitus (33). The reason for the controversy may partly stem from the inability of

TABLE 3 Subgroup analysis of the association between weight-adjusted-waist index and diabetes.

Subgroup	T2DM [OR (95% CI)]	p	p for interaction
Age(years old)			< 0.0001
>=60	1.84(1.64,2.06)	<0.0001	
<60	2.79(2.54,3.07)	<0.0001	
Sex			0.7
Female	1.99(1.80,2.21)	<0.0001	
Male	2.27(2.02,2.56)	<0.0001	
Race/ethnicity			0.83
Non-Hispanic White	2.26(2.01,2.54)	<0.0001	
Mexican American	1.83(1.56,2.14)	<0.0001	
Non-Hispanic Black	1.97(1.76,2.21)	<0.0001	
Others	2.00(1.77,2.26)	<0.0001	
Hypertension			0.01
Yes	2.06(1.89,2.24)	<0.0001	
No	2.20(1.93,2.50)	<0.0001	
stroke			0.49
Yes	1.96(1.53,2.51)	<0.0001	
No	2.14(1.97,2.33)	<0.0001	
CHD			0.33
Yes	2.67(1.94,3.68)	<0.0001	
No	2.11(1.95,2.29)	<0.0001	
Hyperlipidemia			0.48
Yes	2.13(1.96,2.32)	<0.0001	
No	2.08(1.76,2.46)	<0.0001	

Age, sex, race/ethnicity, educational level, marital status, PIR, smoking status, alcohol user, Alt, Ast, UA, creatinine, TC, TG, HDL-C, physical activity,hypertension, stroke, coronary heart disease, and hyperlipidemia were adjusted.
PIR, poverty-to-income ratio; HDL-C, high-density lipoprotein cholesterol; UA, uric acid; TG, triglycerides; TC, total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; T2DM, type 2 diabetes mellitus.

traditional indices to differentiate between fat distribution and muscle mass (34, 35).WWI can accurately reveal centripetal obesity independent of body weight (13). Previous studies have shown that high WWI values are associated with unfavorable body composition outcomes such as high-fat content, low muscle mass, and low bone mass (36). Thus, WWI may serve as a more comprehensible and precise measurement of obesity, reflecting the correlation between obesity and type 2 diabetes mellitus. In recent studies, WWI has been identified as a robust predictor of multiple diseases, superior to BMI and WC (12, 18, 37). WWI is more consistent and reliable in predicting the occurrence of diseases in different races and populations, especially in cross-racial or multicenter studies (37). Regarding other novel indicators such as waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR), which have been used in diabetes research, there is a lack of comparative studies on the comparison with WWI, which may be one of the directions for future studies evaluating obesity and diabetes.

Several underlying mechanisms could account for this positivity in the relationship between WWI and T2DM. Firstly, the increase in WWI may reflect adipose tissue dysfunction, especially the accumulation of visceral adipose tissue, and the large accumulation of visceral fat leads to the release of large quantities of pro-inflammatory factors, including leptin and aldosterone, which lead to insulin resistance and inflammatory responses occurring that can lead to diabetes mellitus (38). Second, adipocytes in centripetally obese individuals are characterized by a hyperlipolytic state that is resistant to the antilipolytic effects of insulin, and the resulting flow of nonesterified fatty acids to the liver not only impairs hepatic metabolism but also leads to an increase in hepatic gluconeogenesis (39). Finally, adipose tissue in obese individuals releases more reactive oxygen species (ROS), and excessive ROS not only reduces the biological availability of nitric oxide (NO) but also hyperoxides tend to react with NO to generate deleterious hydrogen peroxide, which ultimately leads to the development of endothelial cell dysfunction (40, 41). Previous studies have shown that the development of diabetes mellitus is closely related to endothelial dysfunction (42–44).

The advantage of this study is that it is based on NHANES data which were gathered using a graded multi-stage probability sampling strategy, thus making the study more trustworthy and representational. However, our study has several limitations. First, since the current study is a cross-sectional study, causal conclusions cannot be drawn from our results. Second, although we adjusted for various confounders in our model, we could not exclude the effects of other unmeasured potential confounders, such as dietary intake and environmental exposures. Finally, the results may not be extrapolated to other countries because of differences between countries.

5 Conclusions

We report that WWI is strongly associated with the odds of T2DM in U.S. adults and found higher odds of T2DM among young adults and non-hypertensive populations. Future studies should focus on the role of different obesity indices in the development of diabetes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by National Health and Nutrition Examination Survey center in the United

States. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

DZ: Writing – original draft. SZ: Methodology, Conceptualization, Formal analysis, Validation, Visualization, Writing – review & editing. DL: Data curation, Funding acquisition, Supervision, Writing – review & editing. FL: Supervision, Project administration, Validation, Writing – review & editing. ZR: Methodology, Writing – review & editing. XD: Data curation, Funding acquisition, Writing – review & editing. WC: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The impact of weight loss on renal function in individuals with obesity and type 2 diabetes: a comprehensive review

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Obesity and Type 2 Diabetes (T2D) are two highly prevalent diseases that exhibit a complex interplay between them. Obesity serves as a primary risk factor for the development of T2D, and conversely, individuals with T2D often exhibit comorbid obesity. Renal dysfunction emerges as a critical consequence of the convergence of obesity and Type 2 Diabetes, contributing significantly to the overall burden of complications associated with these conditions. Recognizing the profound implications of renal dysfunction in individuals contending with both obesity and Type 2 Diabetes, interventions targeting weight loss have gained prominence as potential therapeutic avenues. Weight loss not only addresses the primary risk factor of obesity but also holds the promise of mitigating the progression of Type 2 Diabetes and its associated renal complications. This comprehensive review aims to explore the impact of weight loss on renal function in individuals contending with the convergence of obesity and T2D.

KEYWORDS

weight loss, obesity, type 2 diabetes, renal function, chronic kidney disease

1 Association between obesity, type 2 diabetes, and chronic kidney disease

The global prevalence of obesity and T2D has reached alarming levels. As of 2021, approximately 529 million individuals were living with diabetes globally, with a standardized prevalence rate of 6.1% (5.8–6.5%) (1). Concurrently, obesity's prevalence and severity continue to rise within the T2D and general populations. Startling statistics from the "National Diabetes Statistics Report (2017)" indicate that an overwhelming 87.5% of adult diabetic patients are either overweight or obese (2).

Obesity is unequivocally established as a critical risk factor for various diseases, including diabetes, hypertension, and cardiovascular disorders (3), all of which have significant adverse effects on kidney health, leading to conditions such as diabetic kidney

disease (DKD) (4, 5) and end-stage renal disease (ESRD) (6). A study by Kamel et al. found that the risk of DKD among individuals with diabetes rises with increasing BMI, ranging from 0.91 for overweight individuals (BMI > 25 kg/m^2) to 2.16 for severely obese individuals (BMI > 40 kg/m^2) (7). These effects are mediated by several factors, including alterations in glomerular hemodynamics, heightened sympathetic nervous activity, hypertension, chronic inflammation, endothelial dysfunction, and so on (8). Intriguingly, even in the absence of diabetes, obese individuals may experience a heightened frequency and severity of proteinuria, with obesity independently contributing to the development of glomerulopathy. Obesity also negatively impacts key Chronic kidney disease(CKD)-associated risk factors, encompassing blood lipid levels, blood pressure, blood glucose control, and the promotion of insulin resistance (9). Notably, CKD tends to be more prevalent and progresses more rapidly in obese individuals with T2D compared to those with normal weight (2).

Given the intricate intertwining of obesity, T2D, and CKD, weight loss interventions have emerged as prospective strategies to enhance renal function and overall health in patients grappling with obesity and T2D (10). Extensive researches underscored the profound benefits of weight loss on kidney health (11–16). Rebecca O'Brien et al. discovered that among patients aged 19–79 with T2DM who underwent bariatric surgery, bariatric surgery was associated with a lower cumulative incidence of diabetic nephropathy(DN) at 5 years (17). Weight loss interventions, including lifestyle modifications, pharmaceutical interventions, and bariatric surgery, have the potential to enhance insulin sensitivity, regulate glucose levels, and control blood pressure (18, 19). Additionally, weight loss has demonstrated efficacy in reducing oxidative stress and inflammation, key factors in kidney injury development (20).

This review aims to provide a comprehensive analysis of the effects of weight loss interventions on renal function in patients with obesity and T2D. By examining the existing evidence, we seek to contribute valuable insights into the potential of weight loss strategies as renoprotective measures and to advocate for the adoption of personalized and multidisciplinary approaches in managing obesity, T2D, and CKD.

2 Obesity and diabetes: primary mechanisms leading to renal dysfunction

2.1 Diabetic kidney disease

Hyperglycemia-induced metabolic dysregulation is a widely recognized primary contributor to the onset and progression of DKD (21). The pathophysiology of DKD is multifaceted, encompassing hemodynamic (RAAS activation, endothelial dysfunction), metabolic (accumulation of advanced glycation end-products), pro-inflammatory (reactive oxygen species generation, tumor necrosis factor-α activation), and pro-fibrotic (stimulation of transforming growth factor-β signaling), but in reality these elements interact locally and systemically to form a complex and dynamic interplay resulting in functional and structural changes to the kidney (22). The Renin-Angiotensin-Aldosterone System (RAAS) plays a pivotal role in the pathogenesis of DKD, partly through its promotion of efferent arteriolar constriction and intraglomerular hypertension, as well as its activation of inflammatory and fibrotic pathways (23). Furthermore, factors such as Protein Kinase C-β, oxidative stress mediators, Advanced Glycation End-products (AGE), as well as various cytokines and chemokines, play significant roles in driving DKD progression in the context of hyperglycemic conditions (21, 22).

Obesity, which often co-occurs with diabetes, poses a substantial risk to renal function (24, 25). Accumulation of visceral fat triggers an adipocyte stress response and pro-inflammatory signaling, leading to metabolic dysregulation. Ectopic lipid deposition within the kidneys and the presence of intracellular lipid metabolites, such as ceramides, contribute to oxidative stress and podocyte insulin resistance, resulting in impairment of the glomerular barrier (26). Obesity also exacerbates conditions like hypertension, insulin resistance, Type 2 diabetes, and atherosclerosis, all of which contribute to renal injury and endothelial dysfunction (24, 26). Recent research indicates that obesity may exert an influence on the composition of gut microbiota, potentially impacting the development of diabetic kidney disease (27). Alterations in gut microbiota associated with obesity, including reduced microbial diversity and shifts in microbial species composition, have the potential to compromise gut barrier integrity and promote inflammation, ultimately affecting renal health (28).

2.2 Obesity-related glomerulopathy

Recent studies identify obesity as an independent risk factor for renal dysfunction, apart from diabetes and hypertension (29). ORG has emerged as a distinctive subtype of CKD, characterized by proteinuria, enlarged glomeruli, and a gradual decline in renal function (30). Obesity-induced alterations in adipose tissue impact renal health through diverse mechanisms. Adipose tissue stress affects adipokine secretion, altering the adiponectin-to-leptin ratio, associated with renal impairment (26). Obesity also impacts renal sodium handling and hypertension development, with elevated leptin levels stimulating the sympathetic nervous system and activating the renin-angiotensin system (26, 30). Mechanical effects of adipose tissue deposition, like perirenal and renal sinus fat accumulation, may also contribute to hypertension and renal injury (31). These changes may slow peritubular capillary blood flow, promote sodium retention, and lead to chronic renal function decline.

The systemic and local disruptions observed in ORG exhibit similarities to the pathogenesis of DKD. These two diseases interact with each other, leading to progressive renal damage.

3 Impact of weight loss on renal function in diabetic patients

Numerous high-quality clinical studies have provided substantial evidence supporting the role of weight loss in alleviating the progression of existing DKD and reducing the long-term incidence of diabetes-related renal complications. As previously discussed, O'Brien et al. conducted a retrospective observational cohort study of 2,205 type 2 diabetes patients who underwent metabolic surgery. A comparison with 11,059 matched non-surgical control subjects revealed a remarkable reduction in the incidence of kidney disease over a 6-year follow-up period. Specifically, metabolic surgery demonstrated a 6.4% incidence in surgical patients compared to 14% in non-surgical controls, with an adjusted hazard ratio of 0.45 [95% CI 0.29-0.71] (17). A matched cohort study by Madsen et al. showed that RYGB surgery led to a significant reduction in T2DM microvascular complications (HR 0.53 [95% CI 0.38–0.73]) and the incidence of type 2 DKD (incidence rate ratio 0.54 [95%CI 0.31–0.94]) (32). Additionally, Glucagon-Like Peptide-1(GLP-1), an incretin hormone, induces weight loss by reducing appetite, delaying gastric emptying, and optimizing insulin and glucagon secretion timing. Studies have demonstrated that Liraglutide not only leads to weight loss but also improves blood sugar control, enhances various cardiovascular markers, and reduces albuminuria. The LEADER RCT (33) confirmed that Liraglutide can slow the occurrence and progression of DKD and alleviate the recurrence of type 2 diabetes after metabolic surgery. Therefore, the use of Liraglutide after metabolic surgery may provide additional renal protective benefits (34). Some medications may potentiate weight loss but are not FDA approved for obesity. Sodium glucose co-transporter 2 inhibitors (SGLT2 inhibitors) also have significant weight-reducing effects. Although their primary purpose is to lower blood sugar by inhibiting renal sodium-glucose co-transporter-2, rather than being a weight loss drug, it has been observed that these agents can substantially improve body weight and exert a potent protective effect on kidney function. A comprehensive meta-analysis has underscored the multifaceted benefits of SGLT-2 inhibitors in patients with T2DM and CKD. These inhibitors not only lowered glycated hemoglobin (–0.29%, 95% CI –0.39 to –0.19) but also demonstrated reductions in blood pressure, body weight, and albuminuria. Furthermore, they mitigated the annual decline in eGFR slope (placebo-subtracted difference of 1.35 mL/1.73 m^2/year, 95% CI 0.78-1.93) and decreased the risk of the composite renal outcome, encompassing doubling of serum creatinine, end-stage kidney disease, or renal death (HR 0.71, 95% CI 0.53-0.95) (35).

3.1 Weight loss interventions primarily alleviate renal damage by improving the following conditions

3.1.1 Diabetes emission

As mentioned earlier, elevated blood glucose levels are a crucial factor in the progression of diabetes patients to diabetic kidney disease. Numerous studies have demonstrated that weight loss interventions can significantly enhance insulin sensitivity, thereby improving blood sugar control and alleviating kidney damage (36). Evidence indicates that early weight loss surgical interventions in T2DM patients can lead to diabetes remission and substantially reduce the residual complications of the disease (36). A notable 10-year follow-up study revealed that 37.5% of patients who underwent surgical treatment sustained diabetes remission throughout the decade. This rate contrasts with 5.5% for medical therapy, 50.0% for biliopancreatic diversion (BPD), and 25.0% for Roux-en-Y gastric bypass (RYGB) (37). Mechanistic research suggests that weight loss primarily reduces intra-abdominal, intramyocellular, and intrahepatocellular lipids under hypocaloric conditions, rather than generalized body fat (38). This loss reverses crucial pathophysiological processes in diabetes, such as enhancing peripheral insulin sensitivity, improving cellular insulin signal transduction, boosting insulin secretion, and decreasing hepatic glucose production (38). The resulting improved insulin sensitivity optimizes glycemic control, thereby preserving glomerular integrity and function, reducing oxidative stress and inflammation, and preserving renal unit structure (39).

3.1.2 Reducing proteinuria and improving kidney structure

Weight loss interventions, particularly surgical procedures, demonstrate sustained remission of albuminuria post-surgery, with significant reductions in albuminuria observed across all baseline albumin-creatinine ratio tertiles, with remission occurring in 78% of patients and parallel studies in Zucker diabetic fatty rats revealed that weight loss and improvements in glycemia following RYGB surgery were accompanied by the normalization of glomerular tuft size, reduced podocyte expression of desmin, and preservation of podocyte foot process morphology (40). Notably, RYGB attenuated podocyte stress and dedifferentiation in the ZDF rat model, likely due to enhanced metabolic control, especially its potent glucose-lowering effect post-surgery (40). Similarly, researchers observed a reduction in urinary protein and the restoration of glomerular injury, attributed to improved glomerular filtration membrane ultrastructure and increased nephrin protein expression (41). Additionally, studies in obese type 2 diabetic patients revealed that calorie restriction also improved glomerular hyperfiltration and various cardiovascular risk factors, while also reducing serum angiotensin II levels, suggesting reduced RAS activity (14). Furthermore, Glucagon-Like Peptide-1 Receptor Agonists (GLP1-RA) like liraglutide can inhibit sodium-hydrogen exchanger 3 in proximal tubular cells, increasing natriuresis and diuresis (42).

3.1.3 Alleviating inflammatory status

Chronic low-grade inflammation is a hallmark of diabetic nephropathy and obesity (43). Researchers have uncovered that the reduction in body weight observed in RYGB rats is associated with a diminished fibrotic Transforming Growth Factor β (TGFβ) signal (44). TGFβ is recognized as a major driver of fibrosis and a key mediator of the hypertrophic and prosclerotic changes in diabetic nephropathy. Consequently, its downregulation appears to be a potential favorable

effect in renal damage in patients with obesity and type 2 diabetes (45). Similarly, Bariatric surgery in mouse models activated Peroxisome Proliferator-Activated Receptor Alpha (PPARα), leading to the inhibition of Reactive Oxygen Species (ROS) generation. This action mitigates oxidative stress and reduces renal apoptosis, showcasing the protective effect of bariatric surgery on kidneys affected by diabetic nephropathy (46). Furthermore, Dietary intervention in mouse models attenuate progressive urinary protein excretion and renal inflammation, suggesting that adiposity drives renal inflammation in DKD (47–51). Reduced expression levels of inflammatory factors such as C-Reactive Protein (CRP) and C-C Motif Chemokine Ligand 2 (CCL2) enhance kidney function, decrease renal fibrosis, and improve inflammation control (52). Furthermore, GLP1-RA have been shown to stimulate pathways that reduce reactive oxygen species in the kidneys. Additionally, they contribute to lowering inflammation by decreasing cytokine production and immune cell infiltration (42). This evidence collectively highlights the intricate interplay between inflammation, weight loss interventions, and the potential protective effects on renal health in the context of diabetic nephropathy and obesity.

3.1.4 Gut microbiota changes

Alterations in gut microbiota following weight loss have been associated with improvements in energy balance, enhanced intestinal insulin release, and weight reduction. Recent meta-analyses have revealed significant alterations in gut microbiota and microbial metabolites following weight loss surgery. These changes are closely associated with improved glucose homeostasis, weight reduction, and modifications in gastrointestinal intake and exercise behaviors (53). A detailed investigation focused on diabetic patients undergoing metabolic surgery has unveiled intricate connections among serum metabolomics, gut microbiota composition, and hormonal profiles, particularly concerning improvements in diabetes and metabolic syndrome. Noteworthy correlations between specific gut bacteria, such as the Eubacterium eligens group, and metabolites like lacosamide glucuronide and UDP-L-arabinose were identified. Positive correlations were observed between Enterococcus and metabolites like glutamic acid and vindoline (54). Furthermore, research has shown that medical treatments also significantly alter intestinal microbiota, with observable changes like increased Proteobacteria and variable Bacteroidetes. These shifts correlate with changes in patient weight, and glucose metabolism (55). Additionally, the dysbiotic state induced by a high-fat diet was ameliorated by transitioning to a lower-fat, higher-fiber control diet, especially when combined with sleeve gastrectomy. This led to increased microbial diversity and shifting relative abundances (56). Moreover, the role of short-chain fatty acids (SCFAs), generated through bacterial fermentation of dietary fiber in the colon, has been crucial in protecting mice against the clinical and histologic manifestations of diabetic nephropathy by activating G Protein-Coupled Receptors GPR43 and GPR109 (57). Targeted dietary fiber supplementation to increase SCFA levels has also been found to contribute to better improvements in hemoglobin A1c levels, partly attributed to increased production of glucagon-like peptide-1 (GLP-1) (58). Hence, alterations in gut microbiota composition following weight loss may contribute to improved blood glucose and blood pressure control by enhancing colonic L cell secretion of GLP-1.

4 Impact of weight reduction on renal function in obese patients

A growing body of evidence suggests that weight reduction yields positive outcomes in non-diabetic patients with obesity, Several longitudinal studies, including a large cohort study (59), observed a substantial decrease in the risk of CKD in obese patients after weight reduction surgery. Notably, improvements in CKD risk categories are observed at 1 and 7 years after surgery, particularly in patients with moderate to high baseline CKD risk. It demonstrated similarities in the effects of weight reduction on renal function in both diabetic and non-diabetic obese patients. These effects encompass reduced adipose tissue, improved lipid metabolism, and ameliorated inflammatory states.

4.1 The benefit of adipose tissue reduction

Post-weight loss, there is a notable decrease in visceral adipose tissue and renal sinus fat. This reduction contributes to a favorable adipokine profile, diminished inflammatory cytokines, and lowered ROS production, potentially resulting in reduced RAAS activation levels (60). Obesity-induced metabolic syndrome disrupts lipid metabolism, leading to abnormal lipid profiles. Weight loss interventions have consistently proven effective in decreasing blood lipid levels and fat deposition, while increasing adiponectin levels, which play a key role in metabolic regulation (15, 61). Adiponectin plays a multifaceted role in metabolic control, promoting fatty acid oxidation through AMP-activated protein kinase (AMPK) activation. AMPK activation enhances insulin sensitivity in crucial insulin-target tissues like skeletal muscle and white adipose tissue, vital for blood glucose control. By stimulating fatty acid oxidation and ceramidase activity, adiponectin counteracts lipotoxicity and oxidative stress (62). Moreover, both intensive lifestyle interventions and bariatric surgery-induced weight loss have been found to reduce lipid accumulation and decrease local and systemic inflammatory microenvironments (60). Additionally, beyond bodyweight reduction, the improvement of the metabolic kidney milieu and restoration of endothelial function could also contribute to the renoprotective effects of regular exercise in chronic diabetic diseases (63). Furthermore, several longitudinal studies exploring the reduction of ectopic renal fat post-weight loss surgery have demonstrated a correlation with improved renal function. Renal sinus fat (RSF), a fat depot at the hilum of the kidney, has been studied for its association with hypertension, and its reduction post-weight loss surgery has been observed. In comparison to the lean control group, obese patients accumulated more RSF (2.3 [1.7-3.1] vs. 1.8 [1.4-2.5] cm^2). Hypertensive patients, when compared to normotensive subjects, had a larger RSF depot (2.6 [2.0-3.3] vs. 2.0 [1.4-2.5] cm^2), even after considering BMI. In combined data, RSF showed a negative correlation with estimated glomerular filtration rate (eGFR) but had no association with systolic or diastolic blood pressure.

After weight loss surgery, RSF decreased along with other obesity markers. The magnitude of RSF reduction was greater in patients who experienced hypertension relief compared to those who still had hypertension (-0.68 [-0.74, -0.44] vs. -0.28 [-0.59, 0] cm2, p = 0.009). The accumulation of RSF appears to be linked to the pathogenesis of obesity-related hypertension, and post-surgery, a significant decrease in RSF was observed, correlating with relief from hypertension (31). Similarly, in a study involving dietary-induced weight loss over an 18-month period, a correlation was observed between RSF and baseline eGFR as well as microalbuminuria. After 8 months, RSF decreased by 6.18%, and this reduction was associated with overall body weight loss. The decrease in RSF was also linked to improvements in lipid profiles and blood glucose control (37).

4.2 Weight reduction and glomerular filtration rate

Studies, including one by Lin et al., demonstrate that bariatric surgery was associated with eGFR preservation in all obese patients and, particularly, in those with moderate-to-high CKD risks. The study revealed a significant negative correlation was evident between an increased eGFR and a reduced BMI (Spearman's correlation -0.229, P < 0.001),and the bariatric surgery group had a significantly lower risk of an eGFR decline ≥25% at 12 months [adjusted HR (aHR) 0.47, P = 0.03). After BS, obese patients with hypertension or albuminuria had significantly lower risks of eGFR declines ≥25% (aHR 0.37, P = 0.02 and aHR 0.13, P = 0.0018, respectively) (64). In a prospective cohort study, researchers observed analogous trends. Twenty-five individuals, comprising both obese and non-diabetic subjects, exhibited considerable stability in unadjusted mean glomerular filtration rate (mGFR) and a noteworthy improvement in adjusted mGFR (65). Caloric restriction-based dietary interventions also exhibit positive effects. Individuals with eGFR < 120 mL experience an overall increase in GFR with weight loss, while those with hyperfiltration witness a substantial decline in GFR, indicating a favorable reduction in obesity-associated glomerular hyperfiltration (66–68). Moreover, utilizing advanced imaging techniques, researchers provided compelling evidence that obesity induces structural, metabolic, and hemodynamic changes in the kidneys. Obese subjects showed higher renal volume but lower radiodensity, suggestive of potential water and/or lipid accumulation. Cardiac output and eGFR were increased by approximately 25% in obese individuals. Total renal blood flow was higher in the obese, and FFA uptake was about 50% higher due to elevated circulating FFA levels. Importantly, following weight loss (26 ± 8 kg), these changes in eGFR, total renal blood flow, kidney volume, FFA uptake, and renal density were partially reversed, thereby mitigating the risk of obesity-induced progression of chronic kidney disease (69).

In conclusion, weight reduction offers multifaceted benefits for both diabetes and obesity, including improved metabolic disturbances, reduced adipose tissue deposition, alleviated chronic inflammation, and restored renal structure and function. For patients with obesity and concurrent diabetes, weight reduction interventions are beneficial for renal health, potentially delaying or preventing renal function impairment (70).

In Figure 1, we elaborate on the specific mechanisms illustrating the impact of weight loss intervention on renal function in obese and diabetic patients.

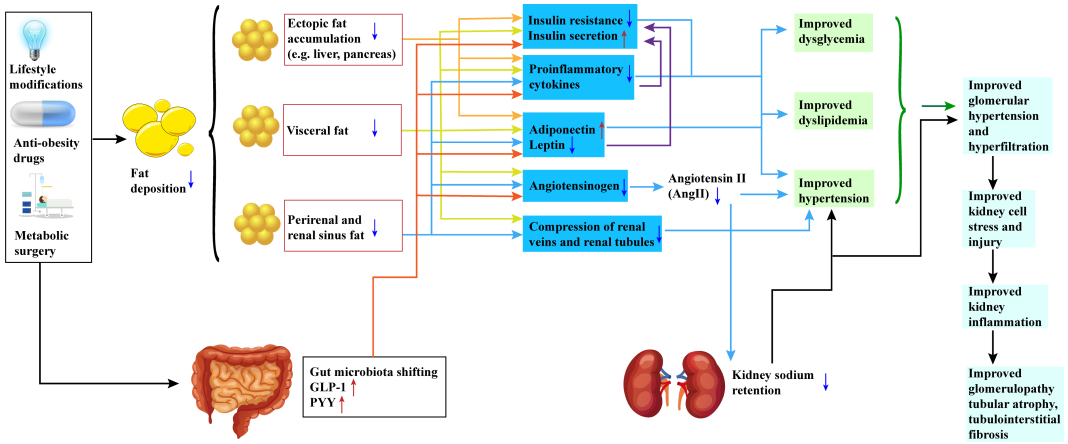

FIGURE 1
Potential Mechanisms of Weight Loss Intervention in Preserving Renal Function in Obesity and Diabetic Patients. All three approaches—lifestyle modifications, anti-obesity medications, and metabolic surgeries—result in a reduction in patient body weight. This reduction is characterized by a decrease in fat deposition, encompassing visceral fat, renal sinus fat, and ectopic adipose tissue. The reduction of fat in these specific regions promotes the restoration of insulin sensitivity, elevation of peripheral insulin levels, suppression of pro-inflammatory factors, and modulation of adipokine release. Simultaneously, alterations in the release patterns of adipokines are observed, marked by an increase in adiponectin levels and a decrease in leptin levels. Furthermore, the renin-angiotensin-aldosterone system (RAS) is inhibited. Additionally, shifts in gut microbiota composition and increased secretion of hormones such as GLP-1 and PYY contribute to these effects. Collectively, these changes culminate in enhanced regulation of blood glucose, blood lipids, and blood pressure. The inhibition of the RAS system also leads to reduced urinary sodium retention, providing a safeguard for the kidneys. These interventions exert a favorable impact on renal function, including the amelioration of glomerular hyperfiltration, preservation of renal cell integrity, and reduction in renal inflammation. Ultimately, these mechanisms translate into enhancements in kidney structure, including the attenuation of glomerular and renal tubular atrophy, as well as a reduction in interstitial fibrosis. GLP-1(Glucagon-Like Peptide-1); PYY(Peptide YY). Red arrows represent an increase, and blue arrows represent a decrease.

5 Perspectives and conclusion

Obesity combined with diabetes poses a significant health challenge globally, with the associated renal issues posing a serious threat to patient health. Weight loss interventions show promising potential in improving renal function, and current research has made some encouraging progress. However, future research directions and key issues still require further exploration to enhance our understanding of this crucial area and ultimately improve patients' quality of life. Firstly, mechanistic studies will continue to be a focal point. Understanding how weight loss interventions impact renal function through biochemical pathways, and delving into the molecular and cellular mechanisms between obesity, diabetes, and renal diseases, is crucial for developing more targeted intervention strategies. These studies are expected to unveil new therapeutic targets and opportunities for drug development. Secondly, long-term effects and safety assessments will be a critical area for future research. Over time, the long-term impact of weight loss interventions on renal function needs more attention, especially regarding their role in the progression of renal diseases. Personalized and comprehensive interventions represent another important future direction. Different patients have diverse genetic backgrounds, lifestyles, and disease stages, making personalized intervention plans crucial. Integrating drug therapy, lifestyle adjustments, even metabolic surgery to optimize renal function improvement will be a key goal of future research and clinical practice.

In conclusion, the impact of weight loss interventions on renal function in patients with obesity and diabetes is a research area of significant clinical importance. With further research and technological advancements, we can not only better comprehend its mechanisms but also formulate more effective treatment strategies, ultimately offering diabetic and obesity patients improved health and quality of life.

Author contributions

XG: Conceptualization, Data curation, Methodology, Writing – original draft. XZ: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. PF: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Probiotic co-supplementation with absorbent smectite for pancreatic beta-cell function in type 2 diabetes: a secondary-data analysis of a randomized double-blind controlled trials

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Introduction: There is growing evidence from animal and clinical studies suggesting probiotics can positively affect type 2 diabetes (T2D). In a previous randomized clinical study, we found that administering a live multistrain probiotic and absorbent smectite once a day for eight weeks to patients with T2D could reduce chronic systemic inflammatory state, insulin resistance, waist circumference and improve the glycemic profile. However, there is a lack of evidence supporting the efficacy of probiotic co-supplementation with absorbent smectite on pancreatic β-cell function in T2D.

Aim: This secondary analysis aimed to assess the effectiveness of an alive multistrain probiotic co-supplementation with absorbent smectite *vs* placebo on β-cell function in T2D patients.

Material and methods: We performed a secondary analysis on a previously published randomized controlled trial (NCT04293731, NCT03614039) involving 46 patients with T2D. The main inclusion criteria were the presence of β-cell dysfunction (%B<60%) and insulin therapy alone or combined with oral anti-diabetic drugs. The primary outcome was assessing β-cell function as change C-peptide and %B.

Results: We observed only a tendency for improving β-cell function (44.22 ± 12.80 *vs* 55.69 ± 25.75; p=0.094). The effectiveness of the therapy probiotic-smectite group was confirmed by fasting glycemia decreased by 14% (p=0.019), HbA1c – 5% (p=0.007), HOMA-2 – 17% (p=0.003) and increase of insulin sensitivity by 23% (p=0.005). Analysis of the cytokine profile showed that statistical differences after treatment were in the concentration of both pro-inflammatory cytokines: IL-1β (22.83 ± 9.04 *vs* 19.03 ± 5.57; p=0.045) and TNF-α (31.25 ± 11.32 *vs* 26.23 ± 10.13; p=0.041).

Conclusion: Adding a live multistrain probiotic and absorbent smectite supplement slightly improved β-cell function and reduced glycemic-related parameters in patients with T2D. This suggests that adjusting the gut microbiota could be a promising treatment for diabetes and warrants further investigation through more extensive studies.

KEYWORDS

probiotics, smectite, absorbent, gut microbiota, type 2 diabetes, pancreatic β-cells, β-cells dysfunction

Introduction

Diabetes is a chronic disease that arises when the β-cells in the pancreas fail to produce sufficient insulin or when the body cannot effectively use the insulin it generates. Around 95% of people with diabetes worldwide have type 2 (T2D). Between 2000 and 2019, there was a 3% increase in age-standardized mortality rates from diabetes (1). T2D belongs to a group of metabolic diseases inherent in hyperglycemia on the background of insulin resistance (IR) and reduction of insulin secretion (2, 3). Chronic hyperglycemia can damage and dysfunctional various organs, such as retinopathy, nephropathy, metabolic and cardiovascular diseases (2, 4). Even though many standard and non-standard schemes for treating T2D have been developed today, the number of new cases does not decrease but instead increases (5). Often, a prerequisite for T2D is obesity and disrupting the microbiota in the large intestine (6, 7).

The gut microbiota is gaining meaningful scientific interest concerning obesity and different associated metabolic disorders to understand obesity's etiology better and find modern methods for its prevention and/or treatment (8, 9). It is established that obesity and T2D are characterized by a chronic state of low-grade inflammation with IR (10). Inflammation is a nonspecific biological response of the immune system to pathogens, toxins, and damaged cells (11). This acute or chronic reaction can lead to tissue damage (12). For several decades, it has been believed that increased inflammatory tone significantly affects glucose metabolism. Therefore, sorbents are indispensable in the fight against chronic inflammation in patients with T2D (13). Chronic inflammation is accompanied by oxidative stress, which results in β-cell dysfunction and α-cell expansion in the pancreas. This leads to the progression of T2D in obese subjects and gut microbiota influences the secretion of these molecules (14–16). Depending on the strain, bacteria can indirectly stimulate pro-inflammatory cytokine production by the host through their metabolites or reduce inflammation by synthesizing anti-inflammatory substances (17). So today, much animal and clinical data suggest the beneficial effects of probiotics in T2D, which mainly focus on their impact on IR, anthropometric parameters, glycemic control and markers of chronic systemic inflammation (18).

Our previous randomized clinical study established that a live multistrain probiotic and absorbent smectite once a day for eight weeks to patients with T2D could reduce chronic systemic inflammatory state, IR, waist circumference and improve glycemic profile (19). Also, it was established that smectite, due to its ability to bind endo- and exotoxins and its capacity to restore the barrier properties of human intestinal cell monolayers, may be beneficial when supplemented with probiotics for NAFLD/NASH development (13, 20).

This work aimed to assess the effectiveness of an alive multistrain probiotic co-supplementation with absorbent smectite *vs* placebo on β-cell function in T2D patients.

Materials and methods

Ethics statement

We conducted a secondary analysis of previously published randomized clinical trials (RCTs) (NCT04293731, NCT03614039) (19, 20). Patient selection was conducted at the Kyiv City Clinical Endocrinology Centre, Ukraine. The primary research protocols were approved by the local Ethics Committee (protocol 2/5_2015 and 2017.19/4) and put into practice on the basis of the Declaration of Helsinki (1975). Before beginning the RCT, the study's objectives and procedures were comprehensively explained to the participants. After the discussion, all patients voluntarily gave their informed consent by signing the required paperwork.

Inclusion criteria

The inclusion criteria were the following: adult participants (aged 18 to 75) with proven T2D diagnosis based on the criteria of the American Diabetes Association (plasma glucose in fasting state ≥7.0 mmol/l; plasma glucose at random measuring ≥11.1 mmol/l; HbA1c ≥6.5% or glucose > 11.1 mmol/l 2 hours after tolerance test with 75 g of glucose) (21); presence of pancreatic β-cell dysfunction which defined as %B<60% and treatment with insulin therapy alone or in combination with oral anti-diabetic drugs (metformin and/or

sulphonylureas) in a stable dose for at least 3 months prior to randomization; HbA1c level 6.5 to 10.0%; a signed informed consent.

Exclusion criteria

The main exclusion criteria were the presence of T1D; intake of anti-diabetic drugs except for those specified in the inclusion criteria (pioglitazone, glucagon-like peptide (GLP-1) analogs, dipeptide-peptidase 4 (DPP-4) inhibitors, etc.); severe diabetes-related complications at screening (ie, end-stage diabetic kidney disease, neuropathy requiring pharmacological treatment, proliferative retinopathy, autonomic neuropathy); regular intake of probiotics, prebiotics or antibiotics for 3 months prior the inclusion; previously diagnosed allergy to probiotics; gastrointestinal disorders including food allergy, gluten-sensitive enteropathy, ulcerative colitis; an uncontrolled cardiovascular or respiratory disease, an active malignant tumor or chronic infections; participation in another clinical trial; pregnancy or lactation.

Study design

The background of secondary analysis was previous RCT were the administration of Probiotic-Smectite was not accompanied by significant changes in the functional activity of β-cells (% B) according to the HOMA2 model (19). The lack of a valid difference and multidirectionality of changes, in the authors' opinion, was preconditioned by the trial design. This is because when the study was scheduled, the functional activity of β-cells was not considered to be an individual preset criterion of inclusion/exclusion and was only assessed as additional parameter in terms of secondary endpoints. For this reason, patients with both hyper and dysfunction of β-cells of various intensities were included in the trial, thus influencing the final interpretation of the results according to this parameter (22). The current study aimed to assess the effectiveness of Probiotic-Smectite combination *vs* placebo in T2D patients with primary β-cell dysfunction. For this purpose, we conducted secondary analysis of published RCTs (19, 20) and after repeated database analysis included patients who responded to updated inclusion/exclusion criteria.

The RCT included T2D patients, who were randomly with an allocation ratio 1:1 prescribed either probiotic or a placebo for 8 weeks. Randomization was double-blind and carried out by a statistical expert with blocks of four using a computer-generated list at www.randomization.com. The groups were homogeneous in terms of age, gender, and diagnosis. The co-investigators distributed the sachets among the participants according to their groups. The group allocation was blind both for the participants and the researchers. In addition to that, with view of supporting the double-blind design the statistics expert did not know the distribution of the participants between the study groups. The code was broken after the analysis had been completed and the database had been closed.

The preliminary randomization period was developed to reduce the effects of diet changes upon metabolic markers. For this purpose, two weeks before randomization all patients were offered to have a one-time session with a dietician to modify their lifestyle. The nutrition program included a corrective diet and drinking regime (natural water daily norm 30-40 ml/kg). Patients were provided with a list of recommended and prohibited products. Any cooking method was recommended except the fry. The last meal was 1.5-2 hours before bedtime. In addition to that the participants were offered to continue their usual intake of anti-diabetic drugs and get a standard average physical exercise for at least 1 hour a day.

Throughout the study, follow-up phone visits were conducted every two weeks to ensure that participants were complying with the protocol requirements and to monitor for any adverse events. In the study, participants who experienced minor negative reactions were given the option to continue or stop taking the supplements, but they still had to attend follow-up appointments. However, patients who reported serious adverse events, such as diarrhea, nausea/vomiting, or sepsis, were not included in the final analysis if they had changes in their previous therapy or had taken antibiotics.

To assess patient compliance, the remaining supplement sachets were counted and an investigator directly asked participants about their adherence to the treatment. Good compliance was defined as consuming more than 85% of the sachets, whereas any participant who missed more than 15% of the recommended doses were excluded from the final results.

Drugs

The sachets containing probiotics supplemented with smectite (Symbiter Forte) or placebo had similar organoleptic characteristics, appearance, texture, weight, and smell. The only difference was on specified number code on them. The "Symbiter Forte" and the placebo were produced and delivered to the study center by "N.D. Prolisok" (Ukraine). The intervention contained a biomass of alive probiotic microorganism symbiosis, colony forming units - CFU/g: *Lactobacillus* – $1.0x10^9$, *Bifidobacterium* – $1.0x10^9$, *Lactococcus* – $1.0x10^8$, *Propionibacterium* – $1.0x10^8$ and *Acetobacter* – $1.0x10^5$; and smectite gel (250 mg). The microbial composition characterized with following richness: 17 strains of microorganisms physiological for mammalian intestines belonging to 11 species. Most strains were taken from All-union Collection of Industrial Microorganisms (ACIM) State Research Institute of Genetics and Breeding of Industrial Microorganisms (Union of Soviet Socialist Republics, Moscow): *Lactococcus lactic ssp. lactis* ACIM B-4305, ACIM B-5387; *Lactococcus lacti* ssp. diacetylactis ACIM B-4303; *Streptococcus salivarius ssp. thermophilus* ACIM B-4304, ACIM B-4741; *Lactobacillus acidophilus* ACIM B-5254, B-5863; *Lactobacillus delbrueckii ssp. bulgaricus* ACIM B-3963, B-5810; *Propionibacterium freudenreichii ssp. schermanii* ACIM B-4544; *Propionibacterium acidipropionici* ACIM B-5800; *Bifidobacterium bifidum* ACIM B-5799; *Bifidobacterium longum* ACIM B-4557, 4635; *Acetobacter aceti* ACIM B-5495. Also two strains were

taken from depositary of D.K. Zabolotny Institute of Microbiology and Virology (IMV) of the National Academy of Sciences of Ukraine (Ukraine, Kyiv): *Lactobacillus helveticus* IMV B-7115; *Bifidobacterium bifidum* IMV B-7113).

We chose this probiotic based on our previous comparative experimental analysis for impact of different probiotic strains diabetes/obesity models. In this animal study, we assess beneficial effects of lyophilized mono-probiotic (*B. animalis VKL*, *B. animalis VKB*, *L.casei IMVB-7280*), the combination of this three strains and multiprobiotic "Symbiter" containing biomass of 17 alive probiotic strains. We have shown that supplementation of probiotic composition, with preference to alive strains, led to a significantly lower prevalence of obesity, improvement of IR, reduction of visceral adipose tissue weight and serum lipid levels as compared to single-strain probiotic (23, 24).

Every patient after randomization received 1 pack (10 g) of probiotic-smectite or placebo per day (QD) for 8-week period. All the participants were instructed concerning the use of the supplementation, i.e. they were told to cut the pack as shown, then dissolve the contents in 15 to 30 ml of boiled drinking water of ambient temperature, stir thoroughly and consume immediate after making ready.

Outcomes assessment and measurement

After obtaining informed consent from the patients, they were requested to provide blood serum samples while fasting. The samples were then promptly frozen at t - 20°C. For each patient, relevant clinical and demographic data were collected.

The primary outcome was the assessment of β-cell function as change C-peptide and HOMA2-β (% B, homeostasis model assessment-estimated β-cell function) which was calculated using HOMA2 calculator. This model can be calculated using the software provided by the Oxford Centre for Diabetes, Endocrinology and Metabolism and available at http://www.dtu.ox.ac.uk/homacalculator/index.php. In addition to that insulin sensitivity (% S) and HOMA-2 index were its secondary outcome. C-peptide was measured using chemiluminescence immune analysis with the help of commercially available sets (Immulite, Siemens AG, Germany) with ng/ml scale.

The secondary outcomes of the RCT that were considered for investigating the efficiency of the probiotic-smectite therapy were glycemic control-related parameters, anthropometric variables and markers of a chronic systemic inflammatory response (TNF-α, IL-1β, IL-6, IL-8, IFN-γ). All parameters were determined following a 12-h fasting period, by the hospital clinical laboratory.

The glucose level in the fasting state was determined by enzymatic Trinder method using an Exan device. HbA1c was measured using immunoturbidimetric analysis on Cobas 6000 (Roche Diagnostics, Basel, Switzerland) with a reference range of 4.8% to 5.9%. HbA1c level was standardized with a reference method in keeping with DCCT (Diabetes Control and Complications Trial) and NGSP (National Glycohemoglobin Standardization Program).

The level of pro-inflammatory cytokines was determined using enzyme-immunoassay with commercially available systems "Sigma" (TNF-α (RAB0476), IL-1β (RAB0273), IL-6 (RAB0306), IL-8 (RAB0320), INF-γ (RAB0222)). Blood samples (5 ml) were taken in a fasting state. Serum was stored at t=−20°C. Cytokine levels under consideration were measured in each sample and expressed in pg/ml.

All the patients underwent anthropometry with the following data accumulated: body height (BH) accurate to 0.001 m; body weight (BW) accurate to 0.001 kg using medical scales. Body mass index (BMI) was calculated by Quetelet formula:

$$BMI = BW/BH^2$$

Waist circumference (WC) was measured using a flexible tape at the belly button level accurate to 0.001 m.

Statistical analysis

Statistical analysis was done using a standard software SPSS version 20.0 (SPSS, Inc., Chicago, Illinois) and GraphPad Prism, version 6.0 (GraphPad Sofware, Inc., La Jolla, CA, USA). Quantitative changes were presented as the mean and standard deviation (M ± SD), qualitative changes were presented as %. To prove the normal distribution hypothesis, Kolmogorov-Smirnov one-sample test was used. To estimate the difference of the incoming quantitative data $\chi 2$ criterion was used. The changes in outcomes of the participants after the initiation of therapy and end of the trial were compared by paired sample t-tests (intra-group). Analysis of covariance (ANCOVA) was used to identify any differences between the two groups after intervention, adjusting for baseline measurements and confounders (BMI and sex) (inter-group).

Results

Totally 105 patients with T2D completed primary RCT and their data were included in intention-to-treat analysis (19, 20). At initial stage we set as inclusion criteria %B as less than 50. From 105 patients only 28 meet the given criterion. After additional analysis of RCT database we found that 18 patients with T2D have %B in range 50-60%. Therefore, at the next meeting of the researchers, a decision was made to extend inclusion criterion % B to less than 60. After the specified modification of the protocol 46 patents meet inclusion/exclusion criteria for the secondary analysis, which greatly increased the statistical power of the study. Twenty-two patients received placebo and twenty-four were included in probiotic-smectite group. The CONSORT Flow Diagram is shown in Figure 1. All participants received standard care that included medical counseling, education in T2D, and lifestyle advice. All patients received more than 90% of the prescribed sachets in the double-blind treatment. The patients were satisfied with the organoleptic features; both additives were tolerated well. The main reported adverse events (AEs) were gastrointestinal

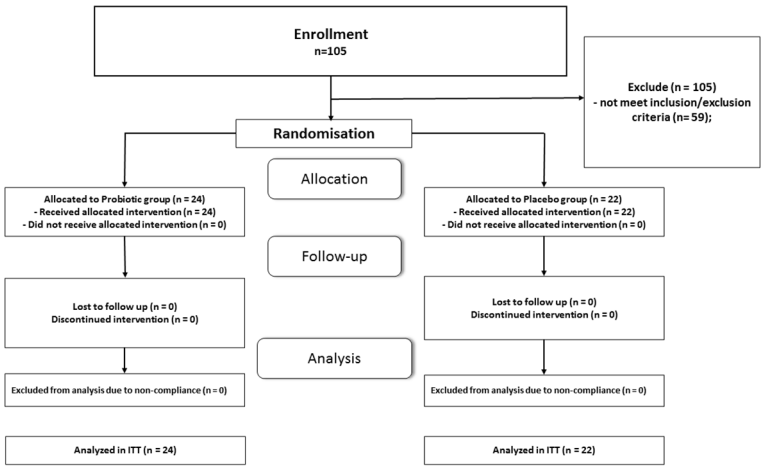

FIGURE 1
Consolidated Standards of Reporting Trials (CONSORT) flow chart-trial protocol.

complaints. In general, prevalence of AE was less than 20%, mild in their intensity and disappeared spontaneously (19, 20).

The baseline clinical parameters (age, gender and T2D duration) of the patients who completed the study are summarized in Table 1. All project participants were on standard treatment, namely oral hypoglycemic drugs, insulin, or combinations. Even though metformin can change the gut microbiota composition (25, 26), we did not exclude patients who took this drug in this study because metformin is currently recognized as a first-line therapy in T2D patients. Participants were randomized to the proportional shares of the patients with a stable metformin dosage 4 weeks before the study started. The two groups had no significant differences in the daily average amounts (Table 1).

Analysis of primary and secondary outcomes of carbohydrate metabolism is presented in Table 2. At placebo group had no significant differences in carbohydrate metabolism parameters, except deterioration by 3% HbA1c (p=0.039), which a decrease in sensitivity (%S) and HOMA-2 can explain. Unfortunately, after 8 weeks of probiotic co-supplementation with absorbent smectite administration, we observed only a tendency for improving β-cell function (44.22 ± 12.80 *vs* 55.69 ± 25.75; p=0.094). Also, ANCOVA analysis didn't show significant differences between the mean changes of the %B (-7.05 ± 21.4 *vs* -11.47 ± 26.04; p=0.642). The C-peptide concentration didn't have statistically significant changes in both groups (Table 2).

TABLE 1 Baseline clinical parameters in examined patients (M ± SD or %).

Parameters	Placebo group (n=22)	Probiotic-Smectite group (n=24)	p
Age, years	54.87 ± 8.59	54.75 ± 8.87	0.971
Duration of T2D, years	13.53 ± 7.97	12.06 ± 6.18	0.569
Metformin, % (n)	59.1 (13)	62.5 (15)	0.813
Metformin daily dosage, mg	1495.0 ± 570.79	1636.36 ± 638.78	0.601
Sulfonilureas, % (n)	31.8 (7)	37.5 (9)	0.686
Insulin daily dosage, IU	31.66 ± 8.54	30.75 ± 7.92	0.839
Insulinotherapy duration, years	7.33 ± 4.84	7.75 ± 5.44	0.885
Insulinotherapy, % (n)	45.5 (10)	50.0 (12)	0.758

However, the effectiveness of the therapy in the probiotic-smectite group was confirmed by other indicators. After 8 weeks of intervention fasting glycemia decreased by 14% (p=0.019), HbA1c – 5% (p=0.007), HOMA-2 – 17% (p=0.003) and increase of insulin sensitivity by 23% (p=0.005). Changes were significant also in inter-group analysis. The mean changes for both groups in ANCOVA analysis were as follow: HbA1c (-0.26 ± 0.45 *vs* 0.41 ± 0.52; p=0.001), %S (0.19 ± 5.38 *vs* -7.58 ± 9.20; p=0.01) and HOMA-2 (-0.05 ± 0.45 *vs* 0.55 ± 0.65; p=0.006) (Table 2).

Both groups' anthropometric parameters changed insignificantly after the intervention (Table 3). The effect of probiotics and absorbent smectite therapy was also analyzed using its influence on the immune system. In the placebo group, all studied parameters did not change significantly. Analysis of the cytokine profile showed that statistical differences after treatment were in the concentration of both pro-inflammatory cytokines: IL-1β (22.83 ± 9.04 *vs* 19.03 ± 5.57; p=0.045) and TNF-α (31.25 ± 11.32 *vs* 26.23 ± 10.13; p=0.041). In the probiotic-smectite group, IL-6, IL-8, and γ-INF concentrations didn't change significantly (Table 4). The between-group ANCOVA analysis didn't find a change in all investigated cytokines.

Discussion

In recent years, more scientific data have pointed out the close connection between intestinal microbial community, nutritional

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TABLE 2 Analysis of primary and secondary outcomes with focus carbohydrate metabolism parameters (M ± SD).

Parameters	Placebo group (n=22)	p1	Probiotic-Smectite group (n=24)	p2	p3	p4
B-cell function, %B						
Baseline value	43.00 ± 10.70		44.22 ± 12.80		0.777	
Post-treatment value	50.05 ± 20.22	0.223	55.69 ± 25.75	0.094		0.642
Mean changes	-7.05 ± 21.4		-11.47 ± 26.04			
C-peptide, ng/ml						
Baseline value	3.11 ± 0.70		3.33 ± 1.05		0.336	
Post-treatment value	3.25 ± 0.91	0.372	3.02 ± 1.10	0.186		0.133
Mean changes	-0.14 ± 0.51		0.31 ± 0.8			
Glucose, mmol/l						
Baseline value	11.11 ± 1.57	0.567	11.44 ± 2.11		0.633	
Post-treatment value	10.70 ± 2.19		9.81 ± 2.29	0.019		0.201
Mean changes	0.41 ± 2.70		1.63 ± 2.45			
HbA1c, %						
Baseline value	8.2 ± 0.86		8.56 ± 1.17		0.338	
Post-treatment value	8.46 ± 0.99	0.039	8.15 ± 1.14	0.007		0.001
Mean changes	-0.26 ± 0.45		0.41 ± 0.52			
Sensitivity, %S						
Baseline value	35.53 ± 8.30		33.42 ± 10.67		0.546	
Post-treatment value	35.34 ± 8.59	0.891	41.00 ± 15.32	0.005		0.010
Mean changes	0.19 ± 5.38		-7.58 ± 9.20			
HOMA-2						
Baseline value	2.95 ± 0.64		3.29 ± 1.03		0.285	
Post-treatment value	3.00 ± 0.77	0.643	2.74 ± 0.98	0.003		0.006
Mean changes	-0.05 ± 0.45		0.55 ± 0.65			

p1-2 - difference in placebo and probiotic-smectite groups before and after intervention (intragroup analysis); p3 - differences between placebo and probiotic-smectite groups baseline characteristics; p4 - difference between groups throughout the study (ANCOVA intergroup analysis). Significance was stated at p<0,05.

habits, lifestyle, and the appearance of various afflictions in the digestive tract, including irritable bowel syndrome (IBS) (27), metabolic diseases and cancer (28). Also, gut dysbiosis enhances the formation and accumulation of specific metabolites with toxic potential that induce the appearance of kidney-associated illnesses (29). Recent studies have shown that altering gut microbiota composition by probiotics, prebiotics and synbiotics can positively treat irritable bowel syndrome (IBS) (27).

Probiotics have been used safely in foods and dairy products for over a hundred years. Recently, there has been increasing interest in

TABLE 3 Analysis of anthropometric secondary outcomes (M ± SD).

Parameters	Placebo group (n=22)	p1	Probiotic-Smectite group (n=24)	p2	p3	p4
BMI, kg/m^2						
Baseline value	33.61 ± 7.12		31.62 ± 4.96			
Post-treatment value	33.54 ± 6.88	0.511	31.56 ± 5.07	0.431	0.371	0.933
Mean changes	0.07 ± 0.44		0.06 ± 0.32			
Weight, kg						
Baseline value	97.17 ± 22.58		95.4 ± 17.32			
Post-treatment value	96.89 ± 21.67	0.419	95.05 ± 17.06	0.213	0.808	0.870
Mean changes	0.28 ± 1.29		0.35 ± 1.06			
WC, cm						
Baseline value	96.93 ± 6.00		95.81 ± 8.82			
Post-treatment value	96.93 ± 5.87	0.999	95.40 ± 8.59	0.109	0.684	0.267
Mean changes	0.001 ± 1.10		0.59 ± 1.11			

p1-2 - difference in placebo and probiotic-smectite groups before and after intervention (intragroup analysis); p3 - differences between placebo and probiotic-smectite groups baseline characteristics; p4 - difference between groups throughout the study (ANCOVA intergroup analysis). Significance was stated at p<0,05.

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TABLE 4 Cytokine profile in patients with T2D (M ± SD).

Parameters	Placebo group (n=22)	p1	Probiotic-Smectite group (n=24)	p2	p3	p4
IL-1β, pg/ml						
Baseline value Post-treatment value Mean changes	20.38 ± 8.17 19.90 ± 6.93 0.48 ± 6.19	0.768	22.83 ± 9.04 19.03 ± 5.57 3.80 ± 6.94	0.045	0.437	0.170
TNF-α, pg/ml						
Baseline value Post-treatment value Mean changes	35.57 ± 9.33 35.18 ± 8.12 0.39 ± 7.80	0.848	31.25 ± 11.32 26.23 ± 10.13 5.01 ± 8.94	0.041	0.257	0.137
IL-8, pg/ml						
Baseline value Post-treatment value Mean changes	71.33 ± 23.25 69.62 ± 22.74 1.71 ± 10.36	0.534	78.06 ± 21.32 72.20 ± 20.54 5.86 ± 11.71	0.064	0.407	0.306
IL-6, pg/ml						
Baseline value Post-treatment value Mean changes	15.29 ± 7.52 15.19 ± 9.42 0.10 ± 5.89	0.949	21.30 ± 9.24 18.15 ± 7.12 3.15 ± 6.99	0.092	0.057	0.201
γ-INF, pg/ml						
Baseline value Post-treatment value Mean changes	160.54 ± 54.52 150.0 ± 66.09 10.54 ± 35.89	0.274	184.46 ± 55.40 177.40 ± 52.20 7.05 ± 28.75	0.342	0.236	0.767

p1-2 - difference in placebo and probiotic-smectite groups before and after intervention (intragroup analysis); p3 - differences between placebo and probiotic-smectite groups baseline characteristics; p4 - difference between groups throughout the study (ANCOVA intergroup analysis). Significance was stated at p<0,05.

their use to prevent, mitigate or treat specific diseases (30). Changes in the gut microbiota composition and its derived metabolites are closely associated with insulin sensitivity (31), and energy homeostasis (32). Thus, the gut microbiota has been attracting much attention in metabolic diseases (33). Today, many clinical trials have investigated probiotics for metabolic diseases such as obesity and T2D (6, 33, 34). It was shown that changes in insulin secretion after lifestyle intervention (3 months of the nutritional program rich in fiber and exercise training) might be mediated via alterations in an insulinotropic polypeptide (GIP) secretion from intestinal K-cells (35).

This group of agents participates in several links of lipid (fat) metabolism at once, helping to accelerate the weight loss process and reduce the toxic load of harmful substances on the body. In this study, the probiotic was combined with absorbent smectite. Smectite (bentonite) is a natural loamy polymineral formed by microscopic particles capable of hydration and displays the most physiologically active properties. The smectite present on the surface of the intestinal tract provides cytomuco-protective therapeutic benefits by supplying energy and plastic materials to epithelial cells. This enhances the strength of the mucosal barrier and facilitates interaction between mineral particles and glycoproteins of the mucosa and the microbial biolayer (36). Smectite is unique in its ability to directly absorb viruses, toxins, radionuclides, heavy metals, and bacterial endotoxins without affecting normal microbiota cells or essential nutrients (19). Due to their absorbent activity and stabilization mucus layer properties, probiotics with smectite can impact the synergistic enhancement of a single effect, significantly increasing insulin sensitivity, improving anthropometric indicators and reducing inflammation (19).

The scientific data regarding smectite administration alone in terms of different metabolic disturbances are very scarce. Smectite has been effectively used in the treatment of several gastrointestinal diseases, including infectious diarrhea and food allergy (37). In experimental colitis models was found that administration of smectite associated with absorption of inflammatory proteins, reduction in systemic markers of inflammation (IL-2, IL-6, IL-8 and IL-12, TNFα, IFNγ) (38–40) and significant improvement in intestinal microbial profile (41). The Dening et al. proposed that spray dried smectite clay particles may be developed as novel anti-obesity treatments (42, 43). These particles had adsorptive capacities for dietary lipids and digestion products (42). When co-administered with a high-fat diet (HFD) to Sprague-Dawley rats, smectite clay particles can reduced the extent of weight gain relative to the negative control treatment group and performed similarly to orlistat *via* an alternate mechanism of action (43).

The systematic analysis of RCTs has supported the potential beneficial effects of metformin in improving β-cell function. This is especially true when it is combined with other anti-diabetic therapies like rosiglitazone, pioglitazone, and vildagliptin. One of the mechanisms behind this improvement is the reduction of pro-inflammatory markers like hs-CRP and TNF-α in patients with T2D (44). Our research aims to develop therapies that have antioxidant properties and reduce inflammation and oxidative stress markers to limit pancreatic β-cell failure in T2D patients and improve blood glucose control.

So, this study investigated the efficacy of probiotics and absorbent smectite for protecting against β-cells damage in T2D patients. Obesity can lead to the expansion of adipose tissue, which in turn produces several pro-inflammatory markers that hasten β-cell dysfunction. These effects are indicative of a compromised immune response, which exacerbates IR and raises blood glucose levels. Concurrently, oxidative stress occurs alongside inflammation, causing disruptions in various biochemical processes that can lead to the death of pancreatic β-cells (44). After 8 weeks of probiotic co-supplementation with absorbent smectite administration, only a tendency for improving β-cell function as a change in %B was found. However, in this group, there was decreased fasting glycemia HbA1c parallel with improvement of insulin sensitivity. Bacteria in the colon use undigested dietary substrates from the small intestines for survival. Carbohydrates fermentation by bacteria produces beneficial metabolites. However, in the case of carbohydrates limitation, bacteria can use alternative energy sources and produce different metabolites, which have a harmful effect on human health. Dietary carbohydrates' main bacterial fermentation products are short-chain fatty acids (SCFAs) and gases (45). Recent studies found that a ligand of toll-like receptor (TLR) 4, activated by metabolites gut microbiota, plays an essential role in the development of IR and obesity as well as in oxidative stress, inflammation, cell proliferation, and apoptosis (46–48). In our study, we also analyzed the effect of the applied therapy on the immune system. Still, the concentration of both pro-inflammatory cytokines were decreased: IL-1β by 17% (p=0.045) and TNF-α by 16% (p=0.041).

In this placebo-controlled RCT, patients were on standard therapy with insulin or oral anti-diabetic drugs, primarily metformin. As mentioned above, patients with metformin were randomly divided into two groups. It has shown that metformin can inhibit microbial bile acids' metabolism by altering gut microbiome symbiosis and blocking gut bile acids' signaling, thereby partially exerting their metabolic benefits (49).

Western diet, which contains a lot of sugars and fat, including trans-fatty acids and cholesterol, leads to dysbiosis. Such a HFD connected with reduced expression of tight junction genes results in increased intestinal permeability. Raised intestinal mucosa permeability with loss of integrity facilitates enteric bacterial pathogens that contain lipopolysaccharides (LPS). Moreover, in mice, LPS increases intestinal permeability both *in vitro* and *in vivo*, suggesting an association between increased intestinal LPS level and the expression of gut tight junction (50, 51). Increased intestinal permeability may contribute to a more significant effect on the immune system of pathogenic antigens and diet, causing mild chronic systemic inflammation and immune-mediated destruction of pancreatic β-cells, which ultimately causes T2D (52–54). That's why sorbents are essential in the fight against excess weight and T2D.

The main limitation of this study was unidentified composition of the intestinal microbiota in the patients at the baseline and post-treatment state with view of defining the personalized impact upon the changes of metabolic parameters. Furthermore, the terms of treatment for 8 weeks may not fully reproduce the changes of HbA1c as it the glycosylation status of RBCs from around 120 days prior to the test date. The latter should be taken into consideration in future studies.

Conclusion

It was found that incorporating a live multistrain probiotic and absorbent smectite supplement positively impacted β-cell function and parallel with reduction of glycemic related parameters in individuals with T2D. This discovery indicates that modifying the gut microbiota could potentially be a successful diabetes treatment and should be explored in more extensive studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Local Ethics Committee at Kyiv City Clinical Endocrinology Centre. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MS: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. DK: Data curation, Investigation, Writing – review & editing. GZ: Formal Analysis, Methodology, Writing – review & editing. DO: Formal Analysis, Methodology, Writing – review & editing. TF: Conceptualization, Writing – original draft, Writing – review & editing. NK: Conceptualization, Investigation, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Mulberry and *Hippophae*-based solid beverage promotes weight loss in rats by antagonizing white adipose tissue PPARγ and FGFR1 signaling

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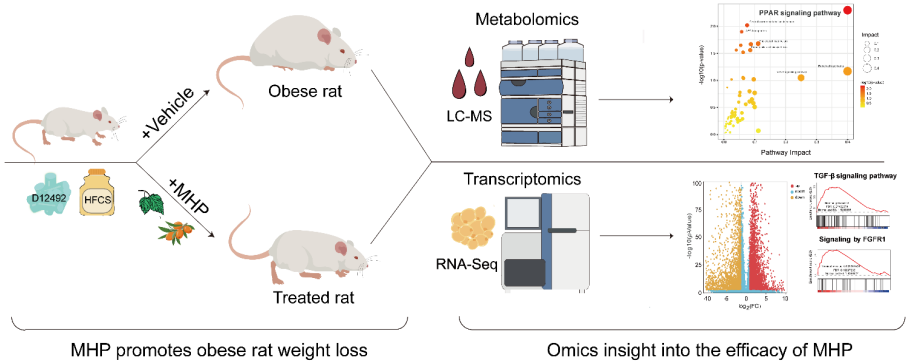
Obesity, a multifactorial disease with many complications, has become a global epidemic. Weight management, including dietary supplementation, has been confirmed to provide relevant health benefits. However, experimental evidence and mechanistic elucidation of dietary supplements in this regard are limited. Here, the weight loss efficacy of MHP, a commercial solid beverage consisting of mulberry leaf aqueous extract and *Hippophae* protein peptides, was evaluated in a high-fat high-fructose (HFF) diet-induced rat model of obesity. Body component analysis and histopathologic examination confirmed that MHP was effective to facilitate weight loss and adiposity decrease. Pathway enrichment analysis with differential metabolites generated by serum metabolomic profiling suggests that PPAR signal pathway was significantly altered when the rats were challenged by HFF diet but it was rectified after MHP intervention. RNA-Seq based transcriptome data also indicates that MHP intervention rectified the alterations of white adipose tissue mRNA expressions in HFF-induced obese rats. Integrated omics reveals that the efficacy of MHP against obesogenic adipogenesis was potentially associated with its regulation of PPARγ and FGFR1 signaling pathway. Collectively, our findings suggest that MHP could improve obesity, providing an insight into the use of MHP in body weight management.

KEYWORDS

obesity, GSEA, PPARγ, metabolomics, transcriptomics, weight-loss



GRAPHICAL ABSTRACT
Efficacy of MHP against diet-induced obesity is validated in rats. Integrated metabolomics and transcriptomics indicate that MHP is able to facilitate weight loss through regulating PPARγ pathway and redressing adipose tissue dysfunction.

Highlights

- MHP promotes weight and adiposity loss of diet-induced obese rats.
- Metabolomics shows MHP efficacy is closely related to PPAR signaling pathway.
- Transcriptomics indicates MHP rectifies white adipose tissue dysfunction.
- Efficacy of MHP is associated with regulation of PPARγ and FGFR1 signaling.

1 Introduction

Overweight or obesity is characterized by an excessive accumulation of adipose tissue and has become a global epidemic (1, 2). Obesity is highly associated with the development of hyperlipidemia, nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM), cardiovascular diseases and some types of cancer (3), posing one of the greatest threats to health. On the other hand, weight management has been shown to provide health benefits. For example, a moderate 5% weight loss has considerable health benefits, including decreased intra-abdominal and intra-hepatic fat and increased multiorgan insulin sensitivity and β cell function (4). Long-term intentional weight-loss also improves the morbidity, mortality, quality of life, and health-care cost (5, 6). Obesity guidelines have recommended weight loss of 5-10% as the goal for medically supervised weight loss (7, 8).

Given the great benefits of weight loss, a number of measures have been applied to realize the goal of 5% or more weight loss and weight maintenance in the past few decades, including active lifestyle modifications and pharmacological and surgical interventions. Among them, noninvasive drugs and dietary supplements have been regarded as promising approaches to address the deficit of lifestyle intervention and reduce the severity of bariatric surgery (9, 10). In the case of dietary supplements, usually no specific guidance from a professional doctor is required and often no strict restrictions are applied – they can be sold as functional beverage or health food (11). Thus, many people turn to weight loss-promoting dietary supplements in the hope to help them achieve their weight-loss goals. However, despite extensive investigations on the role of dietary bioactive substances with weight-loss potentials (12, 13), evidence to support the efficacy of dietary supplements *perse* for weight loss is limited (14). According to the U.S. FDA regulations, all dietary supplements must be safe, but unlike drugs, there are currently no efficacy requirements (13). Consequently, commercially available dietary supplements that are labeled for use in weight loss are multifarious, varying greatly in their efficacy (14, 15). Therefore, there is a pressing need to investigate the claimed efficacy of weight-loss dietary supplements as well as the underlying regulatory mechanisms in metabolic pathways.

In our recent screening tests, a number of dietary supplements are found to have weight-loss activities. Among them, MHP, a solid beverage which is mainly consisted of mulberry leaf aqueous extract and *Hippophae* protein peptides as well as L-arabinose, showed potential protective effects against high-fat diet-induced obesity and hyperlipidemia. Mulberry leaf has been traditionally used to lower blood glucose and lipids, which is supported by their high content of flavonoids and alkaloids (16). *Hippophae rhamnoides* L. has also been shown to have weight-loss and lipid-lowering activities (17, 18). *Hippophae* protein peptides prepared from *H. rhamnoides* seeds have also been proved effective in combating memory impairment (19) and also shown to have hypoglycemic and anti-inflammatory activities (20). These findings suggest that MHP has weight-loss potential, but its efficacy and underlying mechanisms have not been fully investigated.

Variation of endogenous metabolites reflects the alterations of global metabolic homeostasis in cells and organisms, which can be investigated by metabolomics. As a robust analytical tool, metabolomics has been increasingly used in the studies of diagnostic biomarkers, fundamental pathogenic mechanisms, and therapeutic targets (21). By integrating with transcriptomics data

and using such as Ingenuity Pathways Analysis, MetaCore and Reactome software, algorithmically constructed metabolic networks can be generated to provide more insight than phenotypes and biochemical results analyzed individually (22).

In this study, we first evaluated the effects of MHP on a high-fat high-fructose (HFF) diet-induced rat model of obesity and then explored the underlying mechanisms by integrated metabolomics and transcriptomics analysis. Our results showed that MHP rectified white adipose tissue dysfunction and had weight-loss activity, which was related to its regulation on PPARγ (peroxisome proliferator-activated receptor gamma) and FGFR1 (fibroblast growth factor receptor 1) signaling.

2 Materials and methods

2.1 Chemicals and reagents

Liquid chromatography-tandem mass spectrometry (LC-MS) grade methanol (MeOH) and acetonitrile (ACN) were purchased from Fisher Scientific (Loughborough, UK). Formic acid was obtained from TCI (Shanghai, China). 2-Amino-3-(2-chlorophenyl) propionic acid (internal standard) and ammonium formate were obtained from Sigma Aldrich (Shanghai, China). Hematoxylin plus Eosin dye kit (DH0006) was purchased from Leagene (Beijing, China). Ultrapure water was generated using a Milli Q system (Millipore, Bedford, USA).

2.2 MHP preparation and analysis

MHP is mainly consisted of mulberry leaf aqueous extract, *Hippophae* protein peptide and L-arabinose. In the present study, MHP (batch No. 20220623) was manufactured in a Good Manufacturing Practice (GMP) pilot plant following specific quality assurance instructions for the nutritional ingredients, heavy metals and microorganisms (see Supplementary Table S1 in Supplementary Materials).

2.3 Animals experiment

Male Sprague-Dawley rats (5-6 weeks old) were supplied by Guangdong Medical Laboratory Animal Center (Guangzhou, China). Animal study protocols were approved by the Institutional Animal Care and Use Committee of the Guangdong Pharmaceutical University. All animal studies were conducted in accordance with the ARRIVE (Animal research: reporting of *in vivo* experiments) guidelines (23). All animals were maintained under specific pathogen-free conditions, housed with 12 h light/dark cycles at controlled 22-24°C and 60-65% humidity. After acclimatization on a normal chow diet for 7 days, the rats were randomly divided into control group and high calorie diet group. As previously described (22, 24), the control group was fed with standard chow and the high calorie diet group was fed with a high-fat diet plus HFCS-sweetened drink. Two weeks later, an elimination about 20% of obesity-resistant rats was performed and the calorie diet group was divided into HFF group, MHP-H and MHP-L group (n=8 for each group). Except the rats of normal group, all the other rats were fed with a high-fat high-fructose (HFF) diet, and the rats of MHP-H and -L group were supplemented with MHP at a dose of 5.25 g/kg/day and 0.875 g/kg/day by gavage respectively.

The intervention lasted for 8 weeks. Body weight of each rat was measured twice per week throughout the experiment. In addition, food and drink intake were monitored, and the energy intake and energy efficiency (body weight gain(g)/total energy intake (kcal)) of each group were calculated.

2.4 Sample collection

At the end of the experiment, all animals were anaesthetized with pentobarbital sodium after an overnight (12h) fast. Blood and tissues were harvested as previously described (25). Blood was collected for serum metabolomics analysis. The inguinal white adipose tissue (iWAT) was also collected and weighted. Part of iWAT was fixed with 4% PFA for histological examination, while the rest was snap frozen in liquid nitrogen. Samples were frozen at -80°C until analyzed for metabolomics (plasma) or transcriptomics (iWAT tissue).

2.5 Body composition analysis

Body composition analysis was carried out as previously described using an NMR analyzer Minispec LF90II body composition analyzer (Bruker Optics, Billerica, MA, USA) 1 day prior to sacrifice (25). The adiposity, the ratio of lean mass to body weight, and the ratio of fluid mass to body weight were calculated.

2.6 Metabolomics analysis

The untargeted metabolomics analysis was performed as described (26). All the samples were thawed at 4°C. To 100 μL of serum, 400 μL of methanol was added and vortexed for 1 min. The mixture was then centrifuged for 10 min at 12,000 rpm and 4°C, and the supernatant was transferred, concentrated and dried in vacuum. Next, the dry samples were redissolved with 150 μL of 80% methanol solution containing 2-chlorolphenylalanine (4 ppm) and filtered through 0.22 μm membrane prior to LC-MS detection. A small aliquot of each sample was pooled together as the quality control sample.

The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA) using an ACQUITY UPLC® HSS T3 (2.1×100 mm, 1.8 μm) column (Waters, Milford, USA). The column was maintained at 40 °C. The flow rate and injection volume were set at 0.3 mL/min and 2 μL, respectively. For LC-ESI (+)-MS analysis, acetonitrile containing 0.1% formic acid (B1) and water containing 0.1% formic acid (A1) were used as mobile phase, while for LC-ESI (–)-MS analysis, pure acetonitrile (B2) and

5 mM ammonium formate (A2) were used. Chromatographic separation was carried out under the following gradient: 0~1 min, 8% B1(B2); 1~8 min, 8%~98% B1(B2); 8~10 min, 98% B1(B2); 10~10.1 min, 98%~8% B1(B2); and 10.1~12 min, 8% B1(B2). Simultaneous MS and data-dependent MS/MS acquisition of metabolites mass spectrometry data were performed on Orbitrap Exploris120 with ESI ion source (Thermo Fisher Scientific, USA). The sheath gas pressure and auxiliary gas were 40 and 10 arb, respectively. The spray voltage was 3.50 kV and -2.50 kV for ESI (+) and ESI(-), respectively. The capillary temperature was 325 °C. MS^1 scan range of m/z was from 100 to1000 and MS^1 resolving power was set at 60000 FWHM. The number of data dependent scans per cycle was set at 4. The MS/MS resolving power was set at 15000 FWHM. The normalized collision energy was 30 eV.

The raw data were first converted to mzXML format by MSConvert in ProteoWizard software package (v3.0.8789) and processed using R XCMS (v3.12.0) for feature detection, retention time correction and alignment (27, 28). The metabolites were identified by accuracy mass and MS/MS data which were matched with HMDB (http://www.hmdb.ca), KEGG (https://www.genome.jp/kegg/), mzcloud (https://www.mzcloud.org) and the in-house metabolites database of Panomix Biomedical Tech Co., Ltd. (Shuzhou, China).

2.7 Metabolic pathway and function analysis

The metabolic pathway and function enrichment analysis was proceeded as in previous studies (25, 29). PCA (principal component analysis) and OPLS-DA (orthogonal partial least squares discriminant analysis) were applied to discriminate the groups by R ropls (v1.22.0) package (30). In order to screen the differential metabolites (DMs), p value < 0.05 and VIP (variable projection importance) value > 1 were set by fault as the threshold. Using the generated DMs, pathway enrichment analysis was performed using the online metabolomics tool of Biodeep Platform (http://www.biodeep.cn/) to visualize and interpretate the data.

2.8 RNA-Seq

RNA-Seq was performed as reported previously (22). Briefly, RNA from the iWAT was extracted using Trizol reagent kit (Invitrogen, USA) following the manufacturer's instruction. The cDNA library was constructed and sequenced on the Illumina (Novaseq 6000) by Gene Denovo Biotechnology Co. (Guangzhou, China). After the raw data were filtered, the clean reads were used for identification the differentially expressed genes (DEGs) by DESeq2 package. The level of mRNA FPKM (fragments per kilobase of exon model per million fragments mapped reads) value was also calculated. Genes with p value < 0.05 and |Fold change|> 1.5 were categorized as DEGs, and then used for gene ontology (GO) function analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Gene Set Enrichment Analysis (GSEA) was implemented on the Java GSEA platform while gene sets with NES absolute value >1, p value <0.05 and FDR value < 0.25 were considered statistically significant. All the raw data generated by RNA-Seq were submitted to the GenBank's Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/PRJNA1063764).

2.9 Statistical analysis

All data are expressed as means ± standard deviation (SD). A Student's t-test or one-way analysis of variance (ANOVA) was carried out. p value < 0.05 was considered statistically significant. R (https://www.rproject.org) and Graphpad Prism 6.0 software (GraphPad, CA, USA) were used for graphics.

3 Results and discussion

3.1 MHP facilitates weight loss of HFF-diet fed obese rats

In order to address the efficacy of MHP for weight loss, a serial of experiments were performed on a HFF-induced rat model of obesity (Figure 1A). As shown in Figures 1B, C, the body weight of the HFF group rats was significantly increased compared with normal rats, but 8-week intervention with a high dose of MHP ameliorated the body weight gain caused by HFF diet.

Since obesity is associated with excessive fat accumulation, the lean mass and fluid mass of each group rats were also measured. As expected, a high dose of MHP treatment significantly decreased the adiposity and increased the lean index, while there was no difference in the ratio of fluid to body weight (Figures 1D–F). In order to investigate whether the increase of body weight or WAT in rats was related to food, drink or total energy intake, we measured the calory intake and calculated the energy efficiency. It was found that there was no difference in total energy intake between the HFF group and MHP group, suggesting that the weight loss was not due to a reduced total energy intake and that MHP did not inhibit the rat appetite (Figures 1G–J).

3.2 Multivariate statistical analysis of metabolomics data reveals HFF-diet-stressed metabolic alterations

The metabolic profiles of animals are dynamically changing, which can reflect the physiologic or pathologic conditions. Although it is complicated, metabolomics analysis can present some useful hints for mechanistic investigation (31). To study the underlying mechanisms for MHP efficacy, we performed a LC-MS based metabolomic profiling of serum collected from all the three groups of rats. Considering their efficacy, the MHP-H group was chosen for metabolomics analysis. Representative total ion chromatograms of LC-MS profiling of Cont, HFF and MHP groups of rats under positive ion mode are shown in Figure 2A.

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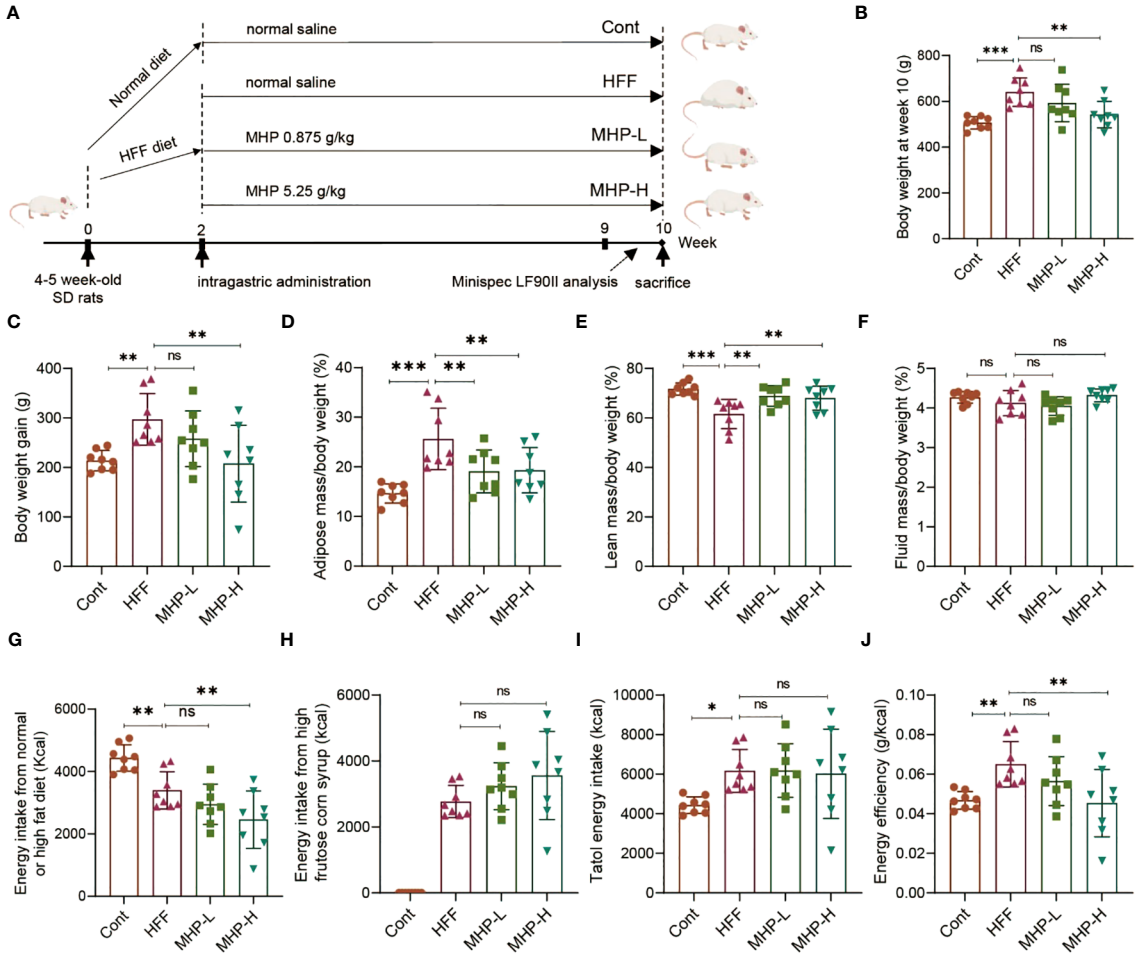

FIGURE 1
MHP facilitates weight loss in a high-fat high-fructose diet-induced obese rats. **(A)** SD rats were fed either a normal chow diet or a high-fat diet plus HFCS-sweetened drink (HFF diet). Two weeks later, an elimination about 20% of obesity-resistant rats from the HFF diet fed group was performed and the rest rats were divided into HFF group, MHP-H group and MHP-L group (n=8 for each group). The control and HFF group of rats received deionized water (vehicle), while the MHP-H and MHP-L groups were supplemented with MHP at a dose of 5.25 g/kg/day and 0.875 g/kg/day, respectively, via oral gavage until the end of the experiment. **(B, C)** Body weight and body weight gain. **(D–F)** Adiposity, lean and fluid index. **(G, H)** Energy intake from normal or high-fat diet and high-fructose corn syrup sweetened drink, respectively. **(I)** Total energy intake. **(J)** Energy efficiency of each group of rats. Cont, control group. HFF, high-fat high-fructose diet fed group. MHP-L and MHP-H are low dose- and high dose-MHP supplemented groups, respectively. MHP, a mulberry and *Hippophae*-based solid beverage. Data are expressed as mean ± SD (n =8). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. HFF group.

Mass spectrometry-based metabolomics studies require quality control to obtain reliable and high-quality metabolomics data (32). For this, the PCA score plots were used for both positive and negative modes (Supplementary Figures S1A, B). The PCA (R^2X = 0.525) and PLS-DA score plots (R^2X =0.505, R^2Y =0.999, and Q^2 = 0.936) generated by multivariate statistical analysis based on acquired data indicated that the metabolome of the three groups of rats were distinct from each other (Figures 2B, C).

The volcano plot of the metabolites represented by MS data also showed obvious difference between the control and HFF group (Figure 2D), so do the HFF and MHP groups (Figure 2G). In order to maximize the discrimination between every two groups and screen out the differential metabolites, OPLS-DA analysis was carried out. As a result, in the OPLS-DA score plot of Cont versus HFF group, clear difference was presented while the cumulative R^2X, R^2Y, Q^2 value was 0.488, 0.998 and 0.762, respectively (Figure 2E). Similarly, HFF group was also different from MHP group in the OPLS-DA score plot with the cumulative R^2X, R^2Y, Q^2 values being 0.479, 0.98, and 0.745 respectively (Figure 2H). R^2X and R^2Y are known to be the cumulative model variation in X and Y, while Q^2 is the cumulative predicted variation. In general, the values of these parameters that are approaching 1.0 indicate that a model is stable and has predictive reliability. A permutation test with 200 iterations confirmed that the constructed OPLS-DA model was valid and not over-fitted, as the original R^2 and Q^2 values to the right were significantly higher than the corresponding permutated values to the left (33). The value of R^2 was 0.99 and Q^2 was 0.09 in Cont versus HFF group (Figure 2F), while R^2 was 0.93 and Q^2 was 0.0 between HFF and MHP group (Figure 2I).

Similarly, the LC-MS data under negative ion mode of metabolic profiles were acquired and analyzed. Representative

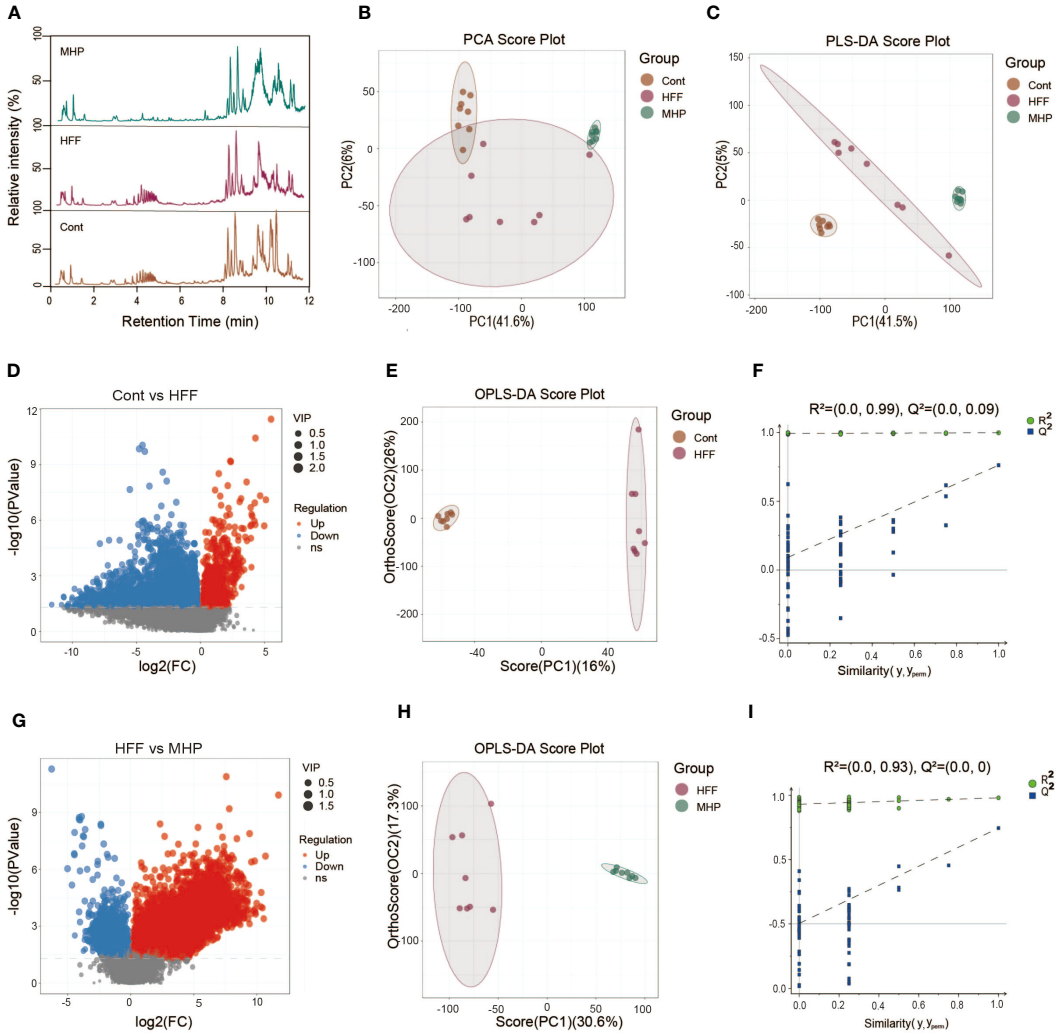

FIGURE 2
Multivariate statistical analysis of the LC-MS data acquired under positive ion mode. **(A)** Representative ESI(+)-MS total ion chromatograms of three groups of serum samples; **(B, C)** Principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) score plots; **(D, G)** Volcano plot of MS^1 significant differential metabolites; **(E, H)** Pair-wise orthogonal projections to latent structures discriminant (OPLS-DA) scores plot; **(F, I)** Statistical validation of the OPLS-DA model by permutation testing.

total ion chromatograms of Cont, HFF and MHP groups were shown in Figure 3A. The PCA (R^2X =0.533) and PLS-DA (R^2X =0.226, R^2Y =0.995 and Q^2 = 0.914) score plots showed clear differences among the Cont, HFF and MHP groups (Figures 3B, C). The volcano plots originated from ESI(-)-MS data also showed distinct difference between Cont and HFF groups (Figure 3D) as well as between HFF and MHP groups (Figure 3G). Following the aforementioned procedure, the OPLS-DA score plot of Cont versus HFF group also showed clear difference with the cumulative R^2X at 0.248, R^2Y at 0.999, and Q2 at 0.876 (Figure 3E). The cumulative R^2X, R^2Y and Q^2 value were 0.231, 0.996, and 0.735 respectively between HFF and MHP groups (Figure 3H). Moreover, a permutation test confirmed that the present OPLS-DA model was valid and not over-fitted. The detailed parameters were as the following: R^2 was 0.97 and Q^2 was 0.03 in Cont versus HFF group (Figure 3F), while R^2 was 0.98 and Q^2 was 0.03 between HFF and MHP groups (Figure 3I).

3.3 MHP efficacy is associated with PPAR signaling pathway as revealed by metabolomics

Metabolome reflects the holistic condition of an organism, presenting a global manifestation by a list of metabolites which are inextricably linked in terms of origination and metabolic pathways involved. Taking VIP > 1 and $p < 0.05$ as threshold values, a total of 73 serum small molecules were identified as the DMs between the control and HFF group rats when the ESI(+) and ESI(-) data were pooled together (Supplementary Table S2 in Supplementary Materials). As shown in Figure 4A, the DMs clustered into two distinct groups in the heatmap, which showed that the serum metabolome was greatly altered after HFF diet consumption.

Individually, each metabolite involves in specific biochemical pathway and plays an important role in the delicate life process. For

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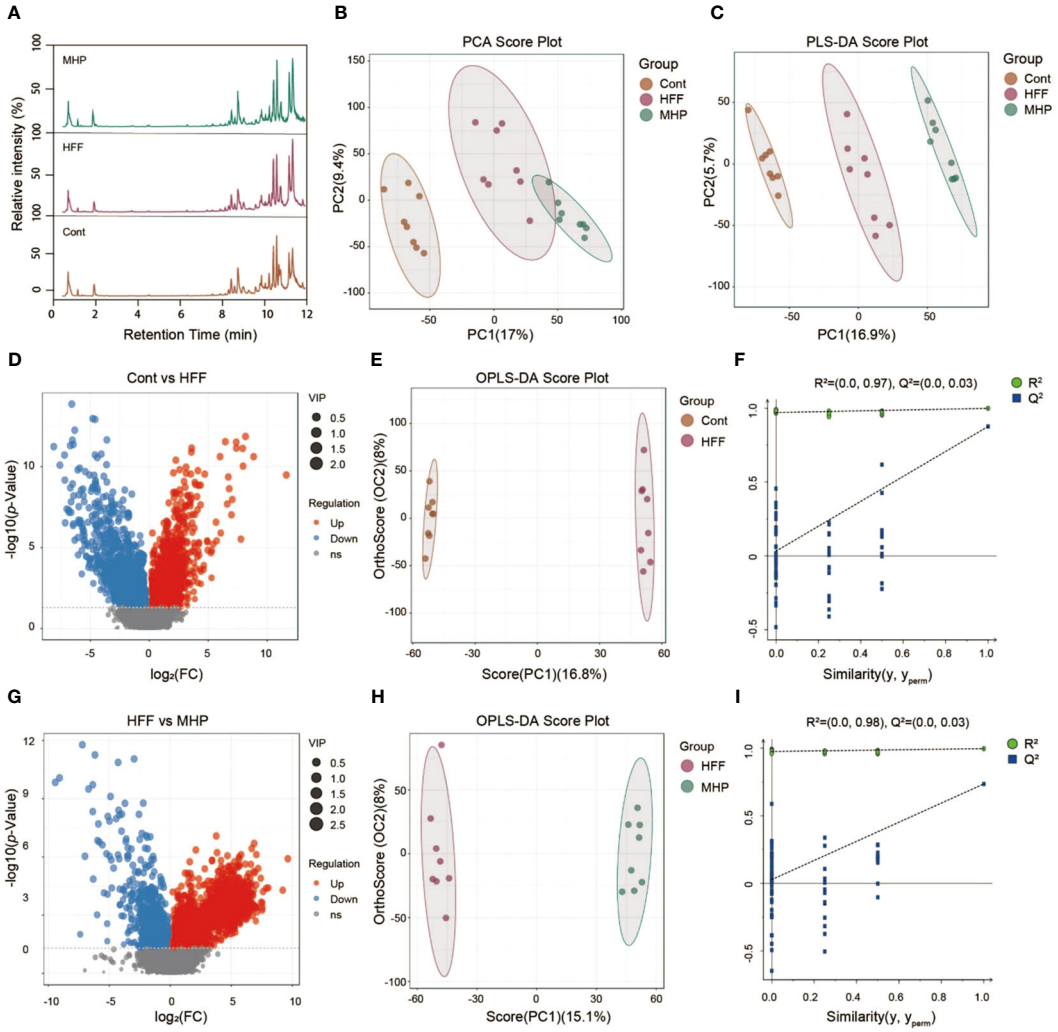

FIGURE 3
Multivariate statistical analysis of LC-MS data acquired under negative ion mode. **(A)** Representative ESI(+)-MS total ion chromatogram of serum samples; **(B, C)** Principal component analysis (PCA) and Partial least squares-discriminate analysis (PLS-DA) score plots; **(D, G)** Volcano plots of MS[1] significant differential metabolites; **(E, H)** Pair-wise orthogonal projections to latent structures discriminant (OPLS-DA) scores plots; **(F, I)** Statistical validation of the OPLS-DA model by permutation testing.

example, when compared with Cont group, the contents of oleic acid and uridine in serum of rats were considerably upregulated (fold change, FC >3.0) while α-tocotrienol was significantly downregulated (FC <0.5) in HFF group. In fact, oleic acid can induce oxidative damage and steatosis in hepatocytes both *in vitro* and *in vivo* (34). Uridine is one metabolite positively associated with obesity, and is also a potential biomarker of insulin resistance (35, 36). Tocotrienols possess powerful antioxidant, anticancer, and cholesterol-lowering properties (37), and tocotrienols supplementation not only inhibits body weight gain but also attenuates oxidation stress in HFD-treated mice (38). On the whole, however, the significantly changed DMs should be taking into account for a living organism. To this end, Cytoscape, MetaboAnalyst, MetaCore and other programs can generate algorithmic networks which can present more insights than the blunt results analyzed individually (25, 39). Thus, in order to illustrate and visualize the underlying metabolic stress induced by HFF diet, the above DMs were further analyzed using MetaboAnalyst 5.0 (www.metaboanalyst.ca) to interpret the apparently independent changes into altered canonical pathways and metabolic regulation networks. As shown in Figure 4B, pathway enrichment results suggest that the changes of metabolic profile in rats were related principally to (1) PPAR (peroxisome proliferator-activated receptors) signaling pathway, (2) linoleic acid metabolism, (3) renal cell carcinoma, and (4) other pathways. The metabolic network generated with DMs is visualized by Cytoscape and shown as Figure 4C. PPARs, including PPARα, PPARγ and PPARβ/δ, are fatty acid-activated transcription factors of the nuclear hormone receptor superfamily that regulate energy metabolism. PPAR signaling pathway is critical to coordinate many cellular events during normal and stress conditions (40).

Likewise, using VIP > 1 and $p < 0.05$ as the thresholds, OPLS-DA generated 76 serum DMs between the HFF challenged rats and MHP supplemented rats based on the integrated ESI(+) and ESI(-)

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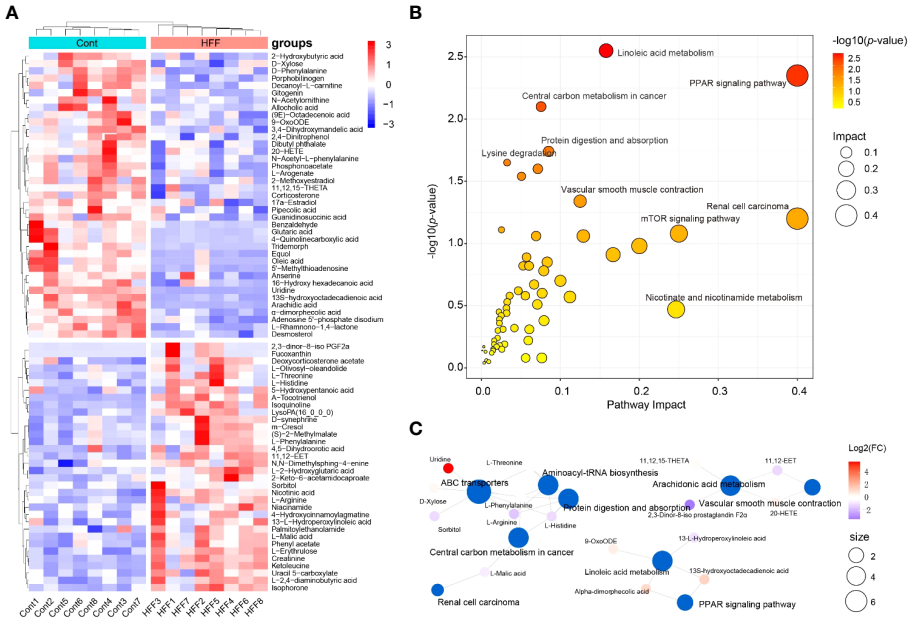

FIGURE 4
Rat serum metabolome is significantly altered when challenged by HFF diet. Untargeted metabolic analysis was performed through Vanquish UHPLC tandem Orbitrap Exploris120 MS system. The compounds were identified and quantitated by accuracy mass and MS/MS data which were matched with HMDB (http://www.hmdb.ca), KEGG (https://www.genome.jp/kegg/), mzCloud (https://www.mzcloud.org) and the in-house metabolites database of Panomix Biomedical Tech Co., Ltd. (Shuzhou, China). **(A)** Relative levels of 73 differential metabolites shown in heatmap. **(B)** Pathway enrichment analysis of the above 73 differential metabolites was performed using the online metabolomics tool of Biodeep Platform (http://www.biodeep.cn/) to visualize the results. **(C)** Metabolic network generated with differential metabolites during HFF versus control. Heatmap was generated by R pheatmap package using the 73 differential metabolites presented by metabolomic profiling of the rat serum samples of control and HFF group. HFF, high-fat diet and high-fructose corn syrup drink.

data (Supplementary Table S3 in Supplementary Materials). The DMs can also be clustered into two distinct groups in the heatmap (Figure 5A), which demonstrates that MHP intervention can ameliorate the metabolism stress induced by HFF consumption. Among them, some individual metabolites are interesting. For instance, compared with HFF group, 6-hydroxyhexanoic acid was remarkably upregulated in MHP group (FC>3.0), which can significantly reduce high-fat diet induced weight gain and improve glucose intolerance and insulin resistance in mice (41). While the content of uric acid was significantly downregulated in MHP group (FC <0.5), and recent studies have confirmed that high level of serum uric acid is associated with obesity and metabolic syndrome (42). However, an algorithmically constructed metabolic network presents more insight than a serial of the individual metabolites (25). Therefore, the above DMs were also used to perform pathway enrichment analysis by MetaboAnalyst 5.0. As shown in Figure 5B, the changes of metabolic profile in rats can be related principally to (1) PPAR signaling, (2) renal cell carcinoma, (3) central carbon metabolism in cancer, and (4) other pathways. The metabolic network generated with DMs between HFF and MHP groups was shown as Figure 5C. Unexpectedly, in both Figures 4B and 5B, PPAR signaling pathway seems to be the most impacted pathway, suggesting that this pathway is critical to coordinate the whole metabolism. Although more evidence may be needed, these results suggest that the efficacy of MHP is closely related to its regulation on the PPAR signaling pathway, which is worthy of further investigations.

3.4 MHP mitigates white adipose tissue transcriptome stress response in HFF-diet induced obese rats

Abnormal WAT is a phenotypic indicator of over-weight or obesity. In the present study, HFF diet intake led to an enlarged iWAT (Figure 6A) and an increase in the ratio of iWAT weight to body weight (Figure 6B). Therefore, we expect that MHP supplementation may attenuate excessive lipid deposition. Indeed, pathohistological examination of iWAT sections, both with H&E staining and Masson's trichrome staining, showed that the rats in MHP-H group had less hypertrophic adipocytes as compared with HFF group (Figures 6C, D) and collagenous fibrosis (Figures 6E, F). These results confirmed the efficacy of MHP on weight loss.

As metabolome changes should originate from its upstream effects, including proteome and transcriptome changes, thus we performed RNA-Seq analysis of iWAT to verify the influence of HFF on the transcriptomic profiles in white adipose tissue (Figure 6G). As shown in Figure 6H, I, an obvious difference was found between the control and HFF groups as well as between the HFF and MHP-H groups. By setting $p < 0.05$ and |Fold change| > 2.0 as thresholds, a total of 5095 DEGs and 7907 DEGs were screened out between control and HFF groups and between HFF and MHP groups, respectively (see Supplementary Figure S2 in Supplementary Materials). Furthermore, KEGG pathway enrichment analysis of these DEGs showed that several pathways, including CAMP signaling, glycolipid metabolism, regulation of

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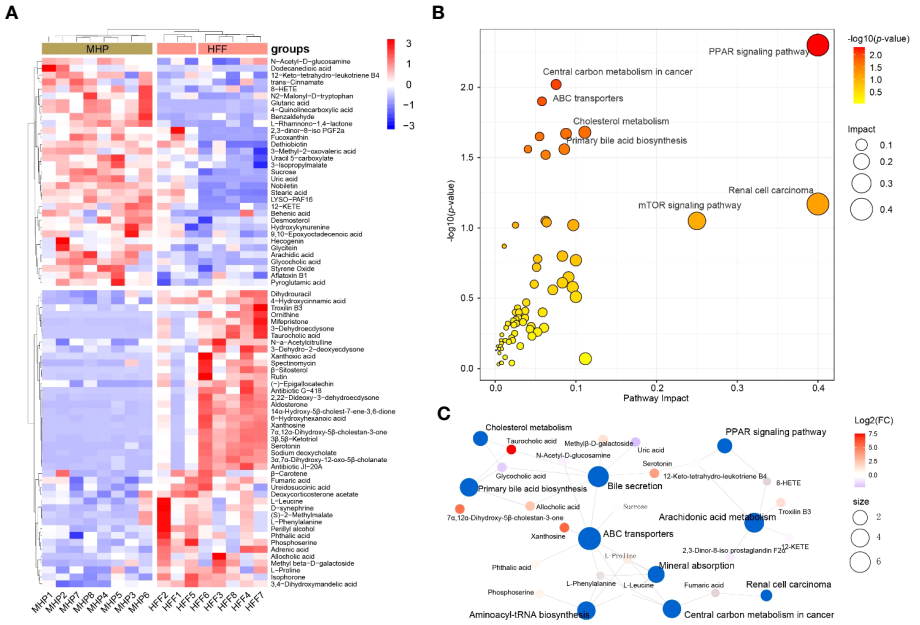

FIGURE 5
Metabolic remodeling in obese rat after a high-dose MHP supplementation. Untargeted metabolic analysis was performed using Vanquish UHPLC tandem Orbitrap Exploris120 MS system. The compounds were identified and quantitated by accuracy mass and MS/MS data which were matched with HMDB (http://www.hmdb.ca), KEGG (https://www.genome.jp/kegg/), mzCloud (https://www.mzcloud.org) and the in-house metabolites database of Panomix Biomedical Tech Co., Ltd. (Shuzhou, China). **(A)** Relative levels of 76 differential metabolites shown in heatmap. **(B)** Pathway enrichment analysis of the above 76 differential metabolites was performed using the online metabolomics tool of Biodeep Platform (http://www.biodeep.cn/) to visualize the results. **(C)** Metabolic network generated with differential metabolites during HFF versus MHP. Heatmap was generated by R pheatmap package using the 76 differential metabolites presented by metabolomic profiling of the rat serum samples of HFF and MHP group. HFF, high-fat diet and high-fructose corn syrup drink.

lipolysis in adipocytes, PPAR signaling, insulin resistance and MAPK signaling, were significantly changed by HFF diet (Figure 6J), confirming that HFF significantly exerted influence on carbohydrate, lipid and energy metabolism of WAT as shown in our previous report on hepatic transcriptome (25). As expected, the high-dose MHP supplementation relieved this situation (Figure 6K) – nearly all involved pathways were redressed after MHP intake, suggesting that MHP could attenuate white adipose tissue transcriptome stress in HFF-diet-induced obese rats.

3.5 MHP facilitates obese rats weight loss partly through PPARγ signaling pathway

Storage of extra calories is an inborn ability of white adipose tissue. In the case of overnutrition, white adipose tissue usually experiences two scenarios, i.e., hyperplasia and hypertrophy. Adipocyte hyperplasia is commonly regarded as a healthy adaptation whereas adipocyte hypertrophy is associated with increased hypoxia which is regarded as an unhealthy state (43). In general, adipocyte hypertrophy is achieved in mature adipocytes via an increase in lipid accumulation or lipogenesis (i.e., triglyceride synthesis), which is synergetic with a decreased lipolysis (i.e., triglyceride breakdown). In contrast, adipocyte hyperplasia relies on a complicated adipogenesis process (44). The nuclear hormone receptor PPARγ is regarded as the master regulator of adipogenesis as it is indispensable for adipocyte differentiation. Mechanistically, TGF-β signals through SMAD3 and directly inhibits PPARγ-C/EBPα complex formation and, in consequence, adipogenesis (45).

TGF-β and PPARγ signaling pathways play a crucial regulatory role in adipocyte hyperplasia and hypertrophy, which may be a key target for the treatment of obesity (Figure 7A). Take PPARγ for instance, some of its ligands have been developed as clinically effective antidiabetic drugs (46, 47). Therefore, GSEA analysis on TGF-β signaling pathway and PPARγ signaling pathway in adipose tissue transcriptome were conducted. As compared to the control group, the HFF group exhibited a significant downregulation trend, which was reversed after MHP treatment (Figures 7B, C). The heatmap of genes related to adipocyte hyperplasia and hypertrophy reflected an obvious difference between the Cont and HFF groups, while, after MHP treatment, mRNA levels of differential genes tended to be normal (Figure 7D). As a transcription factor, PPARγ activates expression of various genes involved in fat deposition, such as *Lpl*, *Fatp1*, *CD36*, *Fabp4*, and *Plin*, by binding a functional peroxisome proliferator response element (PPRE) on their promoter in adipose tissue (48). In this study, MHP significantly suppressed the expression of *Pparγ* and its downstream target genes *Fabp4* and *Plin1* induced by HFF diet, suggesting that MHP may improve fat deposition by regulating the PPARγ pathway (Figure 7E).

In consideration of their structures and functions, the TGF-β superfamily ligands can be divided into two subfamilies: TGF-βs and BMPs (49). In our study, *Tgfβ3*, the key gene involved in adipocyte proliferation, exhibited a significant upregulation trend following MHP treatment (Figure 7E), indicating that MHP may improve the state of

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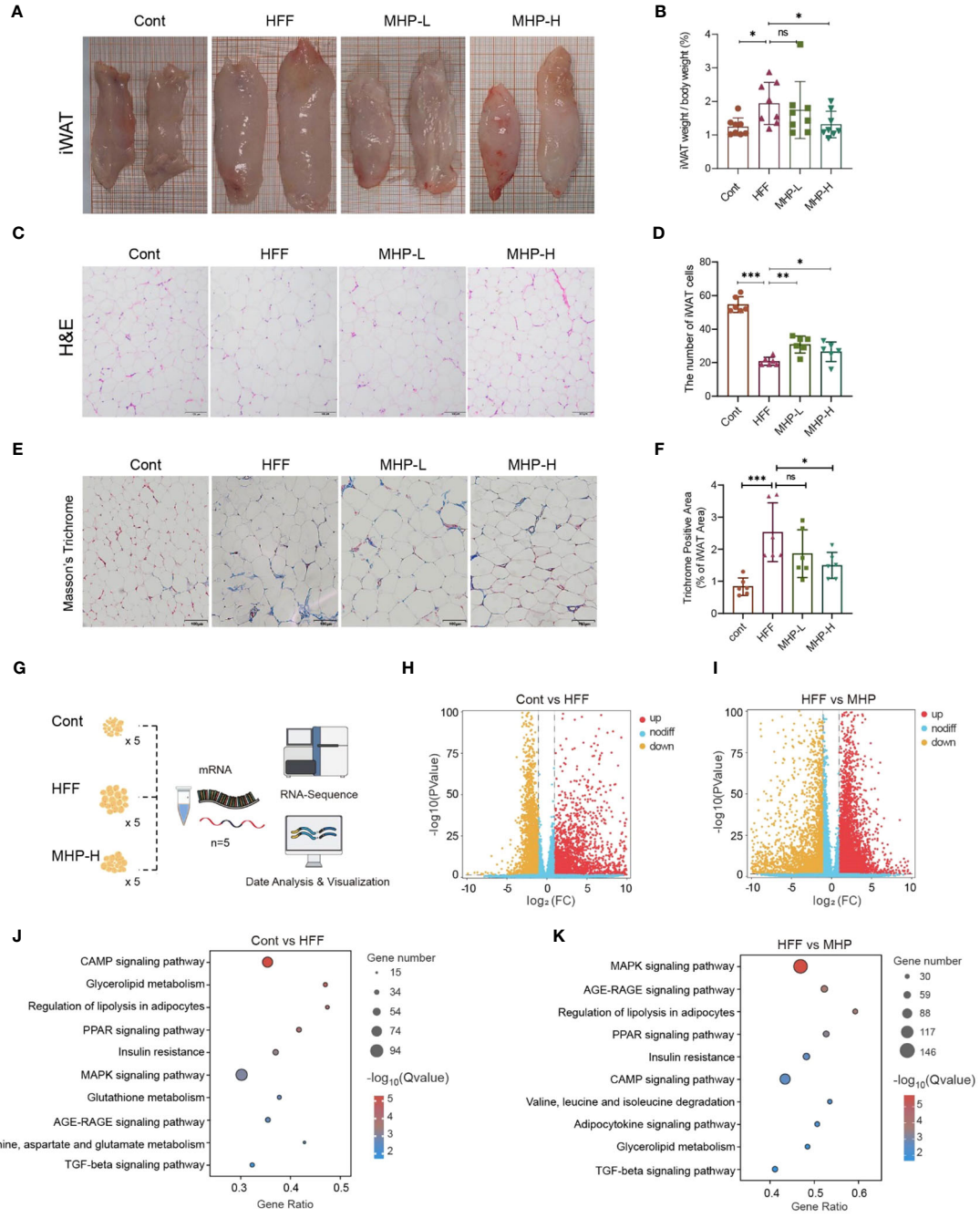

FIGURE 6
MHP improved white adipose tissue transcriptomic stress in HFF-induced obese rats. **(A, B)** Enlarged iWAT and increase in iWAT weight/body weight; **(C, D)** H&E stained iWAT sections and quantitative results of adipocytes. **(E, F)** Masson's trichrome stained iWAT sections and quantitative analysis of collagenous fibrosis. **(G)** RNA-Seq analysis of iWAT; **(H, I)** Volcano plot of genes; **(J, K)** KEGG pathway enrichment analysis of DEGs. Cont, control group. HFF, high-fat high-fructose diet fed group. MHP-L and MHP-H are low dose- and high dose-MHP supplemented groups, respectively. MHP, a mulberry and *Hippo*phae-based solid beverage. GSEA, gene set enrichment analysis. iWAT, inguinal white adipose tissue. The access number of RNA-Seq raw reads is PRJNA1063764. (https://www.ncbi.nlm.nih.gov/sra/PRJNA1063764). Data are expressed as mean ± SD (for iWAT weight/body weight, n =8; for H&E stained and Masson's trichrome stained sections, n=6). *$p < 0.05$, ***$p < 0.001$ vs. HFF group.

adipocyte hypertrophy by inhibiting TGFβ3-mediated adipocyte proliferation. Several genes involved in adipogenesis and lipid accumulation, for example, *Smad*1/2 and *Bmp*2/4, were robustly upregulated in the adipocytes under the HFF diet, but there was a significant inhibition of *Bmp*2/4 expression upon MHP intervention (Figure 7E). BMPs are pleiotropic proteins that regulate processes like cell-fate determination, proliferation, apoptosis and differentiation (50). BMP2 stimulates adipogenesis in 3T3-L1 cells by induction of PPARγ via mainly Smad-1/5/8 and p38 MAPK pathway (51). BMP4 promotes MSCs commitment to the adipocyte lineage and induces adipogenesis in a dose-dependent manner (52). Therefore, our findings suggest that MHP may exert its effects by modulating TGF-β and PPARγsignaling

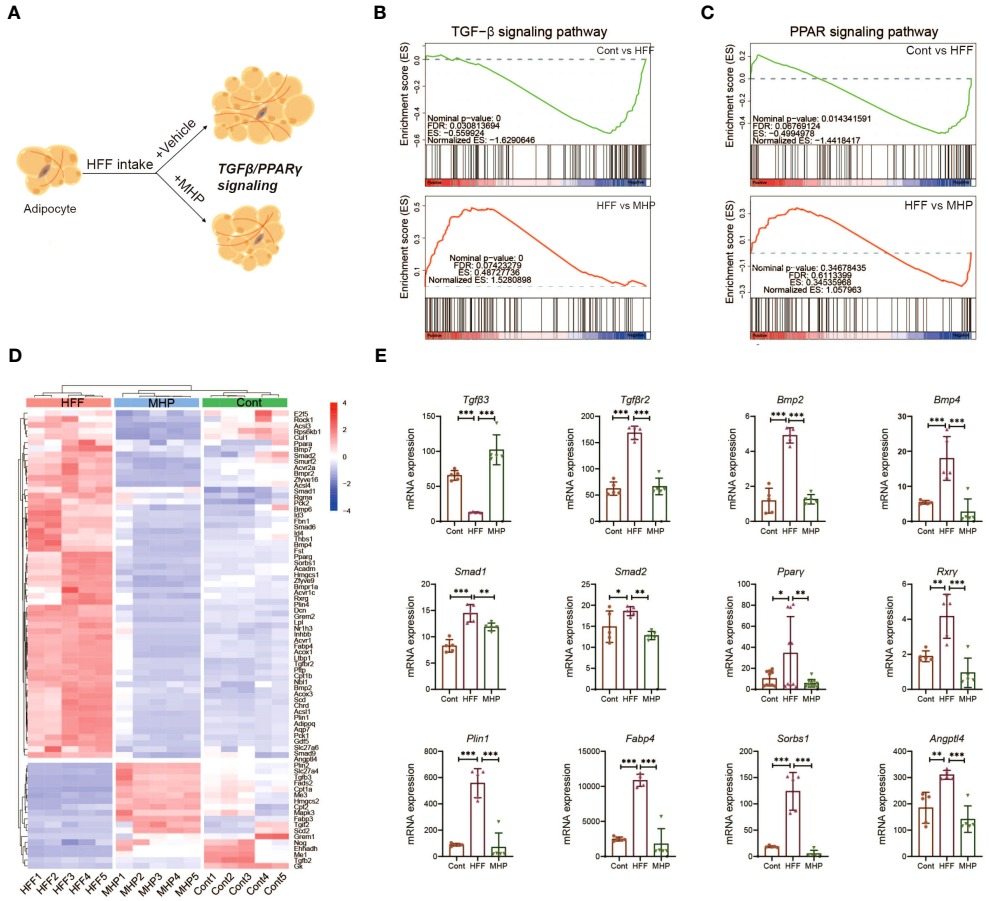

FIGURE 7
MHP promotes obese rats weight loss partly through TGF-β and PPARγ signaling pathways. **(A)** Schematic response of adipocytes to MHP intervention via TGF-β and PPARγ signaling pathways; **(B, C)** GSEA analysis of TGF-β and PPARγ signaling pathways; **(D)** Heatmap of the expressions of genes involved in TGF-β and PPARγ signaling pathway in iWAT; **(E)** mRNA expression level of typical genes. Data are expressed as mean ± SD (n=5). MHP, a mulberry and *Hippophae*-based solid beverage. GSEA, gene set enrichment analysis. iWAT, inguinal white adipose tissue. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. HFF group.

pathways, thereby promoting adipocyte proliferation and inhibiting adipogenesis. Consequently, this could alleviate adipocyte hypertrophy and improve the unhealthy obese conditions.

3.6 Weight-loss effect of MHP is associated with its regulation on adipose fibrosis

Obesogenic adipogenesis is a pathological process which is closely related to ECM (extracellular matrix) (53). In physiological states, a balance between synthesis, deposit and degradation of ECM components exists. However, diet-induced obesity is usually accompanied with ongoing ECM remodeling, which likely results from an excess synthesis of fibrillar components, such as collagens I, III and VI, and an impaired degradation of these proteins (54). Excessive ECM deposition in adipose tissue is a hallmark of fibrosis, which is a major contributor to adipose tissue dysfunction (55). As described, Masson's trichrome staining adipose tissue sections showed a higher content of collagen fiber in HFF-diet challenged rats as compared to the control group, but MHP reversed its progression (Figure 6C). Thus, we reasoned that the health benefit of MHP may also be due to its improvement on the adipose dysfunction (Figure 8A). In other words, MHP intervention may reduce ECM deposition as well as fibroblasts of adipocyte, consequently improving adipose tissue fibrosis challenged by HFF diet.

Since ECM remodeling is a complicated and dynamically changing process, we performed mRNA expression enrichment analysis of the gene set of extracellular matrix organization (Figure 8C). As expected, HFF diet changed the expression of ECM organization pathway genes whereas MHP intervention reversed this condition. As the matrix metalloproteinases (MMP) are regarded as marker proteins involved in adipose ECM remodeling, mRNA expression levels of MMP genes were also compared. As shown in Figure 8G, significant upregulation expression of MMP-2 and MMP-14 genes was found in HFF group rats, while it was normal in MHP group. It has been shown that MMP-2 is a key regulator of adipocyte differentiation, and high-expression of MMP-2 is necessary for adipocytes hyperplasia when responding to excess energy from HFF diet (56). Also, MMP14 is found dramatically upregulated in adipose tissue of obese mice induced by high-fat diet (57). Therefore, the efficacy of MHP is likely due to its protection against adipose tissue dysfunction.

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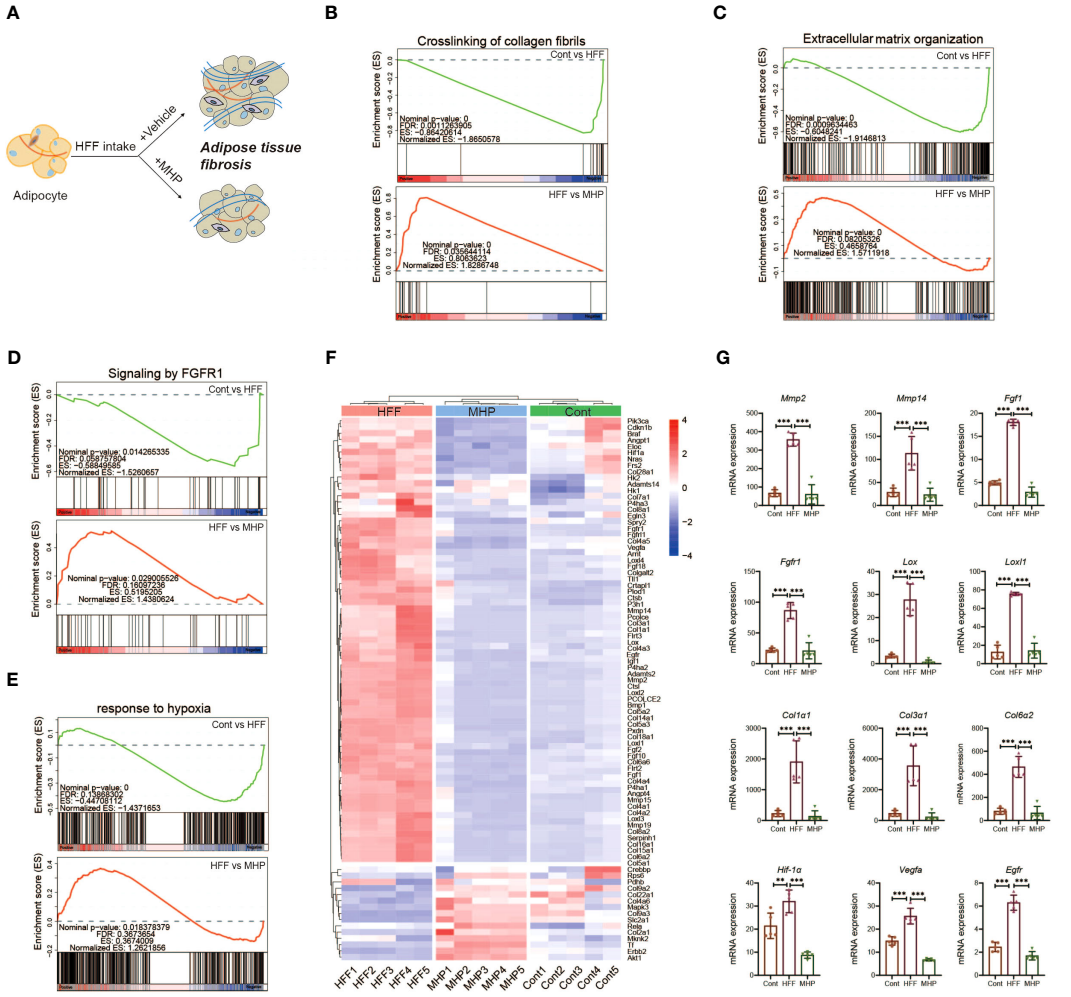

FIGURE 8
Efficacy of MHP on weight loss is associated with its regulation on FGFR1 pathway. **(A)** Schematic response of adipocytes to MHP intervention through improving adipose tissue dysfunction. GSEA analysis of pathways: crosslinking of collagen fibrils **(B)**, extracellular matrix organization **(C)**, signaling by FGFR1 **(D)** and response to hypoxia **(E)**. **(F)** Heatmap of the expressions of genes involved in aforementioned pathways in iWAT. **(G)** mRNA expression level of part of typical genes. MHP, a mulberry and *Hippophae*-based solid beverage. GSEA, gene set enrichment analysis. iWAT, inguinal white adipose tissue. Data are expressed as mean ± SD (n =5). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. HFF group.

Crosslinking of collagen, the main component of connective tissue surrounding adipocytes, is known to affect adipose remodeling, which is crucial for maintaining function and metabolic homeostasis of adipose tissue (58). In fact, increased interstitial fibrosis in WAT may lessen the flexibility of ECM and decrease the plasticity of adipose tissue as obesity progresses, which ultimately leads to adipose tissue dysfunction (55). Through GSEA analysis of the iWAT transcriptome, we found the trend back of crosslinking of collagen fibrils pathway after MHP intervention (Figure 8B). Similar results were also found in collagen formation pathway and assembly of collagen fibrils and other multimeric structures pathway (Supplementary Figures S3A, B).

In addition to ECM remodeling, adipose fibrosis also contributes greatly to adipose tissue dysfunction. We found that the signaling by FGFR1 pathway, which is associated with adipose tissue fibrosis, was markedly downregulated in the HFF group (Figure 8D), so did the downstream signaling of activated FGFR1 pathway (Supplementary Figure S3C). Interestingly, these two pathways were significantly upregulated after MHP treatment (Figure 8D). Further, the heatmap of the DEGs from signaling by FGFR1 pathway and crosslinking of collagen fibrils pathway suggests that MHP was able to improve HFF diet-induced obese adipose tissue fibrosis at the transcriptomic level (Figure 8F). As shown in Figure 8G and Supplementary Figure S3D, the critical genes involved in these two pathways in the HFF group, including *Fgf1*, *Fgfr1*, *Col1α1*, *Col1α3*, *Col6α2*, *Lox* and *Loxl1/2/3/4* showed a marked upregulation trend as compared with the control group, but showed a substantial downregulation trend after MHP intervention.

Fgf1 is unique among the *Fgf* family in its ability to activate all four *Fgfrs* and their isoforms (59). It is reported that adipose *Fgf1* is dramatically induced in HFD-fed mice (60) and expression of *Fgf1* and *Fgfr1* in the adipose tissue of HFD-fed and ob/ob mice is significantly elevated when compared with normal diet-fed and lean control mice (61). In line with these reports, the expression of *Fgf1* and *Fgfr1* was significantly increased in the iWAT of HFF-induced obese rats but was normal after MHP treatment (Figure 8G).

Collagen type I, III, and VI are highly expressed in adipose tissue (62) and diet induced obesity is found to promote the expressions of *Col1α1*, *Col3α1* and *Col6α3* in eWAT of C57/B6J mice (58). Moreover, *Col3α*, *Col6α*, and *Lox* gene expressions are downregulated by metformin and resveratrol, which are shown to decrease collagen deposition in adipose tissue induced by HFD (63). In fact, *Lox* (Lysyl oxidase), which catalyzes the formation of allysine and hydroxylysine, is crucial for collagen fiber crosslinking and thus for fibrosis development (64). Berberine is also found to suppress the expression of *Hif-1α* and *Lox* in eWAT at the state of obesity induced by HFD (65). Therefore, the efficacy of MHP is potentially associated with its protection against adipose tissue fibrosis.

As aforementioned, the adipocyte hypertrophy is usually along with an insufficient local blood supply and a subsequent locally hypoxic microenvironment in adipose tissue (66). Therefore, the genes related to hypoxia response may have some expression alterations in our case. For this, GSEA analysis of the iWAT transcriptome on the response to hypoxia pathway was carried out (Figure 8E). As expected, supplementation of MHP resulted in a strong downregulation trend for the key genes related to hypoxia, including *Hif-1α*, *Vegfr*, *Egfr* and *Igf1* (Figure 8G, Supplementary Figure S3D). Interestingly, an elevated expression of *Hif-1α*, *the* hypoxia-inducible factor-1 *α*, in obese adipose tissue is reported to trigger a potent pro-fibrotic transcriptional program, and inhibition or knockout *Hif-1α* can ameliorate the negative aspects of the obesity-associated fat pad expansion and adipose tissue fibrosis (66, 67).

Rapid tissue expansion during the development of obesity causes the neo-vasculature to struggle to keep up, and the mRNA expression of vascular endothelial growth factor (*Vegf*), a well-known *Hif-1α* target gene, is markedly elevated in HFD fed mice (55). On the other hand, hyperplastic expansion is preceded by angiogenesis but angiogenesis is often insufficient and occurs after hypertrophic adipocyte growth, which can result in hypoxia and tissue dysfunction (68, 69). Previous research has revealed that HFD increases expression of *Egfr* and its ligand amphiregulin in adipose tissue macrophages (ATMs). Furthermore, *Egfr* deletion in ATMs leads to an inhibition of resident ATM proliferation and monocyte infiltration into adipose tissue, which results in decreased obesity and insulin resistance (70). As described above, the expression of hypoxia-related genes was markedly increased in the HFF group, but it was normal after MHP intervention (Figure 8G), suggesting that the efficacy of MHP is related to its improvement on hypoxia condition of the hypertrophic adipocytes.

4 Conclusions

In the present study, body component analysis and histopathologic examination confirm that MHP can effectively facilitate weight loss and adiposity decrease in rat model of obesity. Pathway enrichment analysis with the DMs generated by serum metabolomic profiling and multivariant statistical analysis suggest that PPAR signal pathway was significantly altered when challenged by HFF diet while it was rectified after MHP intervention. Furthermore, the RNA-Seq based transcriptome data indicate that MHP intervention also rectified the alterations of white adipose tissue mRNA expressions in HFF-induced obese rats. Integrated omics reveals the efficacy of MHP against obesogenic adipogenesis was potentially with its regulation of PPARγ and FGFR1 signaling pathway. Collectively, our findings provide reliable evidence that MHP can improve obesity and an insight into the use of MHP in body weight management. Although full understanding of its mechanisms requires further work, MHP is a potential healthy product for body weight management.

Data availability statement

The datasets presented in this study are included in the article/Supplementary Materials. The RNA-Seq data has been linked to the GenBank's Sequence Read Archive here: https://www.ncbi.nlm.nih.gov/sra/PRJNA1063764. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by The Institutional Animal Care and Use Committee of Guangdong Pharmaceutical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

X-TZ: Writing – original draft, Data curation, Formal analysis. A-QZ: Data curation, Writing – original draft, Formal analysis. X-ML: Formal analysis, Resources, Writing – original draft. L-YS: Formal analysis, Data curation, Investigation, Writing – original draft. J-GY: Data curation, Investigation, Writing – original draft. NL: Data curation, Formal analysis, Investigation, Writing – original draft. S-SC: Formal analysis, Data curation, Investigation, Writing – original draft. ZH: Writing – review & editing, Conceptualization, Resources. X-LM: Conceptualization, Resources, Funding acquisition, Writing – original draft. K-PL: Writing – review & editing, Conceptualization, Data curation, Writing – original draft, Funding acquisition, Methodology.

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Conflict of interest

Authors X-ML, J-GY, S-SC, and X-LM were employed by the company Perfect Guangdong Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1344262/full#supplementary-material

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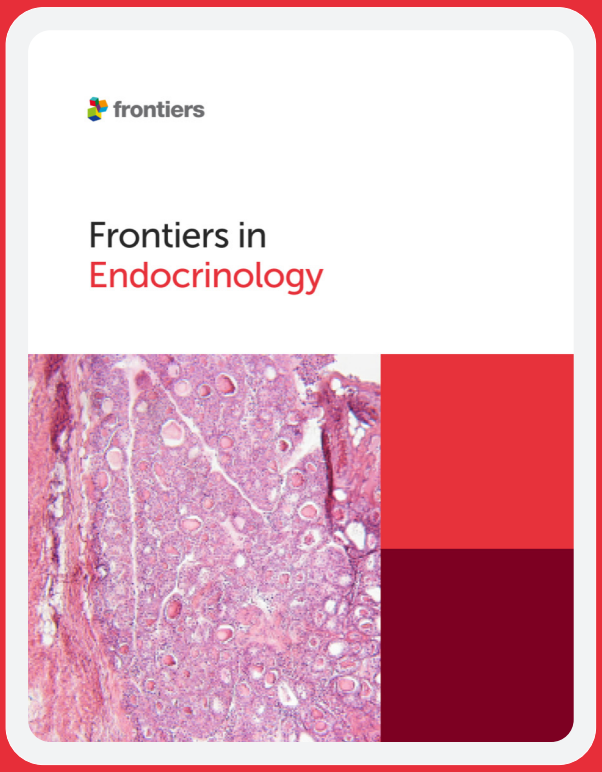

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