INFANT AND CHILD NUTRITION, PHYSICAL ACTIVITY, OXIDATIVE STRESS AND INFLAMMATORY SIGNALING

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Editorial: Infant and child nutrition, physical activity, oxidative stress and inflammatory signaling

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Editorial on the Research Topic
Infant and child nutrition, physical activity, oxidative stress and inflammatory signaling

Two key factors for the correct and healthy development of the child are physical activity and diet. During the childhood, participation in physical activity is particularly important and lack of physical activity at this important stage of life can lead to limited cognitive and developmental disorders (1). In general, the practice physical activity has a positive effect on the motor, respiratory, endocrine, cardiovascular, nervous, and immune systems. Also, physical exercise increases blood circulation, which leads to an improvement in the supply of oxygen and nutrients to the brain and stimulates the maturation of the motor regions of the brain, affecting motor development and increasing the behavioral speed of nervous stimuli (2, 3). In addition, physical activity affects the synaptic plasticity and the excitation of neurons that form these synapses (4), improving cognitive abilities, and self-esteem, along with reduced depression rates (5).

In this sense, school-age children who perform vigorous physical activity report much better cognitive functioning, reduced body weight and body mass index, increasing lean mass and reducing fat mass (Diaz-Castro et al.). It has been shown that children who exercise regularly have better biomarkers related to the molecular functionality of adipose tissue and the brain, such as adipsin, adiponectin, osteocrine, lipocalin-2, nerve growth factor, brain derived neurotrophic factor, and irisin among others (Diaz-Castro et al.). Results showing the usefulness of early interventions based on physical activity in children to reduce risk factors related to sedentary lifestyle. Additionally, an adequate level of fitness during childhood seems to protect against metabolic risk, inflammation and oxidative stress decreasing leptin and nerve growth factor levels, contributing to control adiposity at the prepubertal stage (Llorente-Cantarero, Leis et al.).

In short, school-age children who perform vigorous physical activity report much better cognitive functioning and show better and healthy body development. In this sense, prepubertal metabolically healthy obese children are less sedentary, more active, and they have better metabolic profiles than metabolically unhealthy obese subjects (Llorente-Cantarero, Aguilera et al.). However, currently, factors such as modernization, transportation systems or even the wide variety of electronic equipment and electronic devices, have greatly reduced the need for perform physical work, thus encouraging a more sedentary lifestyle. This phenomenon has special attention among children and adolescents, who spend a lot of time using these devices, a fact even promoted in many cases by the family environment (6). For those reasons, the researchers emphasize that, despite these undoubted benefits, only about a third of children play sports regularly (7) and we cannot forget that the lack of physical activity is one of the main causes of childhood obesity and one of the most important public health problems nowadays. Currently, physical inactivity is considered one of the main risk factors for death worldwide, and a risk factor for diseases such as hypertension, dyslipidemia, and diabetes mellitus (8). Lack of physical activity leads to health problems, including postural problems (such as idiopathic scoliosis), somatic conditions, overweight and obesity, circulation problems, and even premature death (9).

Another factor of great importance to consider for the correct development of children is diet. It is clear that diet is related to multiple factors previously mentioned such as neuronal functionality, metabolism, or body composition. It is also, a very important factor in the development of obesity. Diet and exercise have to be factors that must act together, if what is intended is to achieve a healthy development of children (10, 11). In this regard, it has been indicated that the children practicing exercise showed high adherence to the Mediterranean Diet associated with lower incidence of chronic non-communicable diseases, including obesity, and were more concerned about their diet (Diaz-Castro et al.).

In summary, researching the links between diet, exercise, inflammatory stress, oxidative, and growth factors during childhood is important to understand how they contribute to optimal growth and development, deeply influencing health and the results of the above-mentioned studies and reviews represent an enormous amount of new relevant data on the child nutrition, physical activity, oxidative stress and inflammatory signaling. Despite all the existing literature and evidence related to this extremely important topic, the papers published in this e-book clearly show that there are still many aspects to be clarified and understood such as for example, the engagement of physical activity of girls to assess the influence of physical activity on this group, which is more reluctant to do it. After reading this book, some topics as the development of new physical activity protocols designed specifically for school-age children to maximize the benefits of training and minimize the risk of injury, together with the improvement of nutritional knowledge of foods and diets, enabling to acquire the knowledge and facilitating the development of activities development which will contribute to promote health thanks to the implementation of nutritional knowledge and physical activity protocols at school.

Author contributions

JM-F and JD-C wrote the introduction and the conclusion. JO and MO wrote the central part with comments to the cited papers and references. All authors contributed to the article and approved the submitted version.

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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Implementation of a Physical Activity Program Protocol in Schoolchildren: Effects on the Endocrine Adipose Tissue and Cognitive Functions

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Practicing exercise is one of the best strategies to promote well-being and quality of life, however physical activity in schoolchildren and adolescents is developed in an unpredictable, intermittent way and in short periods. There are relatively few intervention studies investigating the role of physical activity in schoolchildren endocrine function of adipose tissue and cognitive function. One hundred and three boys, divided into two groups: control ($n = 51$, did not perform additional physical activity) and exercise ($n = 52$, performed vigorous physical activity after the regular school classes). The exercise group, developed a 6 months physical activity protocol delivered by the physical education teacher during the second semester of the academic course (6 months). Body composition measurements, adherence to the Mediterranean diet, nutritional intake, hematological and biochemical parameters, endocrine function of the adipose tissue and biomarkers of brain molecular function were assessed at enrolment and after 6 months of intervention. No statistically significant differences between both groups were found for age, height and bone mass. Weight and BMI was lower in the exercise group compared to the control group, increasing lean mass and reducing fat mass. 58.68% of children in the exercise group showed high adherence to the Mediterranean Diet compared to 46.32% of the control group. The exercise group was more concerned about their diet consumed more fiber, vitamin B1, B2, B6, B12, D, Niacin, Folic acid, Fe, Zn, Se and Cu. Triglycerides levels and HDL-cholesterol were higher in the exercise group at the end of the study. Leptin, MCP-1, lipocalin-2, adipsin and PAI-1 levels were lower in the exercise group at the end of the exercise protocol. In contrast, adiponectin and osteocrin markedly increased in the exercise group. Moreover, marked increases were recorded in healthy brain state biomarkers (NGF, BDNF, and irisin) in the exercise group, which could have a positive impact on academic performance. Taken together, all the findings reported are consistent with many benefits of the exercise protocol on adipose tissue and brain molecular function, demonstrating the usefulness of early interventions based on physical activity in children to reduce risk factors related to sedentary lifestyle.

Keywords: children, physical activity, anthropometry, diet, adipose tissue, cognitive function

INTRODUCTION

Practicing exercise is one of the best strategies to promote well-being and quality of life (1). Specifically, systematic and regular physical activity contributes to maintaining and even improving different systems and functions, such as musculoskeletal, osteoarticular, cardiocirculatory, respiratory, endocrine-metabolic, immunological and psychoneurological functions (2). In addition, the improvement of some biomarkers, exercise not only positively affects physical health, but also mental health and quality of life (3, 4). Some studies have shown that patterns of habitual physical activity contribute to improving self-concept, self-esteem, depression and anxiety disorders (5, 6).

In general terms, physical activity in children is developed in an unpredictable, intermittent way and in short periods, which leads to affirm that its influence on health is underrated and even scarcely studied (7). Data from the European Youth Heart Study (8) suggested that the most active children (who dedicate at least 60 min/day of physical activity) have better cardiovascular health, regardless of their adiposity degree. This finding is noteworthy because there is a progressive increase in sedentary lifestyles related to the health problems of children, due to the misuse of free time, generally associated with the use of electronic devices (8–10).

Low levels of physical activity are related to overweight, increase of fat mass and obesity in schoolchildren and children (11) a period in which their practice tends to decrease (12). In addition, obesity has been linked to vascular endothelial dysfunction, which has been identified as a major risk factor for cognitive impairment (13). In this sense, some adipokines levels may be indicators of the effect that physical exercise could have on the endocrine function of adipose tissue. This is why their levels must be taken into account as they play a crucial role in the process. Thus, adiponectin which is an anti-inflammatory adipokine, regulates insulin homeostasis (14), leptin reduces food intake, increases energy expenditure and its levels are inversely correlated with adipose tissue (15), PAI-1 and MCP-1 regulate inflammatory signaling (16) and adipsin links the immune function and the adipose tissue endocrine function through the alternative pathway of complement activation (17), among others. Therefore, school age and adolescence, are crucial stages for the configuration of healthy lifestyle habits that will persist in later life stages (18). The development of unhealthy behavior patterns at these ages is a relatively generalized phenomenon and it is in them that adequate preventive measures have to be adopted that affect a better quality of life for the population (19).

In addition to promoting health and preventing disease, physical activity in schoolchildren is important for growth and development (20). Cognitive function, brain function, and learning outcomes have been reported to be linked to physical activity (21, 22). The association between physical activity, health and intelligence quotients has been shown in previous studies (23). This is why the assessment of some key parameters in cognitive function, such as NGF which enhances neurons differentiation and survival (24) and neurotransmitter biosynthesis, improving overall cognitive function (25), BDNF which has a key role on brain development, neuron proliferation and survival, and cognitive functions such as learning and memory (26, 27) and irisin which regulates the process of neuronal differentiation and maturation (28), is of high importance.

Moreover, there is a positive association between emotional quotient and physical activity (29). Higher emotional quotients have also been associated with longer exercise duration (30). Taken together, these studies suggest that increased physical activity is associated with higher intellectual and emotional cues during childhood. Despite the benefits described, several studies report that the practice of physical activity has suffered a progressive decline in young Spaniards, especially between 12 and 18 years of age (31), which is due by the increase of sedentary habits such as watching TV and playing video games (32). In this sense, there are relatively few intervention studies investigating the role of physical activity in schoolchildren on cognitive function. Furthermore, the results of these previous studies were mainly focused on body composition and psychological tests evaluating cognitive function, concentration, memory, and alertness without providing molecular mechanisms elucidating the role of physical activity in adipose tissue and brain functions. Hence, taking into account all these considerations, the purpose of this study was to determine the impact of a protocol of physical activity on children's endocrine function of adipose tissue and cognitive function biomarkers.

MATERIALS AND METHODS

Subjects

A total of 122 students were asked to participate in the study. During the enrolment phase, 14 students refuse to participate, mainly because they were already performing sports extra-curricular activities several days per week after school hours, and one of them because he had a chronic disease (diabetes). Moreover, 5 students who agreed to participate in the study, finally left it because parents did not complete the informed consent form. The sample size selected for this study was 103 boys, divided into two groups using a simple randomization procedure (computerized random numbers) based on their initial weight to obtain statistically equal groups (ANOVA, $p > 0.05$). Control group, ($n = 51$, did not perform additional physical activity) and exercise group ($n = 52$, performed a more vigorous physical activity, according to the exercise protocol described below). The boys were studying during the second semester in a Center for Primary and Secondary Education in the Malaga region (Spain).

The mean age was 11.21 ± 0.17 years in the control group and 11.16 ± 0.18 years in the exercise group. The study was approved by the Ethics committee of Andalusia Biomedical Research Ethics Portal (ref. 29/01/2018/2/2018). Informed consent was obtained from all the parents with written consent to participate in this study. To avoid an important confounder in this type of trials we performed a 3 days diet questionnaire including 1 day of the weekend to assess the nutritional status of the participants. The information obtained in this survey was evaluated by nutritional software. A blood sample was obtained from each participant and

they completed a medical and health history, physical activity questionnaire and anthropometric measurements.

Calculation of Sample Size

According to earlier findings (33), a minimum sample size of 40 children per intervention is necessary to detect changes due to the physical activity across groups with a power of 80% and $\alpha = 0.05$. As a result, a total of 80 subjects (40 per group) are required. A total of 103 schoolchildren were recruited to allow for a potential loss to follow-up of up to 25%.

Physical Exercise Performance Protocol

The exercise protocol (**Figure 1**) was designed in accordance with a team of experts in sports and physical activity sciences. At the beginning of the study, all the participants (control and exercise groups), performed 3 days per week of training classes of 1 h that consists of three parts (A + B + C): (A) Warm-up (10 min): in which the boys begin with light-intensity movements (e.g., wrist rotations; leg swings). (B) Main part of the exercise (45 min): Technique exercises (15 min): passes, catches, drives, feints, dribbles, shots on goal, control exercises, skill circuits, tactic drills (15 min): rounds, defense drills, attack drills, counterattacks, set plays, superior attack, ball possession drills, pressures, field positions, lines, set pieces, real game situation "match" (15 min). (C) Cool down (5 min): stretching.

The intervention period was developed during the second semester of the academic course (from January to June). Control subjects continued with the routine mentioned above for the 6 months of the study, however, for the exercise group, the intervention consisted of a 6 months physical activity programme delivered by the physical education teacher with specific elements of additional vigorous physical activity, as follows:

- First month: 10 extra minutes of part A (warm up), of the protocol mentioned above are added, which means a total of 70 min of exercise protocol per day (3 days per week), which imp Total duration of the exercise protocol: 70 min. The teacher led an active 10-min warm-up, starting with light-intensity movements (e.g., wrist rotations; leg swings) and progressing through moderate-intensity activity (e.g., arm rotations; walking with a high knee-raise) to include two 4-min periods of vigorous physical activity (e.g., vigorous arm sprints; running on the spot). This warm-up evolves from mild to moderate-intensity activities and incorporates bursts of strong physical activity, which is designed to maximize the training benefit and to minimize the risk of injury. Five boys piloted the warm-up and infusions while wearing heart rate monitors on their chests, which demonstrated that heart rates were in the vigorous zone for the duration of all intervention elements. In total, we got an increase of 10 min of cardiovascular work per session (compared to previous sessions), which means 30 extra min per week, and about 120 min per month. The prescription was made based on the initial determination of the VO_2 peak mL/kg/min and the clinical evaluation. VO_2 peak was calculated with the 20 m shuttle run test using the protocol previously described by Léger et al. (34). The completed laps were noted when a child was unable to follow the pace in two consecutive intervals. For the following months, the volume was maintained with intensity variation (interval work, between 65% and 85% of the reserve heart rate) followed by resistance exercises with standard series.

- Second month: 25 min of aerobic work per session were added to the protocol developed in the first month, which means a total of 85 min of exercise protocol per day (3 days per week). 10 extra min to the part A (warm up) and 15 extra min to the part B (main part of the exercise).

-Third month: 15 min of aerobic work per session were added to the protocol developed in the second month, which means a total of 100 min of exercise protocol per day (3 days per week). 5 extra min to the part A (warm up) and 10 extra min to the part B (main part of the exercise).

- Fourth month: The duration and time of the exercise protocol (100 min per day), was followed as described on the third month, but one extra day per week was added to the exercise protocol (4 days per week) with the same characteristics mentioned in the third month.

- Fifth month and sixth month: The duration and time of the exercise protocol (100 min per day), was followed as described on the third and fourth months, but another one additional day per week was added to the exercise plan (5 days per week) during these 2 months, with the same characteristics mentioned in the third and fourth month.

Blood Sampling

Blood samples were collected from the participants via venous catheter into heparinized tubes. Two blood samples were taken on each group: when enrolled in the study (T1, basal value), and after 6 months (T2). An aliquot od the blood was used for the measurement of hematological parameters and the rest of the blood was immediately centrifuged at 1,750 x g for 10 min at 4° C in a Beckman GS-6R refrigerated centrifuge (Beckman, Fullerton, CA, USA) to separate plasma from red blood cell pellets.

Body Composition Measurements

The assessment of body composition was carried out by electrical bioimpedance (EIB), in the study a multifrequency TANITA MC-980MA equipment (Biológica Tecnología Médica S.L., Barcelona, Spain) with software Suite Biológica 7.1 (Version 368) was used. Following the guidelines supplied by the manufacturer, to carry out the measurement, the subjects were placed in an upright position on the platform and placing their bare feet on the four stainless steel electrodes in such a way that with the tip of the electrodes they made contact with the front electrodes and with the heel with the rear electrodes. The hands held the clamps with the electrodes, keeping the arms stretched along the trunk slightly apart from it. At no time during the test should there be contact with any metal part. An imperceptible multifrequency current between 5 and 1,000 kHz was introduced through the electrodes. The approximate measurement time was 30 s. Height was determined by the height contraction measurement method. The subjects were required to stand still with the feet together and the heels, buttocks, and upper back in contact with the scale. The parameters studied have been: weight, lean mass, fat mass, bone mass, and total body water. Body mass index (BMI) was calculated by dividing body weight in kilograms by height in meters squared ($BMI = kg/m^2$) and height and weight were used

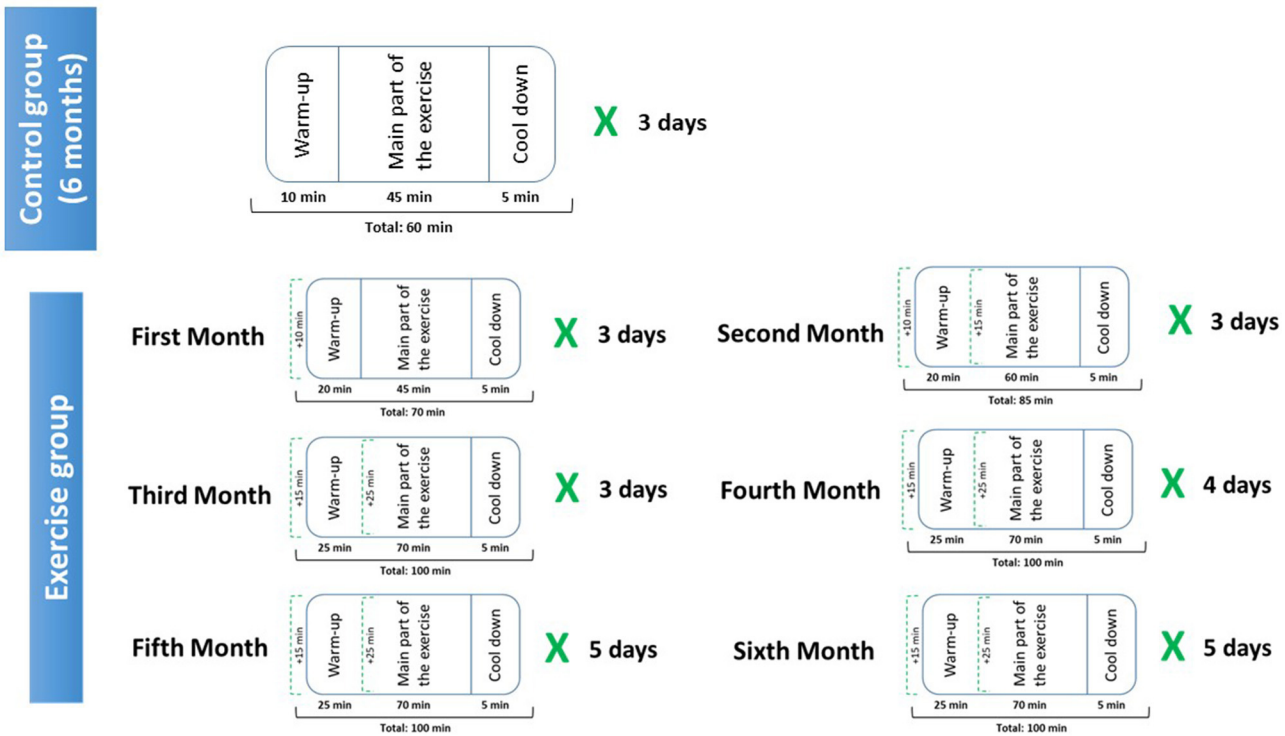

FIGURE 1 | Physical exercise protocol.

to calculate age and gender-specific standardized child body mass index (BMI) (kg/m^2) z-score.

Physical Activity Measurement

The IPAQ-C questionnaire (35) designed to estimate physical activity in children aged 8–12 years was used, in which they asked about their physical activity during different times of the day, such as physical education class transport and free time to later find out the number of metabolic equivalents of task (MET) and determine the level of activity that a person has performed during the last 7 days. The children were classified into three categories (low, medium, high) according to the estimated energy expenditure for each activity: vigorous, 8 MET; moderate, 4 MET; and low, 3.3 MET. It consists of 10 questions that assess different aspects of the levels of physical activity performed by the child using the Likert scale to calculate the final score. MET-min per week, are obtained by multiplying energy expenditure for each activity by the minutes performed and by the days of the week you practice it.

Adherence to the Mediterranean Diet

We assessed adherence to the Mediterranean dietary pattern by applying the KIDMED test of Adherence to the Mediterranean diet (MD) (36), used successfully in different populations (37), among them in Spanish children (36, 38). This instrument consists of 16 dichotomous questions that must be answered affirmatively/negatively (yes /no). The affirmative answers in the questions that represent a positive aspect in relation to the MD (12 questions) add one point, while the affirmative answers in the questions that represent a negative connotation with respect to the MD (four questions) subtract one point. Negative answers do not score. The total score obtained gives rise to the KIDMED index, which is classified into three categories:

a) From 0 to 3: Very low quality diet (low degree of adherence to MD).
b) From 4 to 7: Need to improve the eating pattern to adapt it to the MD (medium degree of adherence).
c) From 8 to 12: optimal MD (high degree of adherence).

Nutritional Assessment

A 24-h recall questionnaire was carried out, it is a retrospective method to calculate the nutrient intake. The 24-h recall consists of asking the interviewed individual about the food consumed, both qualitatively and quantitatively, during a 24-h period. Three 24-h reminders were held including a holiday or a weekend day. The questionnaire was completed through a personal interview with a trained interviewer. To help the surveyed patient fill out this questionnaire and collect data as accurately as possible, a photographic manual was used that includes models of food sizes, prepared dishes, and homemade measurements (39). The duration of the survey was approximately 45 min.

Software for Nutritional Assessment Processing

The data from the food consumption recall survey are processed through the Nutriber computer program (Nutriber, v1.1.1.5.r5, FUNIBER, Barcelona, Spain, 2005) (40), which allows us to know the amount of energy, macro and micronutrients consumed by the subjects and compare them with the recommended intakes for the Spanish population (41).

Hematological Test

White blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) concentration, mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC), haematocrit (HCT), platelets, lymphocytes, monocytes, neutrophils, eosinophils, basophils, mean corpuscular volume (MCV), mean platelet volume (MPV), red cell distribution width (RDW), plateletcrit (PCT) and platelets distribution width (PDW) of fresh blood samples were measured using an automated hematology analyzer Mythic 22CT (C2 Diagnostics, Grabels, France).

Biochemical Parameters

Triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol, uric acid, urea, albumin, creatine kinase-MB (CKMB), creatinine, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), glucose, total bilirubin, total Protein and lactate dehydrogenase (LDH) were measured by standard colorimetric and enzymatic methods, using a BS-200 Chemistry Analyzer (Shenzhen Mindray Bio-Medical Electronics Co Ltd, Shenzhen, China). All of the samples were analyzed in duplicate, and the averages of the paired results were determined.

Endocrine Function of the Adipose Tissue

Adiponectin, adipsin, lipocalin-2/NGAL, total plasminogen activator inhibitor-1 (PAI-1) and resisting were determined using the HADK1MAG-61K MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 assay; leptin and monocyte chemoattractant protein-1 (MCP-1) levels were measured using the HADK2MAG-61K MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 2 assay (Millipore Corporation, Missouri, USA); apelin, follistatin-like protein 1 (FSTL1), osteocrin and osteonectin were determined using the HMYOMAG-56K MILLIPLEX MAP Human Myokine Magnetic Bead Panel assay, based on immunoassays on the surface of fluorescent-coded beads (microspheres), following the specifications of the manufacturer (50 events per bead, 50 μl sample, gate settings: 8,000–15,000, time out 60 s). Plates were read on LABScan 100 analyzer (Luminex Corporation, Texas, USA) with xPONENT software for data acquisition. Average values for each set of duplicate samples or standards were within 15% of the mean. All the analytes in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

Brain Molecular Function Parameters

Brain Derived Neurotrophic Factor (BDNF) and irisin were determined using the HMYOMAG-56K MILLIPLEX MAP assay (Millipore Corporation, Missouri, USA); Nerve growth factor (NGF) levels were measured using the HADK2MAG-61K MILLIPLEX MAP assay (Millipore Corporation, Missouri, USA). Plates were read as mentioned above.

Statistical Analysis

All data are reported as mean values with their standard errors. All variables were tested to see if they followed the criteria of normality and homogeneity of variance using the Kolmogorov-Smirnoff's and Levene's tests, respectively. To compare general characteristics of the subjects in both experimental groups, unpaired Student's *t* test was used. Variance analysis by one-way ANOVA methods was used to compare the differences between periods. Following a significant F test ($P < 0.05$), individual means were tested by pair-wise comparison with Tukey's multiple comparison test, when main effects and interactions were significant. The level of significance was set at $P < 0.05$. Statistical analyses were performed using the SPSS computer program (version 26.0, 2021, SPSS Inc., Chicago, IL).

RESULTS

No statistically significant differences between both groups were found for age, height and bone mass. Weight, BMI and z-score were lower in the exercise group compared to the control group ($P < 0.05$) at the end of the intervention. Fat mass was drastically lower in the exercise group compared to the control group ($P < 0.001$) and the exercise group ($P < 0.001$) at the beginning of the study and the end of the exercise protocol. Lean mass and total water increased in the exercise group compared to the control group at the end of the study ($P < 0.01$) and also with the exercise group at the beginning of the study ($P < 0.01$). Finally, as expected, physical activity markedly increased in the exercise group compared to the control group ($P < 0.001$) after the exercise protocol and to the baseline with the exercise group ($P < 0.001$) (**Table 1**).

Table 2 shows that 58.68% of children in the exercise group showed high adherence to the Mediterranean diet compared to 46.32% of the control group at the end of the intervention ($P < 0.05$).

Regarding energy intake and most of the macronutrients, no statistically significant differences between both groups were found, however the exercise group consumed more fiber ($P < 0.05$), vitamin B1 ($P < 0.01$), B2 ($P < 0.01$), B6 ($P < 0.01$), B12 ($P < 0.05$), D ($P < 0.01$), Niacin ($P < 0.001$) and Folic acid ($P < 0.01$) compared to the control group and also to the same group at enrolment. With regards to minerals, the exercise group consumed more Fe ($P < 0.001$), Zn ($P < 0.05$), Se ($P < 0.01$) and Cu ($P < 0.01$) compared to the control group and also to the same group at enrolment (**Table 3**).

With regards to most of the hematological parameters studied, no statistically significant differences between both groups were found except for the increased in WBC recorded for the experimental group compared to the control group at the end of the study ($P < 0.05$) and RDW which was higher in both groups at the end of the study ($P < 0.01$) (**Table 4**).

TABLE 1 | Anthropometric characteristics and results of the International Physical Activity Questionary in the last 7 days from the control and exercise groups.

	Control group		Exercise group	
	T1	T2	T1	T2
Height (cm)	147.31 ± 1.76	148.01 ± 1.78	145.28 ± 2.09	146.35 ± 2.11
Weight (kg)	41.87 ± 1.88	42.65 ± 1.85^{A}	41.95 ± 1.87	39.01 ± 1.86^{Bd}
Lean Mass (%)	45.65 ± 1.38	46.05 ± 1.61^{A}	46.86 ± 1.45	53.37 ± 1.00^{Bd}
Fat Mass (%)	15.54 ± 1.56	16.25 ±1.48^{A}	15.21 ± 1.43	9.08 ± 0.87^{Bd}
Bone mass (%)	2.56 ± 0.11	2.59 ± 0.12	2.54 ± 0.12	2.51 ± 0.11
Total water (%)	58.66 ± 1.35	58.61 ± 1.42	58.73 ± 1.72	63.68 ± 1.88^{d}
BMI (kg/m^2)	20.66 ± 0.92	20.71 ± 0.91^{A}	19.01 ± 0.87	18.18 ± 0.65^{Bd}
BMI (kg/m^2) z-score	0.90 ± 0.01	0.94 ± 0.08^{A}	0.93 ± 0.05	0.77 ± 0.03^{Bd}
Physical Activity (MET-min per week)	2,105.91 ± 132.05	1,980.32 ± 127.31	2,117.23 ± 115.59	3,387.85 ± 129.33^{Bd}

Mean values among groups with different letters differ ($P < 0.05$).
^{A,B}for T2 by Student's t test between different groups; ^{d}for T1 vs. T2 in the same group by Tukey's test.

TABLE 2 | Results of the KIDMED test evaluating the adherence to the Mediterranean diet from the control and exercise groups.

	Control group		Exercise group	
	T1	T2	T1	T2
Low (≤3, %)	0	0	0	0
Medium (4–7, %)	54.73 ± 0.30	53.68 ± 0.33^{A}	53.25 ± 0.29	41.32 ± 0.39^{Bd}
High (≥8, %)	45.27 ± 0.27	46.32 ± 0.32^{A}	46.75 ± 0.28	58.68 ± 0.34^{Bd}
Mean Value	6.82 ± 0.31	6.25 ± 0.27^{A}	6.85 ± 0.31	7.82 ± 0.37^{Bd}

Mean values among groups with different letters differ ($P < 0.05$).
^{A,B}for T2 by Student's t test between different groups; ^{d}for T1 vs. T2 in the same group by Tukey's test.

Biochemical parameters are shown in **Table 5**. Triglycerides levels were lower in the exercise group compared to the control group at the end of the study ($P < 0.01$) and, in contrast, they increased in both groups after the 6 months period ($P < 0.001$ for the control group and $P < 0.01$ for the exercise group). In the control group HDL-cholesterol decreased after the 6 months period ($P < 0.001$) and, in contrast, in the exercise group increased after the exercise intervention ($P < 0.01$). AP increased in the exercise group compared to the control group after the physical activity intervention ($P < 0.001$). AP also increased in both groups at the end of the 6 months intervention, compared to the beginning of the study ($P < 0.001$).

Endocrine function parameters of the adipose tissue are shown in **Figure 2**. Leptin (**Figure 2A**), MCP-1 (**Figure 2B**), lipocalin-2 (**Figure 2D**), adipsin (**Figure 2F**) and PAI-1 (**Figure 2G**) levels were lower in the exercise group compared to the control group at the end of the exercise protocol ($P < 0.001$ for leptin, $P < 0.01$ for MCP-1; $P < 0.01$ for lipocalin-2; $P < 0.001$ for adipsin; $P < 0.001$ for PAI-1). In contrast, adiponectin (**Figure 2C**) and osteocrin (**Figure 2J**) markedly increased in the exercise group at the end of the study ($P < 0.01$).

Brain cognitive function biomarkers showed marked increases in the exercise group compared to the controls at the end of the physical activity protocol (**Figure 3**) ($P < 0.01$ for NGF, **Figure 3A**; $P < 0.001$ for BDNF, **Figure 3B** and $P < 0.001$ for irisin, **Figure 3C**).

Finally, no statistically significant differences between both groups were found for Natural Sciences, Arts, Social Sciences and Language qualifications, meanwhile Maths and Physical education qualifications were higher in the exercise group compared to the controls at the end of the semester ($P < 0.05$ for Maths; $P < 0.01$ for physical education) (**Table 6**).

DISCUSSION

Previously, several interventions have been carried out to promote physical activity in schools (42–46), however no previous study has examined the influence of exercise on the endocrine adipose tissue and cognitive function biomarkers in young schoolchildren/adolescents as the current study does. In this sense, the current research has several advantages. The physical activity protocol was designed to maximize the benefits of training and minimize the risk of injury, being low cost and really easy to implement with scarce equipment and facilities. By involving teachers in the design and implementation of the protocol, enables them to acquire the knowledge and skills to design and implement protocols for other students and groups, facilitating additional activities development which will contribute to promote physical activity at school.

Overall, the findings indicate that the although children were already active before the exercise protocol, it is encouraging that a marked significant increase in physical activity was observed in the intervention group from baseline to 6 months follow-up, particularly as this is an age when physical activity participation tend to decrease dramatically (47). The findings of the current study are in agreement with a previous report indicating that exercise improves BMI, increasing lean mass and reducing fat mass in children and adolescents (48).

In the current study, 58.68% of children in the exercise group showed high adherence to the Mediterranean Diet compared to 46.32% of the control group at the end of the physical activity intervention, reflecting those children who practice more physical activity are also more concerned about their diet, which

TABLE 3 | Diet composition and nutritional intake analyses from the control and exercise groups.

	Control group		Exercise group	
	T1	T2	T1	T2
Energy (Kcal)	1,864.07 ± 50.76	1,935.12 ± 60.15	1,889.45 ± 63.28	1,945.52 ±63.38
Carbohydrates (%)	45.74 ± 1.25	46.21 ± 1.87	45.39 ± 1.76	45.43 ± 1.29
Protein (%)	17.29 ± 0.53	17.75 ± 0.59	17.11 ± 0.49	17.08 ± 0.55
Lipids (%)	36.97 ± 1.01	37.43 ± 1.09	36.88 ± 1.07	37.42 ± 1.06
Fibre (g)	12.21 ± 0.70	11.97 ± 0.73^{A}	12.02 ± 0.68	13.93 ± 0.51^{Bd}
Vitamin A (μg)	1,114.32 ± 97.60	1,075.27 ± 101.07^{A}	1,121.32 ± 96.51	1,167.78 ± 118.31
Vitamin B_1 (mg)	1.42 ± 0.11	1.39 ± 0.21^{A}	1.47 ± 0.23	1.90 ± 0.19^{Bd}
Vitamin B_2 (mg)	1.25 ± 0.10	1.33 ± 0.14^{A}	1.29 ± 0.12	1.62 ± 0.23^{Bd}
Vitamin B_6 (mg)	1.42 ± 0.12	1.49 ± 0.13^{A}	1.48 ± 0.14	1.89 ± 0.35^{Bd}
Vitamin B_{12} (μg)	5.26 ± 0.98	4.97 ± 0.87^{A}	5.01 ± 0.94	5.91 ± 0.36^{Bd}
Vitamin E (μg)	6.74 ± 0.41	6.32 ± 0.39	6.41 ± 0.49	6.62 ± 0.53
Vitamin C (mg)	110.58 ± 12.96	115.27 ± 11.87	108.32 ± 10.35	107.25 ± 10.11
Vitamin D (μg)	5.92 ± 0.71	5.76 ± 0.65^{A}	5.87 ± 0.39	6.91 ± 0.35^{Bd}
Niacin (mg)	16.74 ± 1.21	17.43 ± 1.03^{A}	16.65 ± 1.23	20.21 ±1.89^{Bd}
Folic Acid (μg)	157.34 ± 17.86	153.45 ± 16.45	158.45 ± 18.31	178.76 ± 16.45^{Bd}
P (mg)	993.77 ± 39.23	1,001.27 ± 41.40	998.98 ± 39.89	1,006.36 ± 49.22
Mg (mg)	160.58 ± 7.97	163.67 ± 6.69	164.39 ± 7.01	165.31 ± 8.05
Ca (mg)	757.34 ± 37.24	755.11 ± 33.23	760.21 ± 31.54	751.98 ± 35.28
Fe (mg)	10.11 ± 0.68	10.15 ± 0.73^{A}	10.98 ± 0.81	12.52 ± 0.71^{Bd}
Na (mg)	1,496.65 ± 119.21	1,503.98 ± 105.22	1,505.42 ± 99.87	1,591.06 ± 101.89
K (mg)	1,582.65 ± 63.97	1,598.43 ± 77.13	1,601.11 ± 65.34	1,688.65 ± 116.25
Zn (mg)	6.37 ± 0.31	6.22 ± 0.41^{A}	6.12 ± 0.29	7.68 ± 0.32^{Bd}
Se (μg)	57.21 ± 6.11	59.22 ± 5.98^{A}	62.25 ± 4.43	75.25 ± 8.39^{Bd}
F (μg)	284.33 ± 21.12	297.22 ± 22.75	289. 45 ± 22.16	301.17 ± 34.25
I (μg)	48.38 ± 4.45	47.45 ± 4.12	46.98 ± 4.01	47.66 ± 6.56
Cu (μg)	672.21 ± 71.30	681.32 ± 68.85^{A}	701.32 ± 70.23	944.35 ± 88.32^{Bd}

Mean values among groups with different letters differ ($P < 0.05$).
^{A,B}for T2 by Student's t test between different groups; ^{d}for T1 vs. T2 in the same group by Tukey's test.

is in agreement with previous results from other studies (37, 38). In addition, Kontogianni et al. (49) also reported that the time dedicated to sedentary activities was lower as the degree of adherence to Mediterranean Diet increased in children and adolescents (3–18 years). Most of the published studies that include Spanish children show a percentage of subjects with adherence to Mediterranean Diet similar to the control group, thus Serra-Majem et al. (50), reported that 48.5% of individuals aged 2–14 years and 44.6% of those aged 15–24 years followed an optimal Mediterranean Diet. Mariscal-Arcas et al. (51), found high adherence to Mediterranean Diet in 46.9% of the subjects aged 8–9 years and in 48.9% of children aged 10–16 years. Ayechu and Durá (2009), found an optimal Mediterranean Diet in 42.9% of 13–16 years old. Rodríguez et al. (52), found a high adherence to the Mediterranean dietary pattern in 42.8% of the children included in the study.

Although total dietary intake patterns may not promote cognitive function during aging (for example, the Western Diet is associated with accelerated aging of the brain), however diet may be beneficial (in this sense, Mediterranean Diet is associated with delayed brain aging and improved cognitive ability) (53). In the current study, the exercise group consumed more fiber which is typically found in many foods of the Mediterranean diet, influencing the relationship between diet and learning through the establishment and maintenance of the gut microbiome by establishing beneficial bacteria in the colon of the human digestive system. The potential role of the "gut-brain axis" in the human body is believed to be fundamentally important. But so far, only animal studies have revealed the possible directions and extent of this relationship (54).

Due to the higher adherence to the Mediterranean Diet, the exercise group consumed more vitamin B complex, vitamin D, folate, Cu, Zn, Se, and Fe. Schoolchildren nutritional status, especially the adequacy of nutrients such as Fe, is positively correlated with various measures of cognitive ability (55). Supplementing children's nutrition with Fe offers many cognitive benefits, including improved working memory, school attendance and learning experience (56). Another micronutrient important for brain development and function in school-aged children is folic acid (57), which is positively associated with academic performance. There is also evidence that vitamin B complex influences memory function and cognitive outcomes,

TABLE 4 | Hematological parameters from the control and exercise groups.

	Control group		Exercise group	
	T1	T2	T1	T2
WBC ($10^3/\mu$L)	5.91 ± 0.28	6.05 ± 0.23^{A}	6.01 ± 0.31	6.73 ± 0.32^{Bd}
RBC ($10^6/\mu$L)	4.59 ± 0.03	4.65 ± 0.04	4.75 ± 0.07	4.84 ±0.08
Hb concentration (g/dL)	13.73 ± 0.19	13.95 ± 0.13	14.06 ± 0.07	14.18 ± 0.22
MCH (g/dL)	29.81 ± 0.27	29.99 ± 0.32	29.83 ± 0.29	29.58 ± 0.28
MCHC (g/dL)	36.38 ± 0.35	37.83 ± 0.24	36.23 ± 0.34	37.72 ± 0.22
HCT (%)	36.83 ± 0.64	36.98 ± 0.39	37.11 ± 0.53	37.81 ± 0.65
Platelets ($10^3/\mu$L)	305.12 ± 10.55	309.12 ± 13.54	289.22 ± 14.21	308.11 ± 13.55
Lymphocytes ($10^3/\mu$L)	2.33 ± 0.10	2.35 ± 0.10	2.45 ± 0.12	2.44 ± 0.13
Monocytes ($10^3/\mu$L)	0.36 ± 0.02	0.32 ± 0.02	0.37 ± 0.03	0.33 ± 0.02
Neutrophils ($10^3/\mu$L)	2.87 ± 0.26	2.91 ± 0.22	2.83 ± 0.18	2.85 ± 0.12
Eosinophils ($10^3/\mu$L)	0.33 ± 0.06	0.35 ± 0.09	0.32 ± 0.02	0.31 ± 0.02
Basophils ($10^3/\mu$L)	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
MCV (fL)	80.40 ± 1.25	79.29 ± 0.88	80.28 ± 0.89	79.41 ± 0.88
MPV (fL)	8.69 ± 0.14	9.23 ± 0.16	8.21 ± 0.17	8.96 ± 0.19
RDW (%)	14.26 ± 0.28^{a}	16.11 ± 0.25^{d}	13.32 ± 0.28^{b}	16.26 ± 0.16^{d}
PCT (%)	0.26 ± 0.01	0.28 ± 0.01	0.24 ± 0.01	0.25 ±0.01
PDW (%)	13.46 ± 0.26	14.01 ± 0.30	13.67 ± 0.22	14.11 ± 0.33

Mean values among groups with different letters differ ($P < 0.05$).
^{a,b}for T1 and ^{A,B}for T2 by Student's t test between different groups; ^{d}for T1 vs. T2 in the same group by Tukey's test.

TABLE 5 | Biochemical parameters from the control and exercise groups.

	Control group		Exercise group	
	T1	T2	T1	T2
Triglycerides (mg/dL)	71.98 ± 10.29	139.36 ± 13.29^{Ad}	69.84 ± 8.71	98.55 ± 11.84^{Bd}
Total cholesterol (mg/dL)	153.01 ± 7.25	151.55 ± 7.57	156.06 ± 8.16	153.87 ± 5.79
HDL-cholesterol (mg/dL)	71.45 ± 5.61	68.43 ± 3.81^{Ad}	73.29 ± 3.32	83.27 ± 4.01^{Bd}
LDL-cholesterol (mg/dL)	32.41 ± 2.22	33.54 ± 3.29	32.54 ± 2.24	31.42 ± 1.92
Uric Acid (mg/dL)	5.37 ± 0.55	5.11 ± 0.40	4.12 ± 0.45	4.19 ± 0.28
Urea (mg/dL)	27.20 ± 2.47	28.00 ± 1.39	28.11 ± 1.93	27.05 ± 3.22
Albumin (g/dL)	4.27 ± 0.07	4.12 ± 0.06	4.25 ± 0.04	4.07 ± 0.02
CK-MB (U/L)	52.64 ± 2.68	37.01 ± 1.99	52.13 ± 2.13	37.24 ± 1.29
Creatinine (mg/dL)	0.62 ± 0.03	0.79 ± 0.03	0.68 ± 0.05	0.77 ± 0.03
AST (U/L)	27.96 ± 2.27	27.13 ± 1.26	27.56 ± 2.01	27.52 ± 1.58
ALT (U/L)	21.57 ± 2.36	24.79 ± 2.36	22.27 ± 2.09	22.91 ± 2.35
AP (U/L)	143.29 ± 25.21	232.98 ± 26.41^{Ad}	144.39 ± 27.20	311.61 ± 29.24^{Bd}
Glucose (mg/dL)	95.03 ± 3.61	95.33 ± 2.88	96.95 ± 3.25	95.32 ± 3.30
Total bilirrubin (mg/dL)	2.05 ± 0.14	1.93 ± 0.09	2.08 ± 0.24	2.11 ± 0.05
Total Protein (g/dL)	8.02 ± 0.16	7.98 ± 00.6	7.83 ± 0.15	8.07 ± 0.23
LDH (U/L)	459.13 ± 27.53	445.11 ± 15.91	446.43 ± 21.33	438.87 ± 22.23

Mean values among groups with different letters differ ($P < 0.05$).
^{A,B}for T2 by Student's t test between different groups; ^{d}for T1 vs. T2 in the same group by Tukey's test.

because they are involved in the formation of synapses, the growth of axons and myelin genesis. In this sense, vitamin B12 is important for cognitive development (58). An association between vitamin B12 status and cognitive function has also been documented from childhood to adolescence. Children fed on a macrobiotic diet low in vitamin B12 featured delayed overall motor, speech, and language development compared with children on an omnivorous diet (59). In adolescence, these children who were fed a low vitamin B12 diet for the first 6 years had lower cognitive test scores than children who were fed an omnivorous diet (60). Vitamin D deficiency is well known to be associated with cognitive impairment because it is involved in

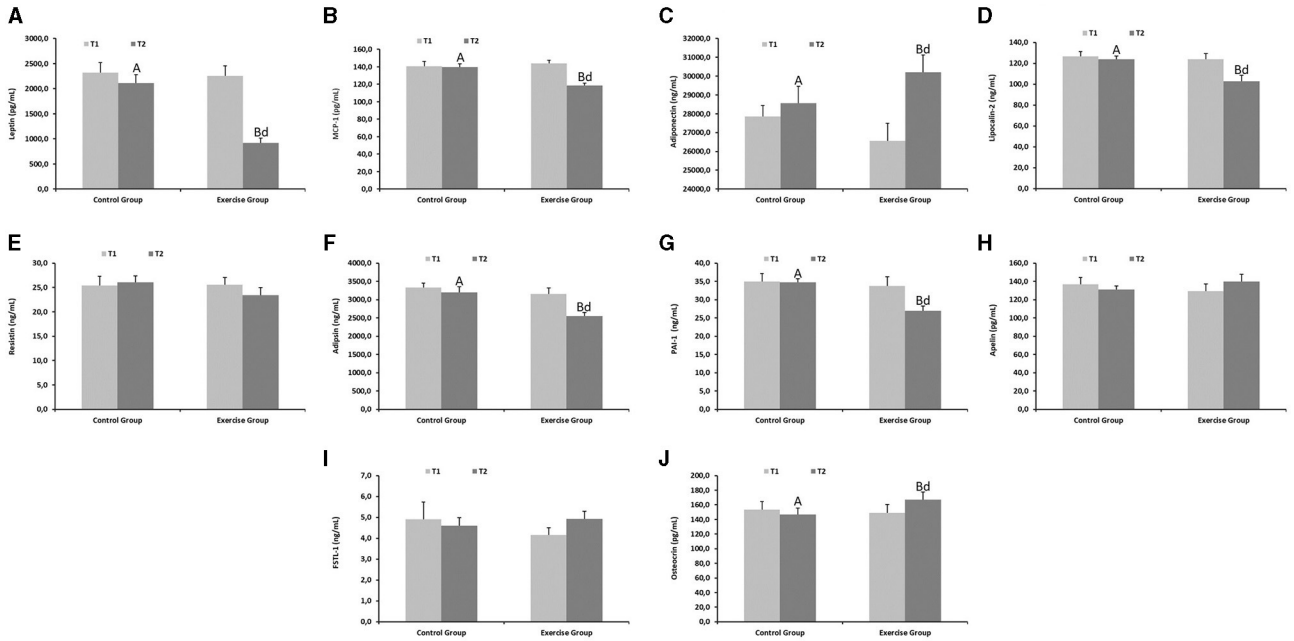

FIGURE 2 | Endocrine function parameters of the adipose tissue from the control and exercise groups. Mean values among groups with different letters differ ($P < 0.05$) (^{a,b}for T1 and ^{A,B}for T2 by Student's *t* test between different groups; ^{d}for T1 *vs.* T2 in the same group by Tukey's test). **(A)** Leptin, **(B)** MCP-1, **(C)** Adiponectin, **(D)** Lipocalin-2, **(E)** Resistin, **(F)** Adipsin, **(G)** PAI-1, **(H)** Apelin, **(I)** FSTL-1, **(J)** Osteocrin. MCP-1, Monocyte Chemoattractant Protein-1; PAI-1, Plasminogen activator inhibitor-1; FSLT-1, Follistatin-related protein 1.

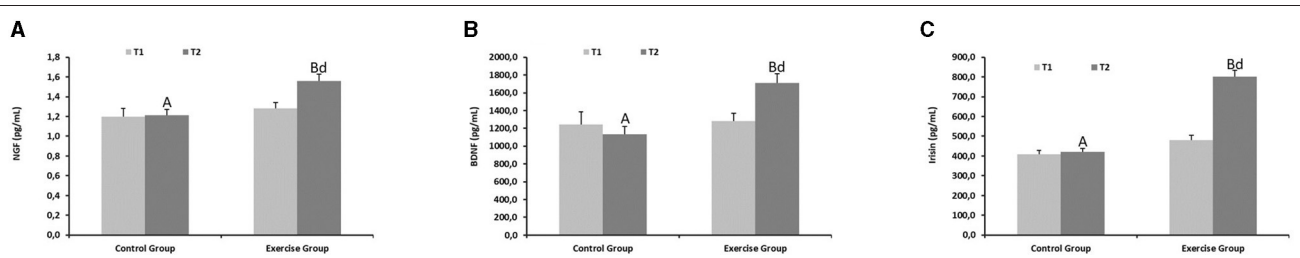

FIGURE 3 | Cognitive function parameters from the control and exercise groups. Mean values among groups with different letters differ ($P < 0.05$) (^{a,b}for T1 and ^{A,B}for T2 by Student's *t* test between different groups; ^{d}for T1 *vs.* T2 in the same group by Tukey's test). NGF, Nerve growth factor; BDNF, Brain-derived neurotrophic factor. **(A)** NGF, **(B)** BDNF, **(C)** Irisin.

TABLE 6 | Academic qualifications from the control and exercise groups at the end of the semester.

	Control group	Exercise group
Maths	6.76 ± 0.42^{a}	7.85 ± 0.55^{b}
Arts	7.57 ± 0.45	7.26 ± 0.47
Language	7.38 ± 0.41	7.58 ± 0.49
Natural sciences	7.53 ± 0.51	7.66 ± 0.52
Social sciences	7.00 ± 0.65	7.08 ± 0.66
Physical education	7.61 ± 0.36^{a}	8.58 ± 0.28^{b}

Mean value ± SEM for ten rats per group.
^{a,b}Mean values among groups with different letters differ ($P < 0.05$) (by Student's t test).

synaptic plasticity, which regulates brain plasticity by interacting with the synaptic network aggregates of the extracellular matrix (61). Zn is necessary for normal brain development and plays an essential role in neuronal migration, neurite formation and synapse formation (62). Although limited, there is some evidence that Zn integration provides better attention and abstract reasoning (56, 63). Zn deficiency is associated with increased rates of neurodevelopmental disorders and attention deficit hyperactivity disorder (54). Dietary zinc, copper, and selenium intake is inversely associated with low cognitive performance in an L-shaped dose–response relationship (64), therefore the higher intake of these minerals in the exercise group might help to the better cognitive performance of the children.

With regard to the hematological parameters, the most outstanding result recorded in the current study was a slight leukocytosis in the exercise group after the physical activity intervention, which can be explained because of the catecholamine-induced demarginating of white blood cells and

increased release from the bone marrow storage reserve during exercise (65). AP increased in both groups after the 6 months follow-up, reflecting the rapid growing peak of the children and increased bone metabolism (66). In addition, AP also increased in the exercise group compared to the control group, because AP is involved in ATP metabolism during exercise, by the participation in amino acid catabolism and the fat hydrolysis trough cell membranes (67).

Previous research has established cross-sectional links between adipose tissue and cognition in preadolescent children, although many of these have been conducted in obese or overweight subjects (68–72), without elucidating the role of the endocrine function of the adipose tissue and the molecular mechanisms involved in brain functions. Our study extends this knowledge by investigating the role of adipokines and the relationship with some molecular function biomarkers in normal weight schoolchildren to establish a relationship between changes in adiposity, physical activity and cognitive function after a physical activity program.

Adiponectin is a potent anti-inflammatory adipokine which also regulates insulin homeostasis, reducing the risk of many chronic diseases, including atherosclerosis, hypertension, nonalcoholic fatty liver, metabolic syndrome, cardiovascular disease, thrombosis, and asthma (14). Leptin reduces food intake, increases energy expenditure and its levels are inversely correlated with adipose tissue (15). In the present study, we have found a reduction in leptin and an increase in adiponectin after the exercise protocol, which is consistent with previous studies of physical activity in children (73). The study conducted by Many et al. (74) reported a decrease in leptin values after physical exercise, although 55% of the subjects continued to have high values when compared with the control subjects, which is explained because in this study they included obese and overweight children. In our study, no weight reduction was found, but a marked decrease in fat mass, which may explain the reduction in leptin and the increase in adiponectin compared to the control group at the end of the exercise protocol, reducing the risk of inflammation and insulin resistance (73, 75, 76). In the current study, the group that performed physical activity featured an improvement in the lipid profile, being the decrease in triglycerides and the increase in HDL-cholesterol statistically significant, which is in agreement with a previous study carried out in children performing aerobic exercise for 8 months (77).

Accumulating evidence supports the belief that exercise can reduce chronic inflammatory response, thus preventing the development of chronic diseases (78). Therefore, exercise may protect nervous system via its anti-inflammatory effects, which is one of the main reasons for the improvement of cognitive function (79). Adipose tissue endocrine function changed profoundly in the exercise group. In our study a lower level of MCP-1 was found in the exercise group, which may explain, at least in part, the reason for their improvement in brain molecular functions. PAI-1regulates several processes, including cell death, senescence, inflammation and it has been identified as key biomarker of several pathological conditions (16). Control of PAI-1 levels and several studies indicate that PAI-1 can be controlled with exercise (80–82), as we have recorded in the current study. Lipocalin-2 is as a pleiotropic molecule involved in a variety of physiological and pathological processes, such as metabolic homeostasis, apoptosis, infection, immune response, or inflammation (83). As previously reported (84), the reduction in lipocalin-2 due to the exercise is linked with other phenomena, like reduction of inflammatory markers, as we also recorded in the current study in the exercise group. Adipsin is an adipokine which links the immune function and the adipose tissue endocrine function, through the alternative pathway of complement activation (17). Our results are in agreement with previous results, indicating that exercise decreases adipsin levels (85). Osteocrin is a molecule released when performing physical activity which improves neuronal function, bone development, and physical endurance (86), prevents inflammatory signaling, cardiac rupture, and heart failure (87). In our study, osteocrin was also increased in the exercise group revealing the intensity of the exercise protocol and additional benefits of the physical activity in the children.

With regard to the brain molecular functions studied, NGF is highly expressed in developing hippocampus and cerebral cortex (88), enhances cholinergic neurons differentiation and survival (24), acetylcholine biosynthesis as well as activity and expression of cholinergic markers, improving overall cognitive function (25). As previously reported, exercise increases NGF production (89), results in agreement with those obtained in the current study. One of the most outstanding results is the increase in the BDNF levels in the exercise group. BDNF is a neurotrophin with a key role on brain development, neuron proliferation and survival, and for cognitive functions such as learning and memory (26, 27). During exercise, skeletal muscle increases expression of hippocampal BDNF (28). In particular, muscle contraction cleaves type III domain-containing protein 5, a protein secreted from the sarcolemma into the bloodstream as irisin. Then, irisin stimulates BDNF expression in the hippocampus and regulates the process of neuronal differentiation and maturation (28). Increased levels of BDNF in the exercise group is an essential mechanism explaining the exercise-induced brain plasticity and cognitive functions enhancement (90–93), revealing a better healthy brain state and a good predisposition for an improvement in cognitive function, which may be influenced by multiple factors, so that it should be confirmed in further studies with longer duration and sample size, as well as together with other biomarkers of cognitive function and some test that allow to better assesses the improvement in the cognitive development.

Our study has also shown differences in the academic performance of children, assessed as part of the academic regular evaluations (papers, exams and assessments performed by the respective teachers of each subject) during the academic course in the school, that could be due to physical activity. These results are in agreement with those obtained previously, in which increased lean mass, decreased fat mass and the amount of weekly physical activity are related to greater cognitive competence in both children and adolescents, especially in Maths (94–98). In this sense, practicing daily physical exercise of moderate intensity with a duration of between 10 and 50 min (98), would be enough to increase academic performance and cognitive performance (94, 99). Taking into account the

positive effects of NGF, BDNF, and irisin on the brain and their functional implications, it is really interesting to promote exercise protocols for maximizing circulating levels of these neurotrophins during the school age, which would have a positive impact on academic performance. Taken together, all the findings reported in the current study are consistent with many benefits of the exercise protocol on adipose tissue and brain molecular function.

This study features some strengths and limitations that should be taken into account. Regarding to the strengths, this study proposes the development of a new physical activity protocol. The physical activity protocol was designed to maximize the benefits of training and minimize the risk of injury, being low cost and really easy to implement with scarce equipment and facilities. By involving teachers in the design and implementation of the protocol, enables them to acquire the knowledge and skills to implement this protocol for other students and groups, facilitating additional activities development which will contribute to promote physical activity at school. Moreover, to our knowledge, this is the first time, that the relationship between adipose tissue endocrine function and cognitive function in physically active school children has been assessed. Finally, this exercise protocol could help to improve the perception of the importance of a healthy lifestyle at a stage of life in which habits are created that last over time. Under the heading of limitations, the following should be noted; the sample size could be larger as well as the duration of the study should be prolonged to a full academic year and not just one semester, in order to observe adipose tissue endocrine and cognitive functions changes in the long term. In addition, the study could have included another sub-population made up of girls to see the influence of physical activity on this group, which is more reluctant to do it, and more cognitive parameters and some test that assesses performance and IQ before and after the physical exercise protocol, should have been measured.

CONCLUSIONS

Overall, the findings indicate a marked increase in physical activity in the intervention group, which is particularly interesting as this is an age when physical activity participation tend to decrease dramatically. At the end of the physical activity intervention, the children were also more concerned about their diet, and due to the higher adherence to the Mediterranean Diet, the exercise group consumed more vitamin B complex, vitamin D, folate, Cu, Zn, Se, and Fe which might help to improve cognitive performance of the children. In our study, no weight reduction was found, but improved BMI and z-score, increasing lean mass and reducing fat mass, which may explain the reduction in leptin and the increase in adiponectin compared to the control group at the end of the exercise protocol, reducing the risk of inflammation and insulin resistance, together with an improvement in the lipid profile. The exercise protocol also reduced inflammatory response, which could also contribute to the improvement of the healthy brain state. Finally, exercise increased NGF, BDNF, and irisin, and taking into account their roles on the brain and their functional implications, it is really interesting to promote exercise protocols for maximizing circulating levels of these neurotrophins during the school age, which would have a positive impact on academic performance. Taken together, all the findings reported in the current study are consistent with many benefits of the exercise protocol on adipose tissue and brain molecular function, demonstrating the usefulness of early interventions based on physical activity in children to reduce risk factors related to sedentary lifestyle.

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 human participants were reviewed and approved by Ethics Committee of Andalusia Biomedical Research Ethics Portal (ref. 29/01/2018/2/2018). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JD-C, JM-F, and JG-V performed the experiments, wrote, and prepared the original draft. JO analyzed and summarized data. MP-J and JT contributed to the data acquisition and critically reviewed the manuscript. JD-C and JM-F supervised project administration, provided resources, funding, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Dynamic Associations of Milk Components With the Infant Gut Microbiome and Fecal Metabolites in a Mother–Infant Model by Microbiome, NMR Metabolomic, and Time-Series Clustering Analyses

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Background: The gut microbiome and fecal metabolites of breastfed infants changes during lactation, and are influenced by breast milk components. This study aimed to investigate dynamic associations of milk components with the infant gut microbiome and fecal metabolites throughout the lactation period in a mother–infant model.

Methods: One month after delivery, breast milk and subsequent infant feces were collected in a pair for 5 months from a mother and an exclusively breastfed infant. Composition of the fecal microbiome was determined with 16S rRNA sequencing. Low-molecular-weight metabolites, including human milk oligosaccharides (HMOs), and antibacterial proteins were measured in feces and milk using ^{1}H NMR metabolomics and enzyme-linked immunosorbent assays. The association of milk bioactive components with the infant gut microbiome and fecal metabolites was determined with Python clustering and correlation analyses.

Results: The HMOs in milk did not fluctuate throughout the lactation period. However, they began to disappear in infant feces at the beginning of month 4. Notably, at this time-point, a bifidobacterium species switching (from *B. breve* to *B. longum* subsp. *infantis*) occurred, accompanied by fluctuations in several metabolites including acetate and butyrate in infant feces.

Conclusions: Milk bioactive components, such as HMOs, might play different roles in the exclusively breastfed infants depending on the lactation period.

Keywords: NMR metabolomics, time-series clustering analysis, breast milk, bioactive component, human milk oligosaccharide, infant gut microbiome, infant fecal metabolites

INTRODUCTION

The gut microbiome plays an important role in the normal growth and development of infants, and it is less diverse and biased toward pathogens in premature infants with necrotizing enterocolitis (1). Perturbation of the gut microbiome could enhance the inflammatory response and lead to the development of necrotizing enterocolitis in infants (2). In contrast, the gut microbiome of healthy infants delivered at full term has a relatively high proportion of the genus *Bifidobacterium*, which confers benefits, such as the prevention of bacterial infections and the maturation of immune functions, to infants (3). In addition to *Bifidobacterium*, anaerobic bacteria such as *Bacteroides* and *Clostridium* have been identified as colonizers during the first 6 months, and are known to have various effects on the development and maturation of infants (4). Generally, bacterial families in exclusively breastfed infants have a higher occupancy of *Bifidobacteriaceae* and a lower occupancy of *Enterococcaceae* and *Enterobacteriaceae* than those in formula-fed infants (5). The infant gut microbiome changes during lactation, and the contents of bacterial genera such as *Bifidobacterium* and *Lactobacillus* fluctuate dramatically even in exclusively breastfed infants (6, 7). Furthermore, the dominant bacterial species are entirely different at different stages of lactation in breastfed infants (8, 9). This suggests that occupancy of the infant gut microbiome continues to change as the abundance of each bacterial species increases or decreases individually during the lactation period. These changes in the gut microbiome could be influenced by the maturation of gastrointestinal function in infants and by the composition of the breast milk ingested by infants. However, because previous studies were based on monthly sampling (6–9), the detailed time-course changes in the gut microbiome, which could be affected by the components in the ingested breast milk, remain unclear.

Breastfeeding is recommended by the World Health Organization and numerous pediatric associations as the best way of feeding infants during at least 6 months of life; breastfeeding is extremely beneficial for the health and development of infants (10). In fact, breast milk, a dynamic biofluid, is recognized as the only food able to meet all of a newborn's nutritional needs. Moreover, it includes various bioactive compounds, such as immunoglobulins (Igs), cytokines, and growth factors, important to prevent infection and promote the development of organs and systems (e.g., the immune system) (11, 12). Additionally, low-molecular-weight metabolites, up to a molecular size of ∼1500 Da, are also contained in breast milk. Recent studies have reported that breast milk components are affected by several factors, including diet, genetic background, lifestyle, and body mass index of the mother, among which the lactation stage has been reported to be the most influential (13, 14). Some of fluctuating milk components are bioactive and affect the gut microbiome of infants. For example, human milk oligosaccharides (HMOs) are unconjugated glycans with a lactose core varying in chain length from 3 to 15 carbohydrates and are assimilated by specific bacteria (15). Fucosylated lactoses such as 2′-fucosyllactose (2′-FL) and 3-FL, sialylated lactoses such as 3′-sialyllactose (3′-SL) and 6′-SL, and oligosaccharides with lacto-*N*-biose structure such as lacto-*N*-tetraose (LNT) and lacto-*N*-fucopentaose (LNFP) I are abundant HMOs in breast milk. These HMOs might play essential roles in supporting the bifidobacteria-predominant gut microbiome in breastfed infants (16). Furthermore, breast milk contains antibacterial proteins such as IgA, lactoferrin (LF), and lysozyme (LYZ), which can contribute to establishing the gut microbiome of infants fed breast milk (17). The gut microbiome modified by bioactive milk components confers various benefits to infants via its metabolites. In fact, a study using an animal model has reported that bifidobacteria could inhibit *Escherichia coli* infection via the production of acetate and the improvement of the intestinal barrier, due to the use of indigestible oligosaccharides (18). In addition, short-chain fatty acids (SCFAs) produced by the gut microbiome have been reported to increase resistance to inflammation and promote sympathetic nervous system maturation (19). However, the relationship between bioactive milk components, such as HMOs and antibacterial proteins, and the gut microbiome and fecal metabolites of breastfed infants remains unclear. This study aimed to clarify whether the infant gut microbiome and fecal metabolites are associated with milk components throughout the lactation period in a mother–infant model using microbiome analysis, NMR metabolomics, time-series clustering, and Spearman correlation analyses. In this study, biological samples were collected approximately every 4–6 days from one mother and an exclusively breastfed infant. Although this study is based on one mother–infant model and is preliminary, it is the first to examine changes in the infant gut microbiome and fecal metabolites weekly over the entire lactation period. Although there exists an omics study of breast milk and infant feces in one mother-infant pair, they have confirmed the influence of complementary foods during the weaning period (20). To our knowledge, there are no studies that have followed the detailed time-course changes in breast milk and an exclusively breastfed infant's feces during the lactation period. Components in breast milk collected weekly to correspond with the sampling time of infant feces were also analyzed. By analyzing these cross-data obtained frequently, dynamic associations between breast milk and infant feces could be determined, and a hypothesis to be confirmed in a large-scale clinical trial would be proposed. We believe that establishing interactions between milk components and fecal metabolites in the gut microbiome will facilitate a better understanding of how breastfeeding affects infant development, as well as the design of nutritional and functional foods for infants at various stages of lactation.

Abbreviations: FL, Fucosyllactose; HMO, Human milk oligosaccharide; Ig, Immunoglobulin; LF, Lactoferrin; LNFP, Lacto-*N*-fucopentaose; LNT, Lacto-*N*-tetraose; LYZ, Lysozyme; OPLS-DA, Orthogonal partial least squares discriminant analysis; OTUs, Operational taxonomic units; PC, Principal components; PCA, Principal component analysis; SCFA, Short-chain fatty acids; SL, Sialyllactose; TSP, 3-(Trimethylsilyl)propionic acid-d4 sodium salt.

MATERIALS AND METHODS

Subject and Sample Collection

The institutional review board at the Japan Conference of Clinical Research approved this study (approval number: BONYU-01),

which proceeded in accordance with the latest Declaration of Helsinki in 2013. The healthy mother of a new-born infant provided written, informed consent for the participation of herself and her infant in all procedures associated with the study. One month after birth, mature breast milk samples were manually by hand-stimulation of the breast collected from one breastfeeding mother once every 4–6 days for 5 months. Breast milk sampling intervals varied between months: 9 times during Month 1 (Day 34–59), 8 times during Month 2 (Day 61–82), 7 times during Month 3 (Day 99–118), 8 times during Month 4 (Day 122–148), and 6 times during Month 5 (Day 155–163). Then, whenever the infant of the mother was breastfed, the first excreted feces sample was collected. This covered the whole lactation period until just before complementary food was introduced. Of note, the infant was exclusively breastfed during the experimental period. The collected samples were temporarily stored at −20°C until 1 month and were subsequently transferred to −80°C storage conditions until analysis.

DNA Extraction

Total DNA was extracted from the feces samples, as described previously (21). Briefly, 200 μL of fecal sample in GuSCN solution was lysed with glass beads (300 mg, 0.1 mm diameter) and 300 μL of lysis buffer (No. 10 buffer, Kurabo Industries Ltd., Osaka, Japan) with a FastPrep-245G (MP Biomedicals LLC, Santa Ana, CA, USA) at a 5 power level for 45 s with 5 min cooling intervals on ice. After centrifugation at 12,000 rpm for 5 min, DNA was extracted from 200 μL of the supernatant using the GENE PREP STAR PI-480 (Kurabo Industries Ltd.) following the manufacturer's protocol.

Microbiome Analysis

After DNA extraction, PCR amplification and DNA sequencing of the V3-V4 region of the bacterial 16S rRNA gene was performed in an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) as described previously (21).

Real-Time PCR

Real-time PCR was performed using an ABI PRISM 7500 fast real- time PCR system (Thermo Fisher Scientific, MA, USA) with TB Green® Premix Ex Taq™ Tli RNaseH Plus (TaKaRa Bio Inc., Kusatsu, Japan) following the manufacturer's protocol. The primers used to amplify the 16S rRNA sequences from *B. breve* were F: 5′-CCGGATGCTCCATCACAC-3′ and R: 5′-ACAAAGTGCCTTGCTCCCT-3′; those used to amplify the 16S rRNA sequences from *B. longum* subsp. *infantis* were F: 5′-GACGAGGAGGAATACAGCAG-3′ and R: 5′-CACGAACAGCGAATCATGGATT-3′; those used to amplify the 16S rRNA sequences from *B. longum* subsp. *longum* were F: 5′-TTCCAGTTGATCGCATGGTC-3′ and R: 5′-GGGAAGCCGTATCTCTACGA-3′. Primers and amplification methods were determined based on previous reports (22, 23). For the determination of bacterial counts, bacterial solutions with already known their counts were used as a standard. All real-time PCR samples were assessed in duplicate.

Measurement of Macronutrients in Breast Milk

The concentration of fat, protein, carbohydrate, and total solid contents in homogenized human milk samples (3 mL) were measured using the Human Milk Analyzer (MIRIS AB, Uppsala, Sweden) via a medium infrared transmission spectroscopy technique. Before analysis, all milk samples were thawed and homogenized using the ultrasonic MIRIS sonicator (MIRIS AB) and were maintained at 40°C prior to measurements.

Measurement of Antibacterial Proteins in Breast Milk and Infant Feces

The total IgA, LF, and LYZ levels in breast milk and infant feces were measured using a human enzyme-linked immunosorbent assays (ELISA) kit (Abcam, Cambridge, UK) according to manufacturer's protocols. Briefly, extracts of infant feces were prepared for ELISA using the same method as the preparation for NMR analysis. All samples were diluted to meet the assay range of the ELISA kits. For IgA ELISA, breast milk samples were diluted to 1×10^5, and infant fecal samples were diluted to 2×10^4. For LF ELISA, breast milk samples were diluted to 2×10^6, and infant fecal samples were diluted to 1×10^5. For LYZ ELISA, breast milk samples were diluted to 5×10^5, and infant fecal samples were diluted to 1×10^2. All assays were performed in duplicate.

Sample Preparation for NMR Analysis

Breast milk samples (1 mL) were centrifuged at 3000 × g for 30 min at 4°C, and the aqueous layer was carefully removed and filtered through a 5 kDa cut-off filter (UltrafreeMC-PLHCC filter; HMT, Tsuruoka, Japan) at 9000 × g at 4°C to remove lipids and proteins. The filtrate (540 μL) was mixed with 60 μL 100% D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) containing 500 mM NaP, 5 mM 3-(trimethylsilyl)propionic acid-d4 sodium salt (TSP), and 0.04% (w/v) NaN_3, and 550 μL of the mixture was transferred to a 5 mm NMR tube (Shigemi, Hachioji, Japan). Fecal extracts were prepared for NMR analysis as follows: fecal samples were mixed with MilliQ water (Millipore, Billerica, MA, USA) containing 50 mM NaP, 0.5 mM TSP, 0.004% (w/v) NaN_3, and 10% D_2O (per 100 mg of feces). This mixture was homogenized at 1800 rpm for 15 min at 4°C using a tube mixer (EYELA CM-1000; Wakenyaku, Kyoto, Japan). The shaken samples were then centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant pH was adjusted to 7.4 ± 0.1 by adding small amounts NaOH or HCl to minimize pH-based peak movement. The 550 μL aliquot was transferred to a 5 mm NMR tube.

NMR Spectra Acquisition and Spectral Data Processing

^{1}H NMR spectra were recorded using a Bruker 600 MHz AVANCE spectrometer (Bruker, Billerica, MA, USA) equipped with a cryoprobe at a proton frequency of 600.13 MHz with the sample temperature controlled at 298 K. The acquisition conditions and data processing procedures were the same as in a previous report (24). Briefly, ^{1}H NMR spectra were recorded using a water-suppressed standard one-dimensional NOESY1D pulse sequence. Each spectrum consisted of 32,768 data points

with a spectral width of 12 ppm. The acquisition time was 2.28 s, and the number of scans was 128. A water-suppressed pulse sequence was used to reduce the residual water signal at the water frequency with a recycle delay [D1 (Bruker notated)] of 2.72 s and a mixing time [D8 (Bruker notated)] of 0.10 s. A 90° pulse length was automatically calculated in the analysis of each sample. All raw spectra were manually corrected for phase and baseline distortions against TSP resonance at $\delta = 0.0$ ppm using the Delta 5.0.4 software (JEOL, Tokyo, Japan) and then analyzed. Spectral binning, multivariate analysis, or direct metabolite relative quantification was performed. The spectra were normalized to the peak area value of the internal standard TSP using the NMR Suite 7.5 Processor (Chenomx, Edmonton, Canada), and the normalized spectral data were further processed. Briefly, in the first round of processing, the 0.0–10.0 ppm chemical shift region was integrated into regions with a width of 0.04 ppm, while the spectral regions related to residual water area (4.68–5.08 ppm) were removed from the multivariate analysis to eliminate the effects of imperfect water suppression. In the second round of processing, metabolite assignment and quantification were determined using the 600 MHz library from Chenomx NMR Suite and previous reports as reference (25–28).

Statistical Analyses

The spectral data matrix was obtained using SIMCA-P 14.0 software (Umetrics, Umeå, Sweden). Concentrations of breast milk components and binned NMR spectra in infant feces during each lactation period were assessed using principal component analysis (PCA). PCA score plots were obtained to visualize the clustering pattern of the samples in the context of two principal components (PC1 and PC2), with each point denoting an individual sample. For microbiome data, weighted UniFrac distance metrics analysis was performed using operational taxonomic units (OTUs) for each sample. Following PCA, supervised classification of orthogonal partial least squares discriminant analysis (OPLS-DA) with group information was also performed to compare the differences between five groups. PCA and OPLS-DA were conducted according to the distance matrix using SIMCA-P 14.0 software. The α-diversity of the microbiome in feces at various lactation points was represented by the Shannon index. A time-series clustering analysis using Dynamic Time Warping in Python version 3.7.3 was performed to verify the similarity between bioactive milk components, the infant gut microbiome, and fecal metabolites in infant feces. After logarithmizing the numerical data, standardization was performed via Z-scoring with the mean and standard deviation set to 0 and 1, respectively. Furthermore, bioactive milk components, the infant gut microbiome, and fecal metabolites showing similar changes over the lactation period were categorized into the same group using the scientific computing package SciPy and the visualization package Seaborne (29). The association of bioactive milk components with the infant gut microbiome and fecal metabolites was explored using Spearman rank correlation coefficient analyses and heatmaps generated using SciPy and Seaborne.

RESULTS

Profiling of Microbiome and Metabolites in Infant Feces

The weighted UniFrac distances based on the OTUs for the microbiome in feces of the breastfed infant during each lactation period were visualized using PCA (**Figure 1A**) and OPLS-DA (**Supplementary Figure 1**). These score plots in months 1–2 and 3–5 postpartum formed clearly separate clusters, respectively. Changes in microbiome α-diversity in infant feces at each lactation point are represented by the Shannon index (**Figure 1B**). As the lactation period progressed, the diversity increased, especially after month 3. The transitions of 15 bacterial species with an average abundance of 0.5% or more during the lactation period are shown in **Figure 1C**. Furthermore, microbiome composition of breastfed infant feces at the 40 species-level at each lactation point is shown in **Supplementary Table 1**. In addition, quantitative PCR was performed to validate the presence of *Bifidobacterium* spp. that showed characteristic and dramatic changes in 16S RNA sequencing analysis (**Supplementary Figure 2**). The changes by quantitative PCR analysis of *B. breve*, *B. longum* subsp. *infantis*, and *B. longum* subsp. *longum* showed similar trends to those of 16S RNA sequencing analysis, which were supported by the results. Representative ^{1}H NMR spectra of infant feces samples at months 1 and 5 postpartum and their enlarged views are shown in **Supplementary Figure 3**. Low-molecular-weight compounds, such as monosaccharides, disaccharides, oligosaccharides, SCFAs, amino acids, and their metabolites were identified, and their chemical shifts are shown in **Supplementary Table 2**. Changes in the feces NMR spectra during each lactation period were visualized using PCA (**Figure 1D**). Because the score plots for month 3 were positioned in the middle of the two clusters, the score plots derived from the NMR spectra for feces did not form clearly separate clusters. OPLS-DA (**Figure 1E**) performed to further compare the differences between five groups showed that the score plots derived from the NMR spectra for feces in months 1–3 and 4–5 postpartum formed separate clusters. Furthermore, the relative concentrations of infant feces metabolites in months 1–5 postpartum are shown in **Supplementary Table 3**. Considering the direct supply of antibacterial proteins from breast milk, the concentrations of IgA, LF, and LYZ in infant feces were measured in months 1–5 postpartum (**Supplementary Table 3**).

Profiling of Components in Breast Milk

The representative ^{1}H NMR spectra of breast milk samples at months 1 and 5 postpartum and their enlarged views are shown in **Supplementary Figure 4**. Low-molecular-weight compounds such as monosaccharides, disaccharides, oligosaccharides, SCFAs, amino acids, and their metabolites were identified, and their chemical shifts are shown in **Supplementary Table 4**. **Supplementary Table 5** shows the concentrations of metabolites, macronutrients (fats, proteins, carbohydrates, and total solids), and antibacterial proteins (IgA, LF, and LYZ) in breast milk at months 1–5 postpartum. Changes in breast milk components during each lactation

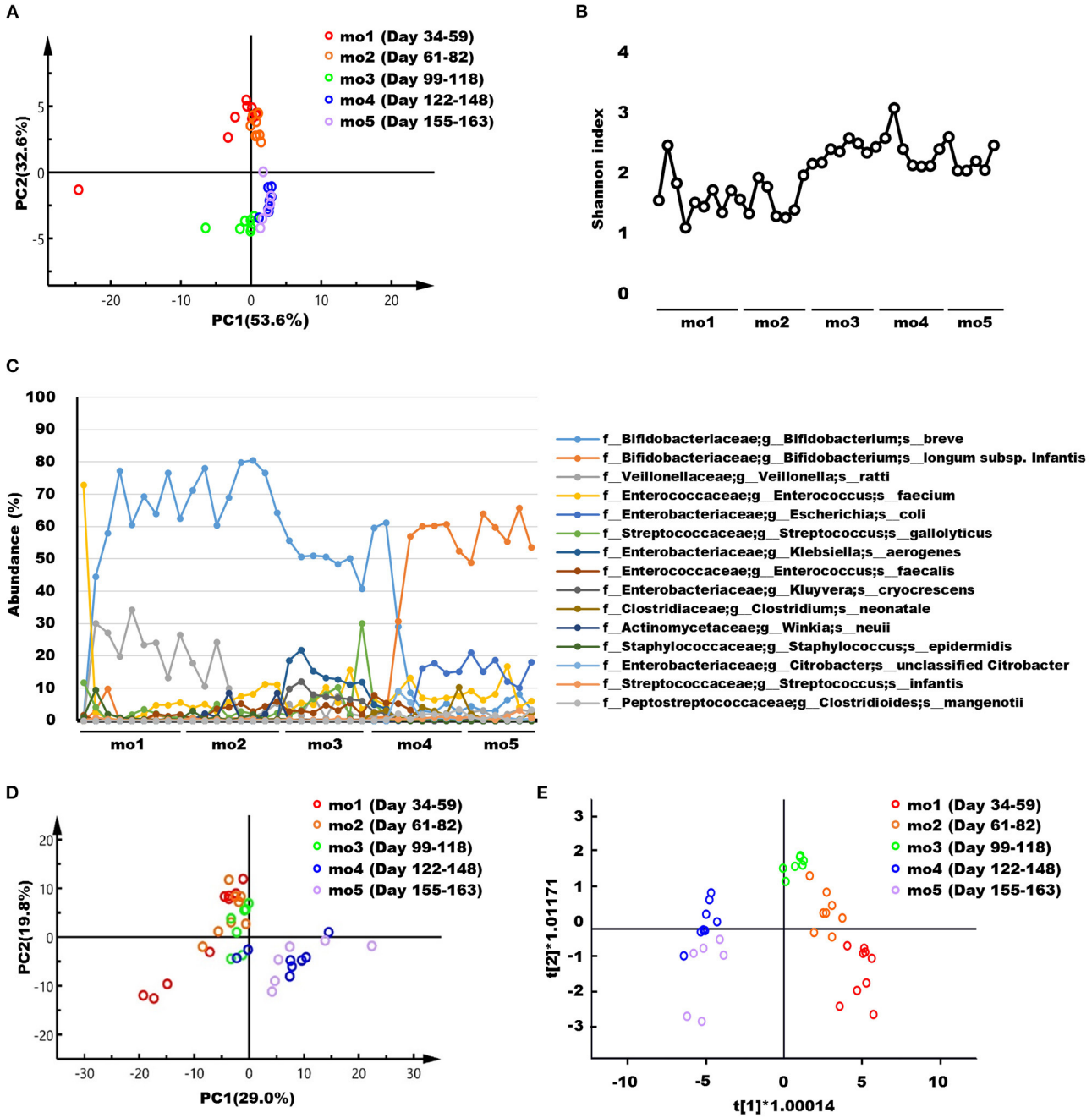

FIGURE 1 | (A) Principal component analysis (PCA) score plots based on the weighted UniFrac distance metric of the microbiome in infant feces samples collected at months 1–5 postpartum. Each symbol represents one individual feces sample. **(B)** Changes in the microbiome α-diversity, as assessed from the Shannon index, in infant feces samples collected at months 1–5 postpartum. **(C)** Transitions of 15 bacterial species with an average abundance of 0.5% or more during the lactation period. **(D)** PCA and **(E)** Orthogonal partial least squares discriminant analysis consisting of five groups score plots derived from the NMR spectra of infant feces samples collected at months 1–5 postpartum. R2X[1] = 0.568; R2X[2] = 0.048. Each symbol represents one individual feces sample. mo, month.

period were visualized using PCA (**Figure 2A**) and OPLS-DA (**Supplementary Figure 5**). These score plots derived from the milk components did not form clearly separate clusters. Furthermore, **Figure 2B** shows fluctuations in the levels of bioactive HMOs and IgA, LF, and LYZ in breast milk. These bioactive components did not also characteristically vary throughout lactation, as shown in **Figure 2B**.

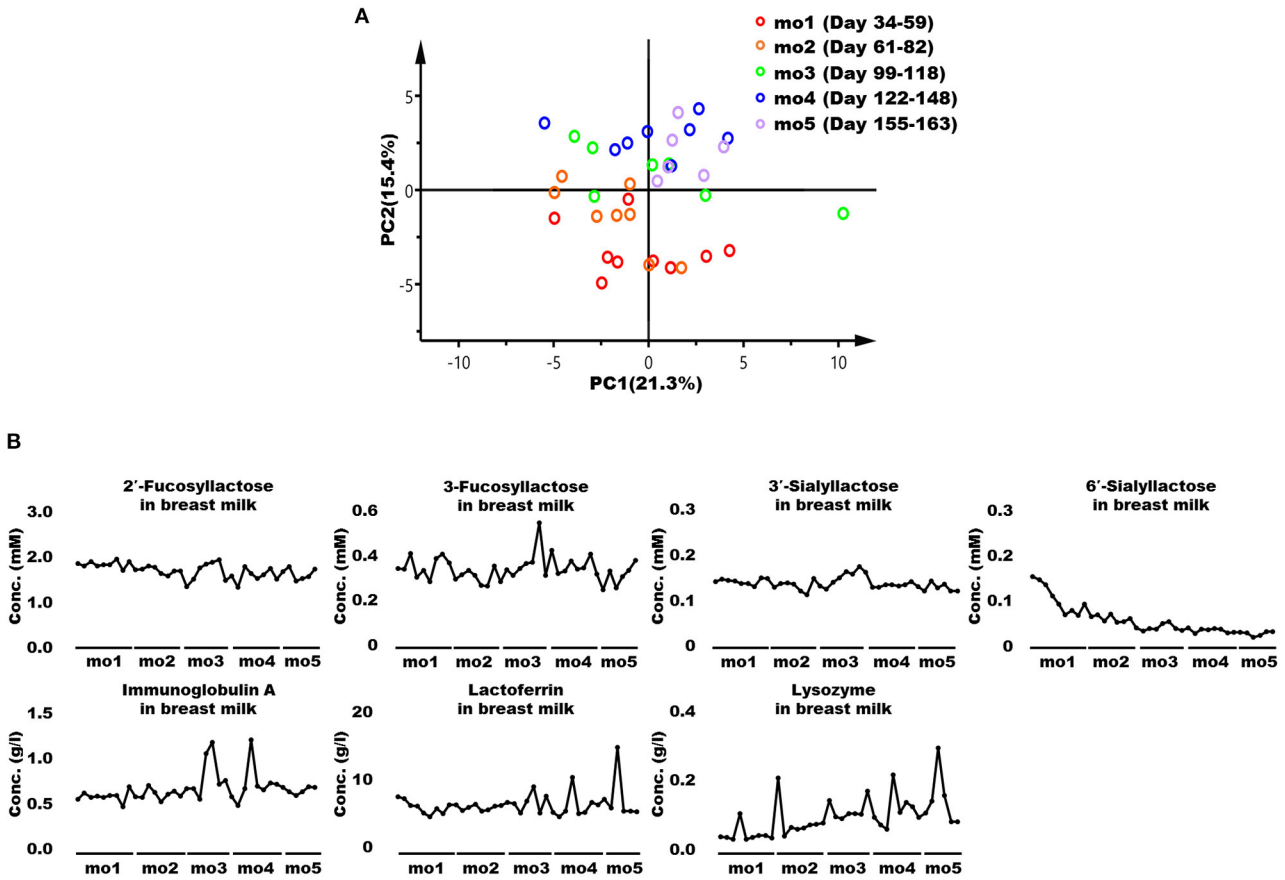

FIGURE 2 | Principal component analysis (PCA) and changes in the bioactive components of breast milk. **(A)** PCA score plots derived from components such as low-molecular-weight metabolites, major nutrients, and antibacterial proteins in breast milk collected at months 1–5 postpartum. Each symbol represents an individual breast milk sample. **(B)** Changes in levels in the human milk oligosaccharides (HMOs) immunoglobulin A (IgA), lactoferrin (LF), and lysozyme (LYZ) in breast milk. mo, month.

Clustering Analysis of Bioactive Milk Components, the Microbiome, and Metabolites in Infant Feces

When bioactive milk components act on the infant gut microbiome via breast milk, bacterial-derived metabolites in infant feces should also fluctuate in association with bacterial changes. Therefore, association of bioactive milk components with the microbiome (at the species level; average abundance > 0.5%) and fecal metabolites of the infant was assessed with time-series clustering analysis using Python software. Three major clusters were defined, showing similar fluctuations over time (**Figure 3**). Values for cluster A were high and low, whereas those for cluster B were low and high during early and late-lactation, respectively, and no characteristic tendency was noted for cluster C throughout lactation. Among compounds and bacterial species classified into the same cluster, those with a particularly high similarity were visually selected. **Figure 4A** shows fluctuations in the levels of four HMOs, antibacterial proteins, urea, and hypoxanthine in infant feces, and **Figure 4B** shows transitions in the abundance of *Bifidobacterium breve* and *Veillonella ratti* in infant feces categorized into cluster A. **Figure 5A** shows fluctuations in the levels of acetate, butyrate, 5-aminopentanoate, and fucose in infant feces categorized into cluster B. **Figure 5B** shows transitions in the abundance of *Bifidobacterium longum* subsp. *infantis*, *E. coli*, *Clostridium neonatale*, and *Clostridioides mangenotii* in infant feces categorized into cluster B.

Spearman Correlation Analysis of Bioactive Milk Components, the Infant Gut Microbiome, and Fecal Metabolites

We used Spearman rank correlation coefficients to extract factors with significant positive or negative correlations among bioactive milk components, bacterial species, and metabolites with high similarity in clusters A and B from infant feces. The results are shown, as heatmaps, for months 1–2 and 3–5 postpartum that were clearly distinguished in microbiome of infant feces by PCA and OPLS-DA (**Figures 6A,B**). Few correlations were identified between bioactive milk components and the microbiome and fecal metabolites in infant feces at months 1–2 postpartum. *Bifidobacterium breve* correlated positively with 3-FL and 3′-SL

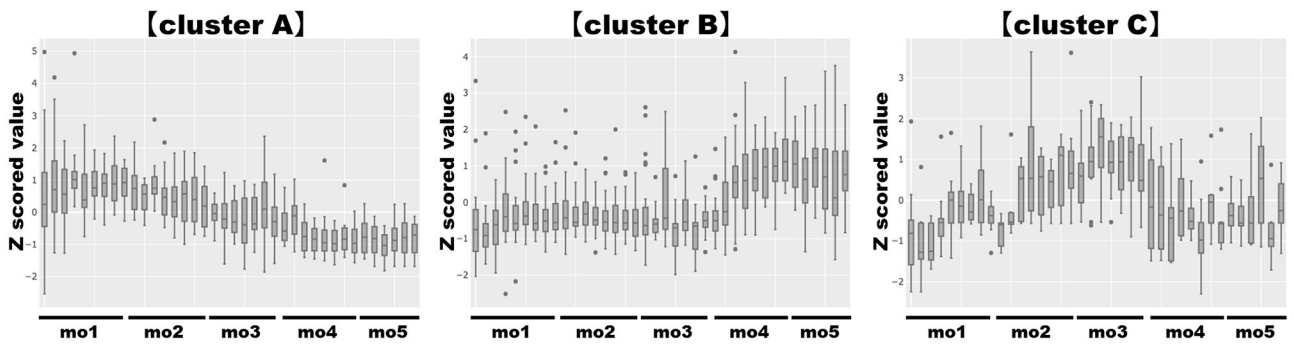

FIGURE 3 | Clustering analysis of bioactive milk components, the microbiome at the species level, and the metabolites in infant feces. Those with similar characteristics (similar fluctuation patterns) were categorized into three clusters, cluster A, cluster B, and cluster C. "Cluster A" showed high values in the early-lactation period and low values in the late-lactation period; "cluster B" showed low values in the early-lactation period and high values in the late-lactation period; "cluster C" did not show a characteristic tendency throughout the lactation period. Categorized factors: (cluster A) 2′-fucosyllactose, 3-fucosyllactose, 3′-sialyllactose, 6′-sialyllactose, formate, hypoxanthine, immunoglobulin A, lactoferrin, lactose, lysozyme, trimethylamine N-oxide, tryptophan, urea, *Bifidobacterium breve*, *Staphylococcus epidermidis*, *Veillonella ratti*. (cluster B) 5-Aminopentanoate, acetate, alanine, aspartate, butyrate, cadaverine, carnitine, choline, creatine, fucose, fumarate, glucose, lactate, o-phosphocholine, propionate, pyruvate, succinate, taurine, threonine, tyrosine, *Bifidobacterium longum* subsp. *infantis*, *Citrobacter* unclassified *Citrobacter*, *Clostridioides mangenotii*, *Clostridium neonatale*, *Enterococcus faecium*, *Escherichia coli*, *Streptococcus infantis*. (cluster C) Glutamate, glycine, isoleucine, leucine, methionine, phenylalanine, valine, *Enterococcus faecalis*, *Klebsiella aerogenes*, *Kluyvera cryocrescens*, *Streptococcus gallolyticus*, *Winkia neuii*. mo, month.

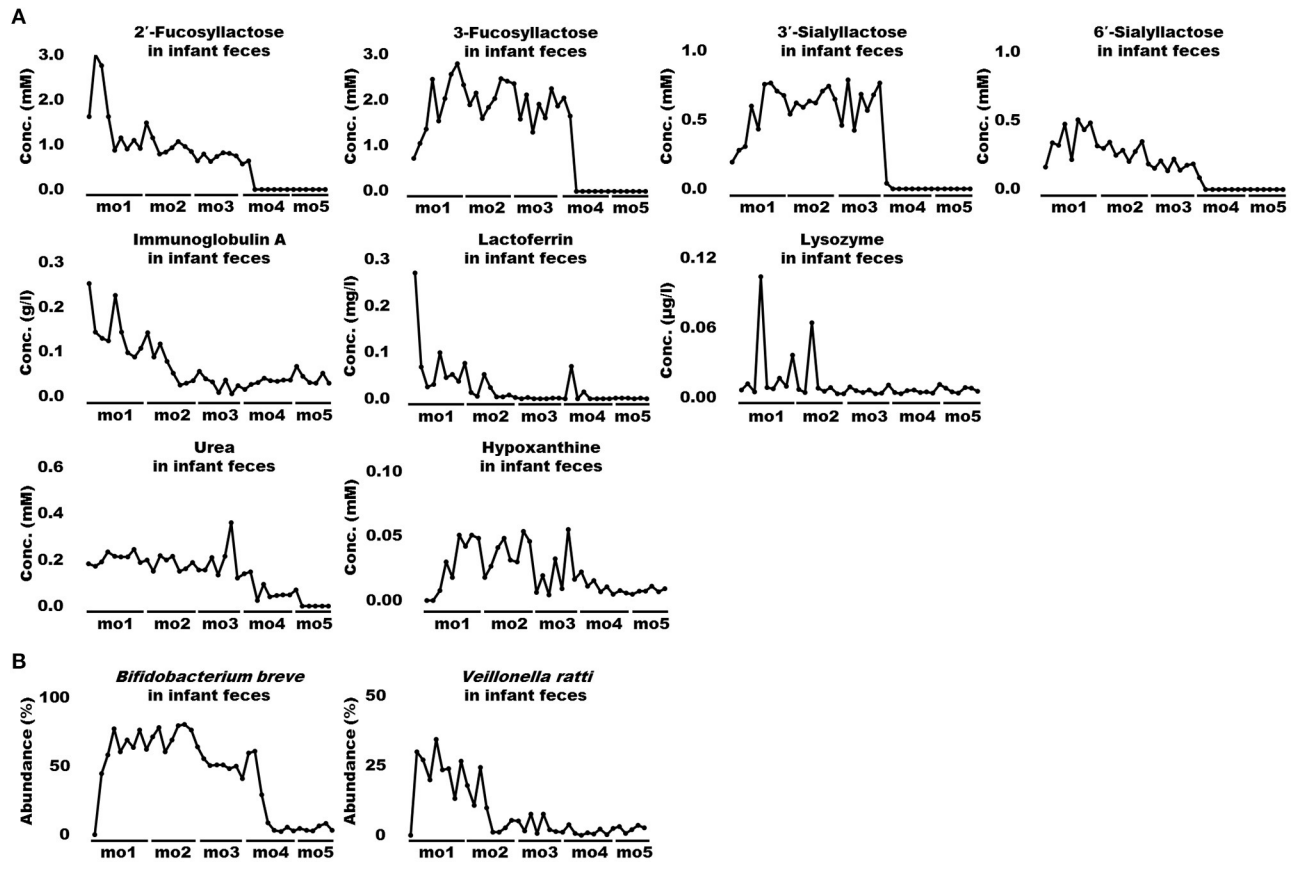

FIGURE 4 | Changes in bioactive milk components, microbiome, and metabolites in infant feces with high similarity in cluster A. **(A)** Human milk oligosaccharides (2′-fucosyllactose, 3-fucosyllactose, 3′-sialyllactose, and 6′-sialyllactose), antibacterial proteins (immunoglobulin A, lactoferrin, and lysozyme), urea, and hypoxanthine in infant feces. **(B)** *Bifidobacterium breve* and *Veillonella ratti*, in infant feces. mo, month.

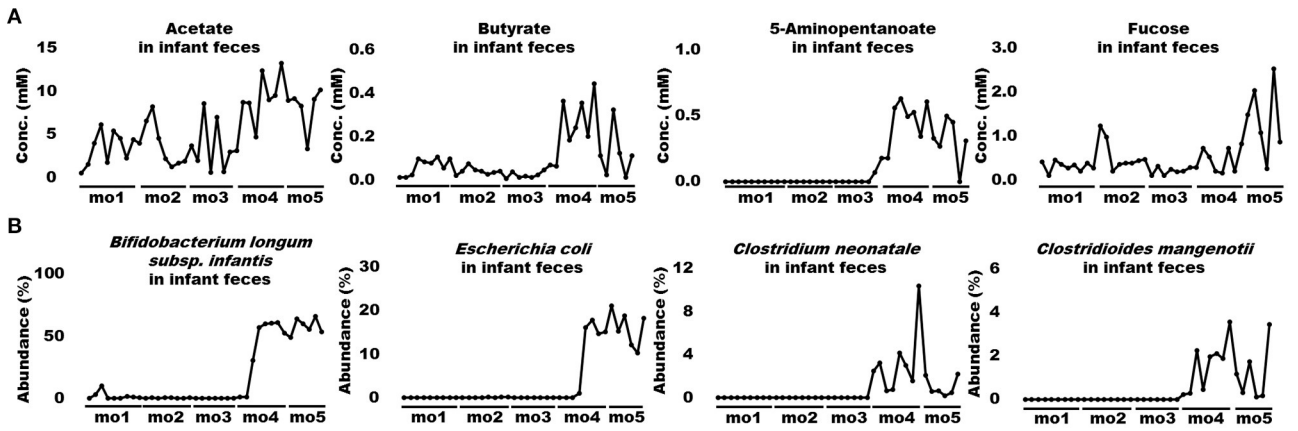

FIGURE 5 | Changes in bioactive milk components, the microbiome, and metabolites in infant feces with high similarity in cluster B. **(A)** Acetate, butyrate, 5-aminopentanoate, and fucose levels in infant feces. **(B)** *Bifidobacterium longum* subsp. *infantis*, *Escherichia coli*, *Clostridium neonatale*, and *Clostridioides mangenotii* in infant feces. mo, month.

($\rho = +0.79$ and $+0.76$, respectively) and negatively correlated with IgA and LF ($\rho = -0.72$ and -0.92, respectively). Correlations were much closer at months 3–5 than at months 1–2. *Bifidobacterium breve* correlated significantly and negatively with *B. longum* subsp. *infantis* and *E. coli* in infant feces at months 3–5 ($\rho = -0.97$ and -0.88, respectively). *Bifidobacterium longum* subsp. *infantis* also significantly correlated with *E. coli* ($\rho = +0.85$). Correlations ($\rho = +0.82$ to $+0.98$) were significantly positive among four HMOs in infant feces. These HMOs were significantly and positively correlated ($\rho = +0.69$ to $+0.94$) with *B. breve* in cluster A in infant feces. *Bifidobacterium longum* subsp. *infantis* and *E. coli* in cluster B in infant feces significantly and negatively correlated with HMOs ($\rho = -0.65$ to -0.96). No bacterial species significantly correlated with the antibacterial proteins IgA, LF, and LYZ, hypoxanthine, or fucose. Acetate and butyrate levels significantly and positively correlated with *C. mangenotii* ($\rho = +0.72$ and $+0.80$, respectively), and 5-aminopentanoate levels correlated positively with *B. longum* subsp. *infantis* and *C. mangenotii* ($\rho = +0.72$ and $+0.70$, respectively). Some metabolites, such as amino acids and other bacterial species, in infant feces were classified into cluster C, which had no characteristic tendency throughout lactation.

DISCUSSION

Breast milk is an ideal nutritional source for infants and contains various bioactive components such as HMOs and antibacterial proteins. However, relationships between milk components and the infant gut microbiome and fecal metabolites throughout lactation remain unclear. Our PCA and OPLS-DA findings revealed different clustering profiles in the infant gut microbiome between months 1–2 and 3–5 postpartum, and the diversity of the gut microbiome also increased 3 months onwards. On the other hand, the concentrations of breast milk components throughout lactation showed some variation but did not show a characteristic tendency. This indicated that the gut microbiome of the breastfed infant fluctuated after month 3 postpartum, even though milk components did not change. These changes in the infant intestinal tract might translate into optimization for the efficient digestion and absorption of breast milk. In fact, it has been reported that after the 3rd month of life, the functional maturation of the gastrointestinal tract progresses rapidly and the fecal properties change dramatically (30). In the OPLS-DA following PCA of the infant gut microbiome and fecal metabolites, score plots of the microbiome formed clusters in months 1–2 and 3–5 postpartum, whereas score plots of the fecal metabolites formed clusters in months 1–3 and 4–5 postpartum. The difference in cluster formation in the postnatal period suggested that the gut microbiome changed first, followed by its fecal metabolites. From a comprehensive point of view, the infant gut microbiota might first respond to changes in the infant's body with gastrointestinal maturation, followed by changes in fecal metabolites.

Interestingly, the concentrations of HMOs such as 2′-FL, 3-FL, 3′-SL, and 6′-SL in breast milk were almost constant, whereas in infant feces these HMOs were no longer detected after the beginning of month 4. Firstly, this change in infant feces suggests that the amounts of HMOs delivered into infant feces via breast milk changed between early and late lactation. The structural characteristics and prebiotic effects of HMOs assimilated by intestinal bacteria protect against pathogens (31). The effects of HMOs might change as infants grow. Secondly, the disappearance of HMOs in infant feces suggest that the infant's gut microbiome began to metabolize these HMOs from month 4 onwards. Importantly, Spearman analysis of infant feces at months 3–5 postpartum revealed significant positive correlations between HMOs and *B. breve*. In contrast, HMOs and *B. longum* subsp. *infantis* were significantly and negatively correlated. Spearman analysis also revealed a significantly negative correlation between *B. breve* and *B. longum* subsp. *infantis* in infant feces in 3–5 months. Several *in vitro* studies have reported that 2′-FL, 3-FL, 3′-SL, and 6′-SL are not metabolized

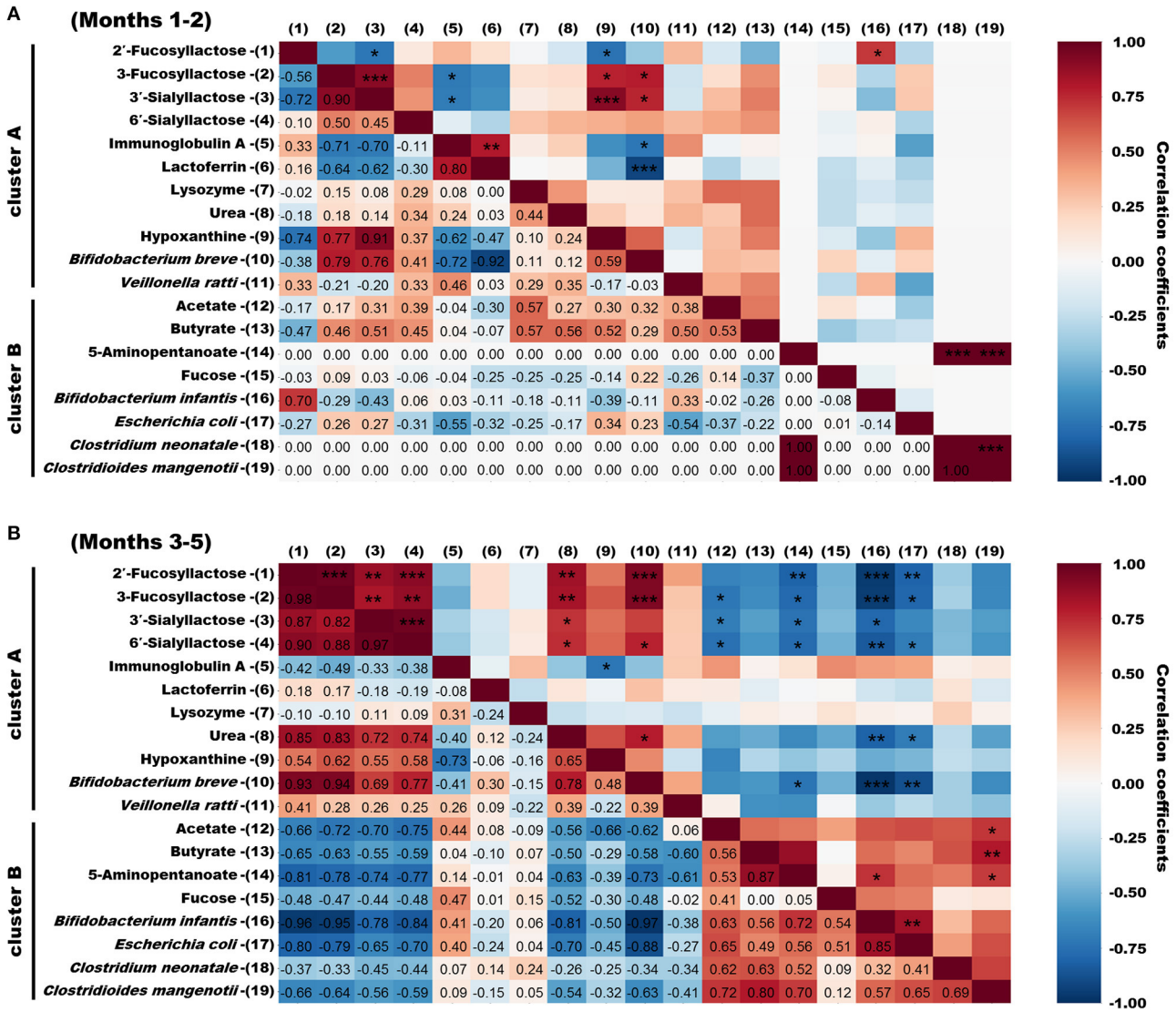

FIGURE 6 | Spearman correlation heatmaps of bioactive milk components, the infant gut microbiome, and fecal metabolites in infant feces. **(A)** Spearman rank correlation coefficients of bioactive milk components, bacterial species, and metabolites in infant feces at months 1–2 postpartum with high similarity categorized into clusters A and B. **(B)** Spearman rank correlation coefficients of bioactive milk components, bacterial species, and metabolites in infant feces at months 3–5 postpartum with high similarity categorized into clusters A and B. Spearman ρ values are shown in different colors and |ρ| values are shown as *0.7–0.8, **0.8–0.9, and *** >0.9. mo, month.

by *B. breve* but are metabolized by *B. longum* subsp. *infantis* due to the difference in the HMO capitalization mechanism. The fucosidase belonging to GH29, which hydrolyzes α-1,3-linked fucosyl lactose, is not present in most of *B. breve*, but only in *B. longum* subsp. *infantis* (32). Besides, *B. breve* and *B. longum* subsp. *infantis* have different structures of ATP-binding cassette transporter of FL with high frequency (33). These differences in enzyme and transporter might also be responsible for the rapid FL utilization of *B. longum* subsp. *infantis* after the beginning of month 4. Besides, it is possible that sialidases which were detected commonly in *B. longum* subsp. *infantis* and *B. bifidum* affect 3′-SL and 6′-SL utilization (34). Given the differences in these multiple HMO capitalization mechanisms, it is inferred that the dramatic decrease in HMOs in the infant feces was probably because of the "species switching" from *B. breve* to *B. longum* subsp. *infantis*. Notably, some strains of *B. breve* are known to metabolize 2′-FL and 3-FL (32). The *B. breve* detected in the infant feces of this study could have been a strain that does not metabolize 2′-FL and 3-FL. *Enterococcus faecalis*, categorized in cluster C, which did not exhibit a characteristic tendency throughout lactation, remained abundant during the switch from *B. breve* to *B. longum* subsp. *infantis*. Exopolysaccharides produced by *E. faecalis* promote the growth of *B. longum* rather than *B. breve* (35). Thus, the growth of *B. longum* could explain the switch from

B. breve to *B. longum* subsp. *infantis* found herein. Furthermore, *E. faecalis* modulates inflammation in the gastrointestinal tract during the first few years of life and affects the development of intestinal immunity associated with allergies (36, 37). These facts indicate that the increase in *E. faecalis* at 2–4 months indirectly and directly contributed to the development of the gastrointestinal tract of the infant studied herein. *Escherichia coli* was relatively significantly and negatively correlated with HMOs in infant feces. Fucosylated and sialylated glycans in breast milk can bind to *E. coli* and suppress infection (38, 39). Therefore, *E. coli* abundance in infant feces might have increased due to the depletion of HMOs. *Escherichia coli* produces acetate via acid fermentation under anaerobic conditions (40). Thus, elevated acetate levels in the infant feces might have been partly due to *E, coli*, even though the correlation coefficient between acetate and *E. coli* was <0.70 at months 3–5 postpartum. Furthermore, supplementing preterm infants with bifidobacteria increases fecal acetate levels via metabolized HMOs and consequently promotes the defense of epithelial and mucosal dendritic cells (41). The genus *Bifidobacterium* has a specific glycolytic system called the "bifid shunt" that efficiently produces lactate and acetate from glucose, a component of HMOs. Thus, the metabolism of HMOs by *B. longum* subsp. *infantis* might have partly contributed to the elevated acetate in infant feces later during lactation in the present study. Several previous studies have reported that the abundance of *B. longum* subsp. *infantis* and *E. coli* are negatively correlated in infants within the first month postpartum or preterm infants (42, 43), contrary to our results. These differences in correlation might be due to the different characteristics of the subjects participated in previous studies and our study. In future clinical trials, these relationships might become apparent by categorizing infants based on the presence ratio of *Bifidobacterium*/*Enterobacteriaceae* in the gut microbiome and following mother-infant pairs individually. Fecal butyrate significantly and positively correlated with *C. mangenotii* at 3–5 months. The results of PICRUSt analysis and an anaerobic culture system have shown that *C. mangenotii* is a potential butyrate producer (44). Therefore, the elevated butyrate levels in the infant feces are thought to be a result of increased *C. mangenotii* abundance during late lactation. Butyrate in the intestinal tract confers benefits, such as anti-inflammatory effects on epithelial cells, to hosts (45). Moreover, it contributes to maturation of the immune system by modulating the differentiation of regulatory T cells in the large intestine (46). However, there are some evidence that differentiation of regulatory T cells in adaptive immune programming is critical within 100 days of age, so the elevated butyrate in the late-lactation period might not be directly involved (47, 48). On the other hand, butyrate also has direct immune-activating functions, such as inhibiting the adhesion of pathogenic bacteria or improving the gastrointestinal barrier (49). Therefore, elevated butyrate levels during late lactation could help immune modulation in infants indirectly and directly. Breast milk contains acetate and butyrate, but they are rapidly absorbed in the upper gastrointestinal tract (50, 51). These metabolites in infant feces are probably produced via fermentation by the gut microbiome.

The changes of antibacterial proteins IgA, LF, and LYZ in breast milk were not characteristic, but those in infant feces were categorized into cluster A, and tended to be higher in month 1–2. These results suggest that antibacterial proteins in breast milk are not necessarily reflected by the content of the influx into the digestive tract of the infant. Secretory IgA is stable against proteolytic enzymes in infant's gastrointestinal tract, and there binds to bacterial and viral antigens, promoting inhibition of attachment to the mucosal lining (17). The presence of high levels of IgA in months1–2 in our study might contribute to the prevention of infection in the gastrointestinal tract of infants with immature mucosal defenses. Although LF and LYZ have their own anti-infection properties, digested forms exert stronger antibacterial activities than the full-length intact forms (52, 53). The disappearance of the LF and LYZ since month 3 in infant feces might mean that the digestion of these intact proteins started at month 3 postpartum, as the digestive capacity of the infant developed. Furthermore, LF in infant feces significantly and negatively correlated with *B. breve* at months 1–2 postpartum. Some peptides isolated from human milk exert strong bifidogenic effects on *B. bifidum*, *B. breve*, and *B. longum* and are resistant to digestive enzymes (54). Thus, digested LF in the gastrointestinal tract might promote the growth of *B. breve* at months 1–2 despite the immature digestive function of the infant. At months 3–5 postpartum, antibacterial proteins (IgA, LF, LYZ) in infant feces did not significantly correlate with any bacterial species detected in the present study. However, antibacterial proteins are supplied via breast milk to the digestive tract, where they modify the infant gut microbiome (55, 56). Further studies are needed to clarify the effects of antibacterial proteins in breast milk on the infant gut microbiome.

Urea and hypoxanthine are metabolites that are respectively generated by amino and nucleic acid metabolism; these were categorized into cluster A, which was respectively abundant and scant during early and late lactation. However, as the amounts of amino acids (or proteins) and nucleic acids required for a developing infant significantly change during lactation, the relationship between the gut microbiome and these nutritional components remains controversial (57). The genus Clostridium produces 5-aminopentanoate from proline (58). Therefore, the increase in 5-aminopentanoate found herein might be due to an increase in the abundance of *C. neonatale* despite the absence of a significant correlation with 5-aminopentanoate. As far as we can ascertain, the function of 5-aminopentanoate in infants is unknown. However, 5-aminopentanoate can be a biomarker of cerebral ischemia (59), and it might therefore play an important role in the development of brain function in infants. Spearman analysis did not identify significant correlations between fecal fucose and any factors. Since fucose is a component of fucosylated HMOs (16), it might have been produced in infant feces via the metabolism of 2′-FL and 3-FL. However, the physiological significance of fucose requires further investigation. In addition, no characteristic fluctuations were shown for metabolites and macronutrients such as fat, protein, and carbohydrate in breast milk in the current study. Since macronutrients have been reported to be directly associated with the gut microbiome of the infant and the modulation of the immune system (4),

further investigation of the nutrients supplied via breast milk is warranted.

The major limitation of this pilot study is that we used a model of only one mother–infant pair. However, this is the first study to examine dynamic associations of breast milk with breastfed infant feces on a weekly basis over the entire lactation period, which have only been studied monthly previously. Frequent sampling data monthly may be used as reference for future large-scale mother–infant studies. For example, given the possibility of dramatic changes in the gut microbiome of other infants during month 4 postpartum, it might be suggested that frequent sampling is necessary during this period. Although it is challenging to collect samples weekly in a clinical study, the dynamic associations between breast milk and infant feces determined in this study will need to be confirmed using a larger sample size. Further, we did not include colostrum and transitional milk secreted during the month 1 postpartum in our analysis (60). This exclusion might explain the limited fluctuations in breast milk components throughout lactation in the present study. However, colostrum and transitional milk contain higher amounts of functional components, such as antimicrobial proteins and immune modulators, than mature milk (61). The consumption of colostrum and transitional milk within month 1 postpartum may have a long-term effect on infant gut microbiome and fecal metabolites. Another limitation is that the involvement of HMOs such as LNT and LNFP I was not investigated. Indeed, these HMOs may be present in breast milk and infant feces, although they could not be detected because of the overlap of NMR spectra derived from compounds with close chemical shift values. Most of the genus *Bifidobacterium* has metabolic pathways and enzymes to utilize LNT and LNFP I, and can capitalize on these HMOs (62). The utilization of these HMOs by the gut microbiome might be involved in the "species switching" from *B. breve* to *B. longum* subsp. *infantis*. Furthemore, these HMOs are also known to be involved in the development and maturation of infants and may explain the fluctuating gut microbiome observed in this study. Therefore, further studies will be needed in the future to confirm the role of these HMOs (63). Finally, another limitation of this study is that the amount of breast milk consumed by the infant was not measured. If the breastfeeding amount were accurately measured, it would be possible to estimate how much HMOs in breast milk are supplied to the infant and utilized in the infant's gut microbiome. In future clinical trials, data on the amount of breast feeding will be needed to verify in detail the dramatic changes in HMOs observed in this study.

In conclusion, microbiome analysis, NMR metabolomics, and Python's clustering and correlation analyses showed that the gut microbiome and fecal metabolites of the infant were dynamically associated with bioactive milk components during a certain lactation period. In particular, HMOs in the breast milk did not fluctuate throughout the lactation period but disappeared from the infant feces after the 4th lactation month. Moreover, at the same time species switching from *B. breve* to *B. longum* subsp. *infantis*, *E. coli*, and *C. mangenotii* occurred, accompanied by an increase in the levels of metabolites, such as acetate and butyrate, in the infant feces. Importantly, our data suggest that the changes in metabolites in the infant feces might be linked to benefits such as the maturation of immune function and protection against infection at a certain period during lactation. Therefore, bioactive milk components, such as HMOs, might play different roles in the exclusively breastfed infants depending on the lactation period. In particular, the increase in metabolites such as acetate and butyrate in the late-lactation period might reflect the switch from developmental benefits to the infant via breastfeeding to benefits conferred through metabolism in the infant or its own gut microbiome. It has also been reported that the microbiome in breast milk, and bacteria on the mother's skin and the infant's oral cavity, might contribute to changes in the infant gut microbiome (64). To develop a mechanistic understanding of the complex interactions among microbial species, more detailed studies are required, such as co-culturing the bacterial species that fluctuated in this study. However, the present findings could contribute to understanding the relationship between bioactive milk components and the infant gut microbiome and to the development of supplementary nutritional foods for breastfed infants during lactation.

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 at: DDBJ; Accession PRJDB12676.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Japan Conference of Clinical Research (Approval Number: BONYU-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YK, HI, YT, SN, and TA designed the study. YK, DK, YO, and SN analyzed the data and interpreted the result. NS contributed to discussions including the experimental schedule. YK conducted the experiments and wrote the paper. YK is primarily responsible for the final content. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.813690/full#supplementary-material

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"GENYAL" Study to Childhood Obesity Prevention: Methodology and Preliminary Results

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Objective: This article describes the methodology and summarizes some preliminary results of the GENYAL study aiming to design and validate a predictive model, considering both environmental and genetic factors, that identifies children who would benefit most from actions aimed at reducing the risk of obesity and its complications.

Design: The study is a cluster randomized clinical trial with 5-year follow-up. The initial evaluation was carried out in 2017. The schools were randomly split into intervention (nutritional education) and control schools. Anthropometric measurements, social and health as well as dietary and physical activity data of schoolchildren and their families are annually collected. A total of 26 single nucleotide polymorphisms (SNPs) were assessed. Machine Learning models are being designed to predict obesity phenotypes after the 5-year follow-up.

Settings: Six schools in Madrid.

Participants: A total of 221 schoolchildren (6–8 years old).

Results: Collected results show that the prevalence of excess weight was 19.0, 25.4, and 32.2% (according to World Health Organization, International Obesity Task Force and Orbegozo Foundation criteria, respectively). Associations between the nutritional state of children with mother BMI [$\beta = 0.21$ (0.13–0.3), p (adjusted) < 0.001], geographical location of the school [OR = 2.74 (1.24–6.22), p (adjusted) = 0.06], dairy servings per day [OR = 0.48 (0.29–0.75), p (adjusted) = 0.05] and 8 SNPs [rs1260326, rs780094, rs10913469, rs328, rs7647305, rs3101336, rs2568958, rs925946; p (not adjusted) < 0.05] were found.

Conclusions: These baseline data support the evidence that environmental and genetic factors play a role in the development of childhood obesity. After 5-year follow-up, the GENYAL study pretends to validate the predictive model as a new strategy to fight against obesity.

Clinical Trial Registration: This study has been registered in ClinicalTrials.gov with the identifier NCT03419520, https://clinicaltrials.gov/ct2/show/NCT03419520.

Keywords: pediatric obesity, early intervention, single nucleotide polymorphisms, machine learning, nutrition

INTRODUCTION

Obesity is a complex, chronic and multifactorial disease, originated as an interaction between genetic and environmental factors (1). The prevalence of overweight and obese children is rising every year. Specifically, in compliance with the WHO, the number of overweight and obese children aged 0–5 years increased from 32 million globally in 1990 to 41 million in 2016. And it is expected to increase to 70 million by 2025 if these trends continue (2). The situation in Spain is also alarming, with a prevalence of 23.2% of overweight (22.4% boys and 23.9% girls), and 18.1% of obesity (20.4% boys and 15.8% girls) according to data from the ALADINO study carried out by the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (3).

Childhood obesity usually leads to adulthood obesity, which increases the risk of developing certain diseases, such as hypertension, type 2 diabetes and cardiovascular diseases, prematurely (4–6). This early age has been identified as a key point for the implementation of healthy dietary and lifestyle patterns. Thus, the home and schools provide a useful environment to develop educational and lifestyle interventions for school-age children (7).

There is no doubt about the multifactorial etiology of obesity in which socio-cultural, dietetic, environmental and genetic factors are involved (8–11). However, current knowledge is still insufficient to determine the relative importance of these different factors, having a complex network of associations between them (12). In this regard, machine learning techniques represent a powerful prediction tool through their great ability to big data analysis. Thus, Machine learning represents a tool based on a set of algorithms that can characterize, adapt, learn, predict, and analyze data, increasing the knowledge of obesity and offering possibilities of predicting the disease with unprecedented precision. These techniques have been proposed as a potential tool to predict a future excess of body weight and its comorbidities. There are several predictive machine learning algorithms such as neural networks, decision tree analysis or random forest. Each of them should be used according to the purpose and nature of the study variables (13).

Considering all the above-mentioned aspects, the main objective of the GENYAL study is to design and validate a machine learning-based predictive model that identifies children who would benefit most from actions aimed at reducing the risk of obesity and its complications, considering both environmental and genetic factors, and applicable at the beginning of the school stage. The nutritional education developed in the intervention's schools will be also evaluated as part of the predictive model. This article describes the methods and analyses that will be applied. In addition, it summarizes some preliminary results obtained after the first year of the data collection.

Abbreviations: ACM, Autonomous Community of Madrid; SNPs, Single Nucleotide Polymorphisms; IPAC, Individual Physical Activity Coefficient; PAC, Physical Activity Coefficient; EW, Excess Weight.

METHODS

Type of Study and Duration

The present study is a cluster randomized clinical trial with 5-year follow-up intervention based on nutritional education, annual anthropometric measurement evaluations and data collection from questionnaires. Saliva samples were collected for all the schoolchildren in the initial evaluation (2017) in order to obtain genetic information. The final evaluation will be carried out 4 years after the initial intervention, which corresponds to the end of the primary school (**Figure 1**). The study is therefore expected to last 5 years, from 2016 to 2017 academic year to 2021–2022. **Table 1** provides a schedule of activities and interventions throughout the study.

Recruitment, Sample Size, and Sample Characteristics

Due to the nature of the study as a clinical trial, the large number of variables necessary for the design of the preventive model with machine learning (each of them of a very different nature), and the duration of 5 years, a statistically robust sample size could not be implemented. Furthermore, the *Consejería de Educación e Investigación de la Comunidad de Madrid* was responsible for the selection of six representative schools of the Autonomous Community of Madrid (ACM) (Spain) (two in the north, two in the center and two in the city's south zone), considering the number of students per center and the average socioeconomic level of the districts and neighborhoods. Therefore, the selection was representative of the average income of the ACM households (14). All the School Boards approved the participation in the study and included a total of 569 potential children participants from different districts of Madrid: Chamberí, Hortaleza, Carabanchel, Puente de Vallecas, and Moncloa-Aravaca.

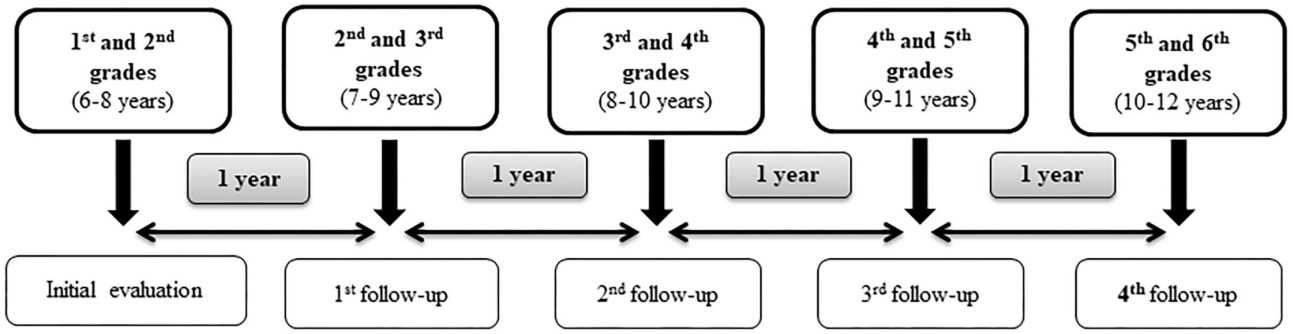

FIGURE 1 | Timetable of the study.

TABLE 1 | GENYAL study timeline.

	Study period in years						
	Enrolment (T-1)	**Allocation (T0)**	**T1**	**T2**	**T3**	**T4**	**T5**
Enrolment							
Study approval	x						
Schools recruitment	x						
Informed consent	x						
Group allocation		x					
Assessments							
Sending of questionnaires			x	x	x	x	x
Anthropometric measurements			x	x	x	x	x
Saliva samples collection			x				
Questionnaires collection			x	x	x	x	x
Machine learning model results							x
Intervention group							
Sending educational material for parents, teachers, and schoolchildren					⟷	⟷	
Nutrition education workshops and talks					⟷	⟷	

T-1 (enrolment) and T0 = 2016/2017; T1 = 2017/2018; T2 = 2018/2019; T3 = 2019/2020; T4 = 2021/2021; T5 = 2021/2022.

Inclusion and Exclusion Criteria

The inclusion criteria to participate in the study were: being in 1st or 2nd grade of primary school and having an informed consent signed by at least one of the parents.

Exclusion criteria were not attending school during the evaluation days or having planned not to stay at the school the following years.

Randomization

In order to avoid cross-contamination between intervention and control subjects, randomization was carried out by school center instead of individually. Thus, participating schools were randomly and proportionally stratified into two groups: intervention schools and control schools, considering the number of participants per center, their geographic area and their socioeconomic status.

The randomization procedure was carried out with the statistical software R version 3.4 (www.r-project.org).

Ethical Aspects and Data Processing

Protocols and methodology used in the present study comply with the ethical principles for research involving human subjects laid down in the Declaration of Helsinki (1964) and its modifications. The study was approved by the Research Ethics Committee of the IMDEA Food Foundation (PI:IM024; Approval date: March 29th, 2016) and it has been registered in ClinicalTrials.gov with the identifier NCT03419520. School centers and families were informed in detail about the different stages of the project both, orally and in writing. Signed informed consent from at least one of the parents were collected by the researchers prior to the first evaluation. This document included a specific consent to DNA extraction and the evaluation of polymorphisms from the saliva samples. In addition, it included a section on the storage of the remaining samples as a collection registered, according to Spanish legislation (Royal Decree 1716/2011, of November 18th).

Data compiled along the study are going to be processed using a web application that applies dissociation criteria making the volunteers' data anonymous, in compliance with the current

Spanish legislation (Organic Law 15/1999 of December 13th, on the protection of Personal Data) and may be used for scientific purpose as publications and conferences. Only the researchers directly related to the study will be allowed to access data.

Selection of Single Nucleotide Polymorphisms

A total of 26 single nucleotide polymorphisms (SNPs) associated with a higher risk of early-age onset of obesity and its comorbidities were selected. The selection was made considering the biological activity of each SNP, Caucasian allele frequencies and the scientific evidence that supports the association between the presence of the polymorphism and the risk of developing overweight, obesity or its complications. The sum of the risk alleles will further be used to design a genetic risk score.

Different databases such as 1,000 Genomes, HapMap, Pubmed, GWAS Central, GWAS Catalog or Ensembl were used. **Table 2** shows the selected 26 SNPs, which will be included in the predictive model.

Questionnaires

Different questionnaires were designed based on other surveys used in similar studies to facilitate the comparison of the results. All of them are annually sent to families by email or in the paper format according to the parents' preference and are filled by at least one of the parents. The information collected is summarized in **Table 3**.

Regarding social, health and demographic data, parents annually complete a self-reported questionnaire that includes different personal questions based on the surveys used in the ALADINO and ELOIN studies (3, 112).

Dietary information is gathered using a 48-h food record of 2 non-consecutive days, a weekday and a weekend day, as recommended by the European Food Safety Authority guidelines (113). Afterwards, the data are tabulated and analyzed using the DIAL software (*Alce Ingeniería*, Madrid, Spain) (114) in order to obtain information about macro and micronutrients.

Moreover, the adherence to the Mediterranean diet pattern is assessed using the "KIDMED Mediterranean Diet Quality Index" in addition to general questions about the dietary habits of the children and their parents. The KIDMED questionnaire consists of a total of 16 dichotomous questions that must be answered affirmatively or negatively to obtain a score (115).

Physical activity and free time data about the children and their parents are gathered using a questionnaire with different sections adapted and modified from the ALADINO (3) and the ELOIN (112) studies. In addition, a 48-h physical activity record is collected, corresponding to 24 h of a weekday and a complete weekend day (116). In the physical activity record, parents had to specify the time that their children spent during 24 h of a week day and 24 h of a weekend day doing different activities, including resting hours and activities with a variable level of intensity (very light, light, moderate and intense). The time spent doing each activity is multiplied by the corresponding activity coefficient defined by the WHO (117), added and divided by 24, obtaining the Individual Physical Activity Coefficient (IPAC). Then, the IPAC corresponding to a weekday is multiplied by 5 and the weekend IPAC by 2, both results are added and divided by 7, thus, obtaining the median physical activity per individual. Afterwards, it is necessary to convert the IPAC into a Physical Activity Coefficient (PAC) according to sex, therefore an equivalence is made between the IPAC and the PAC proposed by the Institute of Medicine (118). Finally, participants are classified into sedentary, lightly active, active and very active in line with their PAC.

All these data and information are collected every year on equal terms.

Anthropometric and Blood Pressure Measurements

These data are collected in the school centers, early in the morning, by previously trained nutritionists, following standardized protocols and WHO international instructions for this age group (117). For the anthropometric measurements, children had to wear a T-shirt and gym shorts. All measures are taken twice, and the average is used for the analyses.

Height is determined using a Leicester height rod with an accuracy of 1 mm (Biological Medical Technology SL, Barcelona, Spain). Body weight and fat mass percentage are assessed using a BF511 Body Composition Monitor (BF511- OMRON HEALTHCARE UK, LT, Kyoto, Japan). Furthermore, fat mass percentage is classified according to the tables offered by OMROM Healthcare (119). Waist and brachial circumferences measurements are taken using a non-elastic tape (KaWe Kirchner & Wilhelm GmbH, Asperg, Germany; range 0–150 cm, 1 mm of precision). The waist circumference measurements obtained are classified by percentiles in compliance with Fernández et al. (120). Triceps skinfolds are taken following the International Society for the Advancement of Kinanthropometry guidelines (121) using a mechanic caliper (HOLTAIN LTD. CRYMYCH UK 10 g/mm^2 constant pressure; range 0–39 mm and 0.1 mm of precision) and the results obtained are ranked according to percentiles proposed by Frisancho AR (122).

Using these data, other variables of interest are calculated. In particular, BMI is calculated as the body weight divided by the squared height (kg/m^2). There is not a universal technique to classify the BMI values in the pediatric collective, therefore, the results are ranked according to the percentiles of Faustino Orbegozo Eizaguirre Foundation, reviewed in 2011 (120), International Obesity Task Force reviewed in 2000 (123), and WHO reviewed in 2007 (124). The results of overweight and obesity rates are unified as a single category called excess weight (EW). The arm muscular and fat areas are obtained using the equations proposed by Mataix Verdú and López Jurado (125) and López-Sobaler and Quintas Herrero (126), respectively. The protein and caloric reserves are calculated by Frisancho AR equations (122). Waist/height ratio is calculated as waist circumference (cm)/height (cm) and it was classified according to Panjikkaran et al. and Ashwell investigations (127, 128). Height/age index is rated in percentiles according to Fernández et al. (120).

For blood pressure monitoring, an automatic digital monitor is used (OMRON M3-Intellisense) using a cuff suitable for

TABLE 2 | Single nucleotide polymorphisms selection.

Gene[a]	Name	Function	Location[b]	Alleles		WT	MAF[b]	Frequency[b]	Association	References
APOA5	rs662799	Upstream gene variant	Chromosome 11:116792991	G	A	A	0.16 (G)	G: 0.076/A: 0.924 G|G: 0.010; A|A: 0.859; A|G: 0.131	Overweight and obesity, lipid profile, CVD, MS	(15–19)
BDNF	rs925946	Intron variant	Chromosome 11:27645655	T	G	G	0.25 (T)	T: 0.338/G: 0.662 T|T: 0.141; G|T: 0.394; G|G: 0.465	Overweight and obesity, calcium consumption, MS, waist/hip index, abdominal obesity	(20–25)
ETV5	rs7647305	Intron variant	Chromosome 3:186116501	T	C	T	0.24 (T)	T: 0.227/ C: 0.773 T|T: 0.101; C|T: 0.253; C|C: 0.646	Overweight and obesity, early menarche, BP	(20, 24, 26, 27)
FTO	rs3751812	Intron variant	Chromosome 16:53784548	G	T	G	0.22 (T)	G: 0.556/T: 0.444 G|G: 0.303; G|T: 0.505; T|T: 0.192	Overweight and obesity, lipid profile	(28–31)
FTO	rs7190492	Intron variant	Chromosome 16:53794840	A	G	G	0.30 (A)	A: 0.343/G: 0.657 A|A: 0.131; A|G: 0.424; G|G: 0.444	Overweight and obesity	(28, 29, 32)
FTO	rs8050136	Intron variant	Chromosome 16:53782363	C	A	A	0.32 (A)	C: 0.556/A: 0.444 C|C: 0.303; A|C: 0.505; A|A: 0.192	Overweight and obesity, T2DM	(28, 29, 33–36)
FTO	rs9939609	Intron variant	Chromosome 16:53786615	T	A	A	0.34 (A)	T: 0.556/A: 0.444 T|T: 0.303; A|T: 0.505; A|A: 0.192	Overweight and obesity, extreme childhood obesity, T2DM, MS, early menarche, fat mass	(20, 27, 37–41)
GCKR	rs1260326	Missense variant	Chromosome 2:27508073	T	C	C	0.29 (T)	T: 0.429/C: 0.571 T|T: 0.172; C|T: 0.515; C|C: 0.313	Overweight and obesity, MS, lipid profile, glycemic profile	(42–45)
GCKR	rs780094 a	Intron variant	Chromosome 2:27518370	T	C	C	0.30 (T)	T: 0.409/C: 0.591 T|T: 0.162; C|T: 0.495; C|C: 0.343	Overweight and obesity, lipid profile, MS, glycemic profile, fatty liver, T2DM	(42, 43, 46–50)
GNPDA2	rs10938397	Intergenic variant	Chromosome 4:45180510	A	G	A	0.33 (G)	A: 0.576/G: 0.424 A|A: 0.333; A|G: 0.485; G|G: 0.182	Overweight and obesity, extreme childhood obesity, early menarche, T2DM, glycemic profile, fat mass, waist circumference, waist-height ratio, central obesity	(20, 27, 40, 41, 51)
KCTD15	rs368794	Intergenic variant	Chromosome 19:33829547	T	A	A	0.42 (T)	T: 0.298/A: 0.702 T|T: 0.081; A|T: 0.434; A|A: 0.485	Overweight and obesity, fat mass	(20, 41, 52, 53)
LEPR	rs1137101	Missense variant	Chromosome 1:65592830	A	G	A	0.42 (A)	A: 0.515/G: 0.485 A|A: 0.253; A|G: 0.525; G|G: 0.222	Obesity, lipid profile, glycemic profile, caloric intake, T2DM	(54–58)

(Continued)

TABLE 2 | Continued

Gene[a]	Name	Function	Location[b]	Alleles		WT	MAF[b]	Frequency[b]	Association	References
LPL	rs328	Stop gained	Chromosome 8:19962213	C	G	C	0.09 (G)	C: 0.874/G: 0.126 C|C: 0.768; C|G: 0.212; G|G: 0.020	Lipid profile, CVD	(59–63)
MC4R	rs17782313	Intergenic variant	Chromosome 18:60183864	T	C	T	0.24 (C)	T: 0.742/C: 0.258 T|T: 0.515; C|T: 0.455; C|C: 0.030	Overweight and obesity, extreme childhood obesity, MS, T2DM, abdominal obesity, fat mass, waist circumference, waist-height ratio	(22, 41, 64–69)
NEGR1	rs2568958	Upstream gene variant	Chromosome 1:72299433	G	A	G	0.32 (G)	G: 0.364/A: 0.636 G|G: 0.141; A|G: 0.444; A|A: 0.414	Overweight and obesity, extreme childhood obesity, fat mass.	(20, 41, 70)
NEGR1	rs3101336	Upstream gene variant	Chromosome 1:72285502	T	C	T	0.32 (T)	T: 0.364/C: 0.636 T|T: 0.141; C|T: 0.444; C|C: 0.414	Overweight and obesity, severe early-onset obesity.	(70–72)
NPY	rs16147	Upstream gene variant	Chromosome 7:24283791	T	C	T	0.48 (T)	T: 0.485/C: 0.515 T|T: 0.273; C|T: 0.424; C|C: 0.303	Overweight and obesity	(73–75)
PPARγ	rs1801282	Missense variant	Chromosome 3:12351626	C	G	C	0.07 (G)	C: 0.904/G: 0.096 C|C: 0.828; C|G: 0.152; G|G: 0.020	Overweight and obesity, glycemic profile, CVD, T2DM, Skin folds	(76–82)
SEC16B	rs10913469	Intron variant	Chromosome 1:177944384	T	C	C	0.23 (C)	T: 0.758/C: 0.242 T|T: 0.576; C|T: 0.364; C|C: 0.061	Overweight and obesity, waist circumference, fat mass, early menarche	(27, 28, 30, 55, 83, 84)
TCF7L2	rs7903146	Intron variant	Chromosome 10:112998590	C	T	T	0.23 (T)	C: 0.687/T: 0.313 C|C: 0.525; C|T: 0.323; T|T: 0.152	IMC, T2DM, MS, glycemic profile, height, lipid profile in DM patients	(85–93)
TMEM18	rs2867125	Intergenic variant	Chromosome 2:622827	T	C	C	0.14 (T)	T: 0.162/C: 0.838 T|T: 0.051; C|T: 0.222; C|C: 0.727	Overweight and obesity	(30, 94, 95)
TMEM18	rs4854344	Intergenic variant	Chromosome 2:638144	G	T	G	0.15 (G)	G: 0.167/T: 0.833 G|G: 0.051; G|T: 0.232; T|T: 0.717	Overweight and obesity, waist circumference, visceral fat and SBP	(83, 96–98)
TMEM18	rs6548238	Regulatory region variant	Chromosome 2:634905	T	C	C	0.12 (T)	T: 0.167/C: 0.833 T|T: 0.051; C|T: 0.232; C|C: 0.717	Overweight and obesity, extreme childhood obesity, fat mass, early menarche, waist circumference,	(20, 27, 41, 55, 65, 97, 99, 100)
TMEM18	rs7561317	Intergenic variant	Chromosome 2:644953	A	G	A	0.16 (A)	A: 0.162/G: 0.838 A|A: 0.040; A|G: 0.242; G|G: 0.717	Overweight and obesity	(21, 22, 24, 33, 40, 101, 102)

(Continued)

TABLE 2 | Continued

Gene[a]	Name	Function	Location[b]		Alleles	WT	MAF[b]	Frequency[b]	Association	References
UCP2	rs659366	Upstream gene variant	Chromosome 11:73983709	C	T	C	0.41 (T)	C: 0.636/T: 0.364 C|C: 0.414; C|T: 0.444; T|T: 0.141	Overweight and obesity, glycaemia	(103–105)
UCP3	rs1800849	5 prime UTR variant	Chromosome 11:74009120	G	A	G	0.19 (A)	G: 0.768/A: 0.232 G|G: 0.586; A|G: 0.364; A|A: 0.051	Overweight and obesity, glycaemia, lipid profile, waist hip ratio	(103, 106–110)

[a] *Approved name (111).*

[b] *Ensembl (https://www.ensembl.org/index.html). Location, For forward strand.*

WT, wild type; MAF, Minor allele frequency; Frequency, Allele and genotype; APOA5, apolipoprotein A5; BDNF, brain derived neurotrophic factor; ETV5, ETS variant 5; FTO, FTO, alpha-ketoglutarate dependent dioxygenase; GCKR, glucokinase regulator; GNPDA2, glucosamine-6-phosphate deaminase 2; KCTD15, potassium channel tetramerization domain containing 15; LEPR, leptin receptor; LPL, lipoprotein lipase; MC4R, melanocortin 4 receptor; NEGR1, neuronal growth regulator 1; NPY, neuropeptide Y; PPARγ, peroxisome proliferator activated receptor gamma; SEC16B, SEC16 homolog B, endoplasmic reticulum export factor; TCF7L2, transcription factor 7 like 2; TMEM18, transmembrane protein 18; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3; CVD, cardiovascular disease; MS, metabolic syndrome; BP, blood pressure; T2DM, type 2 diabetes mellitus; SBP, systolic blood pressure.

TABLE 3 | Content of the questionnaires used in the study.

Questionnaire	Variables
Characteristics of parents and family	
Social, health and demographic	People living with the schoolchildren, number of siblings, family structure, level of education, employment situation, profession, country of birth, years living in Spain, income level, diagnosis of disease, smoking habit, body weight, height and date of birth.
Diet	Daily intakes, frequency of breakfast per week, breakfast foods, reasons for stop breastfeeding, desire to extend the breastfeeding period, perception of their child's diet.
Physical activity	Distance between school and home, weekly hours of physical activities, frequency of physical activity shared with their child, town conditions for doing outdoor activities.
Characteristics of children	
Social, health and demographic	Country of birth, years living in Spain, adopted or foster child, diagnosis of disease, body weight and height reported by their parents and date of birth.
Diet	Diet changes over the last year and reasons, number of daily intakes, frequency of breakfast per week, place where they have breakfast, breakfast foods, place where they have lunch, frequency of eating in fast food restaurants, breastfeeding, beikost's start date.
	48-h dietary record
	KIDMED questionnaire
Physical activity	Sleeping hours, type of transport used to go to school and back home, outdoor activities, time usually spend watching TV, playing video games or using the computer, presence of TV or computer games in their bedroom, weekly hours of physical activities and children's physical activity level according to their parents
	48-h physical activity questionnaire

children. The results are classified according to the percentiles established by the Spanish Association of Pediatrics (129).

All these measurements are repeated every year on equal terms.

Compiling Saliva Samples, DNA Extraction and Genotyping

Buccal smears were collected for DNA extraction following standardized protocols. For this purpose, a sterile swab free of human RNAse, DNAse and DNA (300263DNA-Hisopos Deltalab polystyrene and polyester) was used. Children had to have their mouth clean and avoid eating or drinking 30 min prior to collection. Three samples were taken per children, each one identified with the number corresponding to the order of extraction, to ensure traceability. As the samples were collected, they were directly stored in refrigeration until all the children were evaluated. Immediately after, they were frozen at −80°C until their processing.

Genomic DNA was extracted from the buccal swabs using the INVISORB® SPIN TISSUE MINI KIT (Stratec), according to the manufacturer's instructions. Samples were lysed in the presence of proteinase K and a specific lysis buffer. The lysate was then purified and finally, it was eluted in a free EDTA solution.

TABLE 4 | Educational components of GENYAL study.

	Format	Module	Objective
Children	Notebook activities	Food and nutrients	Essential nutrients: definition, function and sources
		The food pyramid	To identify the different food categories and explore which foods are in each group
	Workshops	The food pyramid	To draw a food pyramid and to create healthy dishes with the different food categories
Parents	Nutritional guide	Introduction	To know the bases and objectives of the study
		Food and nutrients	Essential nutrients: definition, function and sources
		The food pyramid	To identify the different food categories and which foods are in each group
	Workshops	Preliminary results	To know about the preliminary results of the study and the recommendations derived from them
Teachers	Nutritional guide	Introduction	To know the bases and objectives of the study
		Food and nutrients	Essential nutrients: definition, function and sources
		The food pyramid	To identify the different food categories and which foods are in each group
	Workshops	Preliminary results	To know about the preliminary results of the study and the recommendations derived from them

For genotyping, the DNA samples were loaded in TaqMan® OpenArray® Real-Time PCR plates (Life Technologies Inc., Carlsbad, CA) already configured with the specific selected SNPs with specific waves for each allele, marked with a different fluorophore to determine the genotype. This process was made using the OpenArray® AccuFill™ System (Life Technologies Inc., Carlsbad, CA). Once it was charged, a PCR performed and the chips were read in the QuantStudio® 12K Flex Real-Time PCR Instrument (Life Technologies Inc., Carlsbad, CA). Results were analyzed using the TaqMan® Genotyper software (Life Technologies Inc., Carlsbad, CA), which automatically assigns the genotype to each sample according to the amount of detected signal for each fluorophore.

The duplicate analysis was used to validate the genotyping result.

Design of Educational Tools and Implementation of the Nutritional Education Programme

For the implementation of the nutritional education programme in the "intervention schools", three different kinds of guides were designed aimed at parents, children and teachers. All this information was developed and adapted to the participants' age by the nutritionists from the IMDEA Food Foundation. This material is sent to parents and educational centers in different modules adapted to parents, students, and teachers. The same modules include different activities and topics each year according to the children's growth. The sending strategy follows a protocol, and it will be maintained until the end of the study, through email or regular delivery, as the receiver may prefer.

Moreover, some workshops are being carried out and are summarized in **Table 4**.

The validation of this tool is expected to be carried out through the impact generated over the years of the study, measured as the evolution of anthropometric variables and the dietary habits, between control and intervention schools. Moreover, parents and teachers along the study will evaluate all the material.

Statistical Analysis

Descriptive analyses of the baseline data were performed by computing for the categorical variables the class's absolute and relative frequencies, and for the quantitative variables the mean, median, standard deviation, interquartile range, maximum and minimum. To check the homogeneity of the two groups in the case of quantitative variables, *t*-tests were used for normally-distributed variables, or Mann-Whitney *U*-test as non-parametric alternative. In the case of categorical variables, Chi-Square or Fischer exact tests were used. The association between anthropometric and dietary, social, health and SNP variables were performed by linear or logistic regressions. The Bonferroni correction was applied for multiple tests. In addition, for the SNPs variables, the Hardy-Weinberg equilibrium condition was tested by means of Chi-Square tests. All analyses were conducted with R Statistical Software version 3.41. Statistical tests used a 0.05 significance level, in two-tailed tests.

Regarding Machine Learning models, they will be derived to predict the BMI from all the analyzed variables *after the 5-year follow-up*. Both classification (after dichotomization of the BMI) and regression models will be considered, and Random Forest will be applied. It has been observed that the use of Random Forest improves the predictive model's performance, creating a more effective predictive model than the one that could be obtained using decision tree or logistic regression techniques (13). The predictive power of the models will be evaluated and internal cross-validation and external validations with external datasets will be implemented. Variable importance analyses will be performed in order to quantify the relative weights of the different variables in the prediction of BMI. The model will be iteratively improved by refitting with new data along with the successive yearly evaluations during the study.

RESULTS

Parents of 224 children (116 girls and 105 boys) accepted to participate in the study and signed the informed consent. It shows a collaboration rate of 39%. Finally, 221 children were evaluated, since three did not attend the initial evaluation (**Figure 2**).

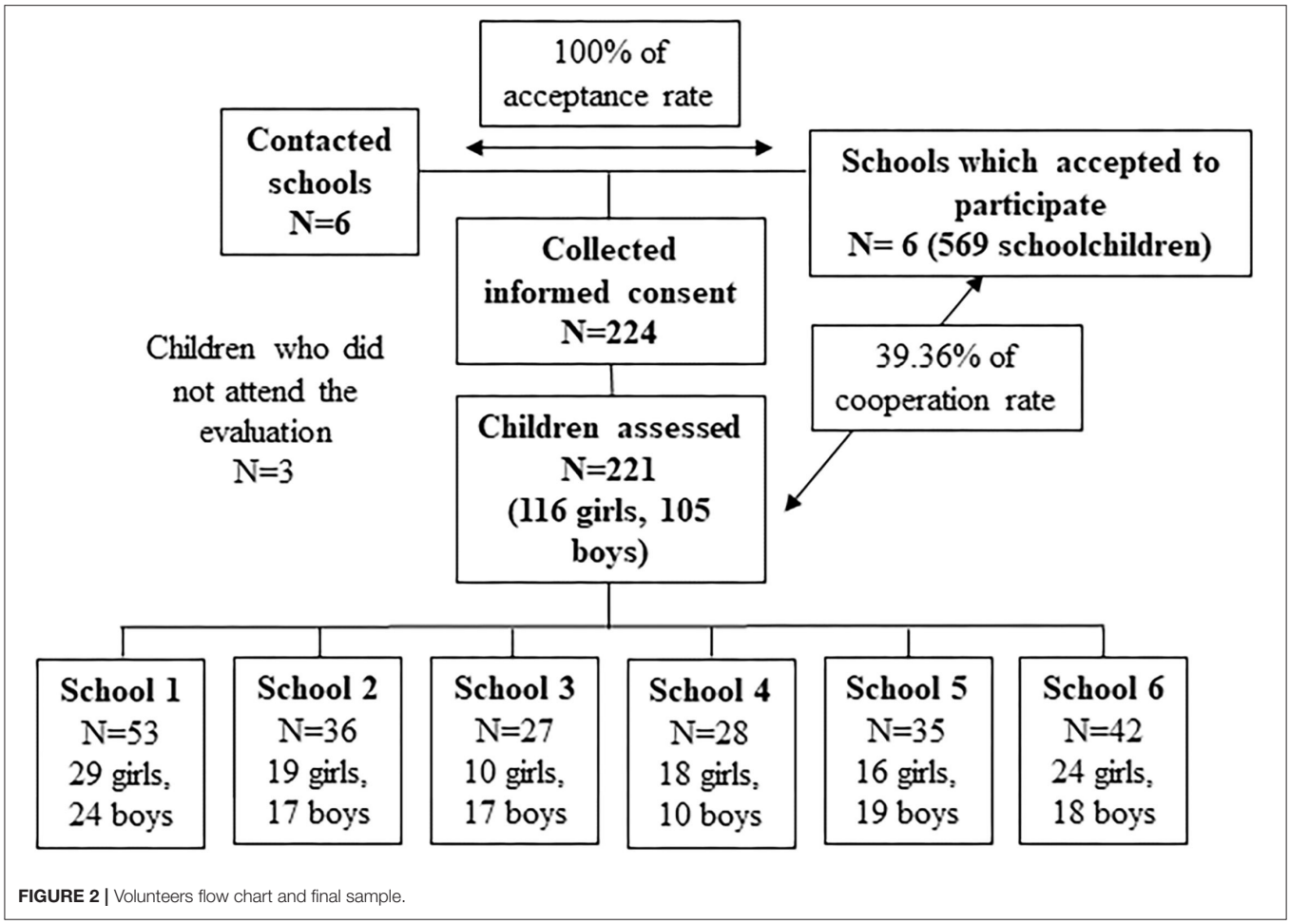

FIGURE 2 | Volunteers flow chart and final sample.

Among the total number of students enrolled in the study, 115 belonged to intervention schools while 106 were in control schools. **Tables 5**, **6** show the basal main characteristics of the study sample according to the corresponding control or intervention group.

According to the preliminary results obtained after the first year of the evaluation, 32.2% of the students presented EW, taking into account the WHO criteria. These figures were higher when IOFT standard (25.4%) or the national criteria of the Orbegozo Foundation (19.0%) were applied.

A significative association between the nutritional state of children and mother BMI [$\beta = 0.21$ (0.13–0.3), p (adj) < 0.001], geographical location of the school [OR = 2.74 (1.24–6.22), p (adj) = 0.06], dairy servings per day [OR = 0.48 (0.29–0.75), p (adj) = 0.05] and eight of the total SNPs studied [rs1260326, rs780094, rs10913469, rs328, rs7647305, rs3101336, rs2568958, rs925946; p (not adj) < 0.05] were found (data adjusted for sex and age).

DISCUSSION

The GENYAL study to prevent child obesity is, to our knowledge, the first interventional trial in Spanish schoolchildren aiming to provide preventive and therapeutic approach based on a high degree of evidence for early obesity through machine learning.

The baseline data and the associations observed after the first analysis support the evidence that environmental and genetic factors play a role in the development of childhood obesity.

According with the results, for each point in the BMI of the mother, the BMI of the child was increased 0.21 kg/m^2. It shows that among the multiple risk factors for the development of obesity in children, parental obesity is one of the most impactful as a result of both genetic and environmental interactions. Children imitate their parents, therefore, the parents' dietary habits and PA are more likely to be reproduced by their descendants (130).

With reference to the location of the school, that represent the socioeconomic level, presented a close relationship with the presence of EW, with a decreasing distribution of risk from south to north area. These results are consistent with other studies that have shown how the socioeconomic status of the school correlates with the prevalence of overweight and obesity as it increases the likelihood that schoolchildren will follow a diet rich in energy-dense, low-cost foods, as well as fewer opportunities to practice sport (131).

TABLE 5 | Main socio-demographic and anthropometric characteristics of the schoolchildren by groups (intervention schools vs. control schools).

	Intervention group (n = 115)	Control group (n = 106)	p
Socio-Demographic			
Spanish (%)			
– No	1.90	3.00	0.678
– Yes	98.10	97.00	
Spanish mother (%)			
– No	35.00	23.50	0.074
– Yes	65.00	76.50	
Anthropometry			
Age (years)	6.73 ± 0.76	6.77 ± 0.69	0.505
Sex (%)			
– Girls	52.20	42.50	0.148
– Boys	47.80	57.50	
Fat mass (%)	21.23 ± 7.56	19.84 ± 6.71	0.159
Muscle mass (%)	27.97 ± 3.00	28.01 ± 2.97	0.924
BMI (kg/m^2)	17.21 ± 2.99	16.68 ± 2.24	0.319

TABLE 6 | Main dietary and physical activity characteristics of the schoolchildren by groups (intervention schools vs. control schools).

	Intervention group (n = 115)	Control group (n = 106)	p
Physical activity			
Physical activity rate	1.61 ± 0.11	1.55 ± 0.09	<0.001
Number of hours dedicated to moderate and vigorous activities	3.53 ± 1.72	3.96 ± 1.89	0.068
Diet			
Total caloric value (kilojoules):	7,695.97 ± 1,962.52	7,678.59 ± 1,463.50	0.378
– Carbohydrates %	45.00 ± 5.15	43.94 ± 5.43	0.154
– Lipids %	38.40 ± 4.91	39.52 ± 5.09	0.116
– Proteins %	16.57 ± 2.25	16.52 ± 2.09	0.971
Total fiber (g)	18.54 ± 6.37	17.78 ± 5.19	0.481
KIDMED score	6.33 ± 1.98	6.67 ± 1.82	0.251

Regarding dietary aspects, dairy servings per day showed a protective effect against EW. It could be related to several factors such as if this food is a source of calcium, peptides, bioactive compounds, etc. They have been studied due to their relationship in the appetite control and other mechanisms involved in controlling weight (132, 133). These results highlight the important role that this group of foods could have for the prevention of weight overload.

The study of these factors in the child population and the social context of Madrid is minimal, having found a single study with similar sociodemographic characteristics (134). Nevertheless, no intervention was performed in this study, nor was genetic data collection, thus the GENYAL project is shown as a novel study in this regard.

In this study, a significant association (data adjusted for sex and age) between the nutritional status of schoolchildren and 8 SNPs was found (rs1260326, rs780094, rs10913469, rs328, rs7647305, rs3101336, rs2568958, rs925946). In previous studies, these polymorphisms have been associated with adiposity traits and their related comorbidities (**Table 2**). Genetic factors play an essential role in the development of obesity (135). Thus, knowing which ones are associated with excess weight early in life could contribute to obesity early detection.

Conversely, it is important to note that current knowledge is insufficient to determine the relative importance of these different factors. Therefore, new techniques are needed to be used as predictive instruments (12). Currently, machine learning is considered an extremely valuable tool in the medical field, since it is capable of providing diagnostic and early detection strategies for diseases through the analysis of large datasets (136). Prevention plays a crucial role in controlling the high obesity prevalence, so machine learning techniques have already been used for the prediction of the BMI in children (137). However, the current predictive model would be the first to include obesity-related SNPs as genetic information, as well as anthropometric, social and lifestyle variables.

Regarding the last results from the Commission on Ending Childhood Obesity, the implementation of integral programs that promote healthy environment in schools is recommended with the objective of ensuring that children grow well and develop healthy habits (138). Nevertheless, although Spain is one of the countries where more intervention studies to prevent obesity have been developed (139), the politic strategies to prevent chronic illnesses such as overweight and obesity are not defined, and even show very low evidence of efficiency, according to the last data revised by the Cochrane Database (140).

According to the latest scientific research, the intervention studies in schools which include family and community spheres, implementing actions to promote healthy food and physical activity, are the most effective (5, 46). This study has been designed to elaborate strategies and to work as a multidisciplinary team reinforcing the educational sphere of the participating children and their environment, school, and family.

One of the strong points of our study consists in the implementation, and subsequent validation, of educational tools for students, their parents and teachers, applying a nutritional education method that promotes healthy dietetic habits and physical exercise, both in schools and outside. The importance of the validation of these educational strategies lay on a large number of studies with contradictory results, which might be partly explained by the fact that many researches may have lacked statistical power to detect changes in the results of interest related to adiposity or children's nutritional status (5). In the present study, the educational approaches to the intervention schools will be held for 5 years, in line with the annual anthropometric assessments. Their utility will be evaluated taking into account the evolution of the anthropometric and dietetic results annually collected. Moreover, the presence or absence of the educational program (control and intervention schools) will be included as an input dichotomous variable in the predictive model, evaluating its influence on the predicted BMI. This will

allow us to detect differences in the body composition between intervention and control schools, enabling us to assess the impact of educational support.

Another strength of the present study is the selection of 26 SNPs for early prevention of obesity, by making an extensive bibliographic research, as shown in **Table 2**. The nutritional genomic tools would be very useful in the research and prevention of obesity, and they would be an important support in public health applications. Obesity is a multifactorial illness, where the genetic variants involved are dispersed along the whole genome. Although SNPs have been cataloged as the best indicators to predict obesity risk (141), several studies suggest that much remains to be discovered. There is a lot of interest in predicting the appearance of chronic diseases at an early age (142). According to the last review about precision nutrition (143), the creation of a genetic risk score may let us determine the risk of developing obesity or other chronic pathologies related to the individual genetic component, and even be able to predict the expected weight gain as a consequence of exposure to different variables, such as specific diets.

On the other hand, we consider that the sample size used is one of the weak aspects in our study, pointing to the necessity to include new schools to increase the number of children. Nevertheless, from this first phase of the study, we expect to calculate the sample size needed to increase the statistical power and, consequently, to find solid associations between the studied variables. Furthermore, the classification of schools according to the area and the socioeconomic level widens the scope of the research, making it more representative of the city of Madrid. Similarly, the use of dietary and physical activity questionnaires may lead to reporting bias, but in the absence of better tools with low cost and high throughput, these records can offer valuable information, although it should be interpreted with caution.

After 5 years of follow-up, the GENYAL study aims to validate the machine learning predictive model that considers environmental and genetic factors in the obesity development, as well as the educational tools, to obtain new and potentially valuable data to increase our knowledge of the precipitants of childhood obesity and their relative importance to design preventive protocols at an early age based on machine learning models. With a view to the future perspective of continuity of this study, in addition to increasing the sample size to validate the results obtained, the possibility of implementing personalized nutritional education interventions is proposed to improve adherence and efficacy by applying the novel concepts provided by studies on precision nutrition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Fundación IMDEA-Food. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

VL-K was the principal investigator and responsible for the study and protocol design. HM-P helped designing the protocol and drafting the manuscript. HM-P, EA-A, RI, and IE-S were responsible for data collection. GC conducted statistical analysis of the data. SM contributed to genetic samples management. JM, GR, and AR supervised the final compilation of the manuscript and provided scientific advice and consultation. All authors read and approved the final manuscript.

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RNA-Seq Analysis Reveals the Potential Molecular Mechanisms of Puerarin on Intramuscular Fat Deposition in Heat-Stressed Beef Cattle

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To investigate the effect of *Puerarin* on intramuscular fat deposition in heat-stressed beef cattle and its underlying mechanism. Thirty-two healthy Jinjiang bulls were randomly divided into four groups and dietary with 0 (Control), 200 (Pue200), 400 (Pue400), and 800 (Pue800) mg/kg *Puerarin* in the feed concentrate. The results showed that *Puerarin* treatment enhanced the concentration of crude fat, fatty acid (C14:1 and C17:1), and the activity of fatty acid synthase in *Longissimus thoracis* (LT), but decreased the levels of blood leptin ($P < 0.05$). High-throughput sequencing of mRNA technology (RNA-Seq) was used and the analysis showed that 492 genes were down-regulated and 341 genes were up-regulated in LT, and these genes were significantly enriched to the pathways related to lipid metabolism. These results indicated that dietary supplemental with *Puerarin* enhanced intramuscular fat deposition by regulating lipid metabolism of heat-stressed beef cattle.

Keywords: puerarin, beef cattle, heat stress, intramuscular fat deposition, lipid metabolism, RNA-seq

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INTRODUCTION

Heat stress caused by high temperature is one of the most critical environmental stressors challenging in cattle production (1), which leads to endocrine disorder, abnormal nutrient metabolism and changes in body tissue composition (2, 3). Among the main components of the body, body fat is the most changeable. Many reports have shown that heat stress can inhibit the growth of beef cattle and reduce the deposition of body fat, especially intramuscular fat (4, 5).

Intramuscular fat is one of the main factors used to determine the beef quality grade in many countries due to its beneficial effect on the tenderness, aroma, juiciness, and palatability of beef (6). Intramuscular fat deposition is the result of comprehensive effects of animal growth, body fat distribution, fatty acid composition, key genes of fat metabolism and transcription regulators (7, 8).

Puerarin is the main active component of *Pueraria lobata*, and the latter is a traditional Chinese herbal medicine in China that play an important role in relieving muscle, alleviating pain and reducing fever (9, 10). Several reports have demonstrated that *Puerarin* has a protective effect on regulating lipid metabolism, anti-oxidative and anti-inflammation (11, 12). A previous study revealed that *Puerarin*, like estrogen, could affect the hormone secretion levels, thus improve the production performance of animals (13).

Moreover, our previous study has found that *Puerarin* enhanced the immune function and antioxidant capacity of beef cattle in summer, and improved the growth performance and meat quality of heat-stressed beef cattle (14).

However, little attention has been paid to the effect of *Puerarin* on intramuscular fat deposition. In light of the above considerations, the objective of this study was to evaluate the potential efficacy of the supplementation of *Puerarin* on intramuscular fat deposition and analyze its mechanism by RNA-Seq sequencing technology combined with bioinformatics.

MATERIALS AND METHODS

Animal Ethics

All the experimental procedures applied in this study were reviewed and approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-20190015). All procedures involving live animals handling, management, and health care followed the regulations of laboratory animals used for scientific purposes and were implemented within it.

Puerarin, Animals, and Experimental Design

Puerarin was purchased from a company in Xi'an, whose content was 98.1% by analysis of liquid chromatography. The experimental cattle's feeding and management have been described in detail in our previous study (15). In brief, thirty-two Jinjiang bulls at 15-month-old (291.65 ± 8.84 kg) were randomly divided into four groups (n = 8): control group, Pue200, Pue400, Pue800 group (200 mg/kg, 400 mg/kg, and 800 mg/kg *Puerarin* in the feed concentrate), respectively. The composition and nutrient levels of the basal diet were shown in **Table 1**. The feeding trial lasted for 70 days including a 10-day adaptation period and another 60-day experimental period (July 1 to September 8, and the temperature, relative humidity, and humidity index in cattle house were 30.68°C, 68.05%, and 81.81, respectively).

TABLE 1 | Composition and nutrient levels of the basal diet (air-dry basis, %).

Ingredients	Content	Nutrient levels	Content
Wheat	56.50	DM	89.42
NaCl	0.50	NE_{mf}/(MJ/kg)[b]	5.45
$NaHCO_3$	1.00	CP	11.19
Premix[a]	2.00	NDF	30.08
Rice straw	20.00	ADF	15.18
Brewer's grains	20.00	Ash	7.80
Amount	100.00	Ca	1.11
		P	0.67

[a]*The premix provided per kilogram of diet: 3,200 mg of iron as iron sulfate, 1,500 mg of manganese as manganous oxide, 2,000 mg of zinc as zinc oxide, 650 mg of copper as copper sulfate, 35 mg of iodate as calcium iodate, 10 mg of selenium as sodium selenite, 10 mg of cobalt as cobalt chloride, 130 g of calcium as calcium carbonate, 30 g of phosphorus as calcium hydrogen phosphate, 45 mg retinyl acetate, 40 μg cholecalciferol, and 3.0 mg DL-α-tocopheryl acetate.* [b]NE_{mf} *were calculated values, while others were measured values.*

Serum Biochemical Indexes Analysis

On day 60, blood samples (15 mL each, n = 6) were collected at 14:00 from the jugular vein and then serum was prepared immediately. The concentrations of serum insulin (INS), triiodothyronine (T3), thyroxine (T4), cortisol (COR), adiponectin (ADPN), and leptin (LEP) were determined by using radioimmunoassay kits (Beijing Sinouk Institute of Biological Technology, China). The levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) in serum were measured by using spectrophotometric kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Muscle Sample Collection and Analysis

According to the results of blood samples, at the end of the experiment, four bulls with medium body weight were selected from control, Pue400 groups, Pue800 group, respectively. These bulls were transferred to the slaughterhouse and sacrificed at a commercial abattoir following the standard procedures. And then, approximately 200 g of the *longissimus thoracis* (LT) muscle samples from the right half-carcasses between the 12th and 13th ribs were quickly separated, 20 g samples were frozen immediately in liquid nitrogen and stored at −80°C until RNA isolation, and 50 g samples were stored at −20°C for the analysis of the fatty acid composition and the activity of the fatty metabolizing enzyme, other remaining samples were used to determine the contents of moisture, crude protein (CP), crude fat (CF), crude ash (CA), calcium(Ca), and total phosphorus (P) according to Association of Official Analytical Chemists (AOAC). In brief, the content of CA was obtained by incinerating the samples in a muffle furnace at 550°C for 3 h; CP was calculated by quantitative analysis of nitrogen using the Kjeldahl method with copper sulfate and potassium sulfate as catalysts; CF was extracted with diethyl ether using a Soxhlet extractor; Ca and P were determined colorimetrically.

Fatty Acid Composition and Fatty Metabolizing Enzymes in Muscle Analysis

Briefly, the crude fat in the LT muscle was extracted, and positive hexane, sodium methanol and methyl ester were added in crude fat in turn for fat esterification, and then ethyl acetate was added to obtain fatty acid methyl ester. Fatty acids were expressed as percentages of the total fatty acid methyl esters, which analyzed by a gas chromatograph (Shimadzu, Japan) and the method was referenced to Wang (16). The enzyme activity of fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), hormone sensitive lipase (HSL), and lipoprotein lipase (LPL) in LT muscle of beef cattle were tested using ELISA kits (Delivery code number: ml077321; ml061000; ml061693; ml076623) purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd., (Shanghai, China). Both the in-batch and interbatch coefficients of variation were less than 10%.

RNA-Seq Library Preparation and Data Analysis

The 60 g of *longissimus thoracis* samples were randomly selected from each of the control and Pue400 groups for RNA-Seq analysis. Total RNA was isolated from eight LT samples from control and Pue400 groups by Trizol reagent (Invitrogen, Waltham, MA, United States) according to the manufacturer's instructions. The quantity and quality of total RNA were assessed using the Agilent 2100 Bioanalyzer (Agilent, CA, United States). Then, transcriptomic sequencing was performed by Shanghai Majorbio Biopharm Technology Co., (Shanghai, China).

Raw reads of all eight samples were pre-processed through the removal of containing adaptors-read with more than 17% unknown nucleotides. The valid reads of each samples were aligned to the *Bos taurus* genome assembly.[1]

To analyze gene expression, the number of unique-match reads was calculated and normalized to FPKM (Fragment Per Kilo base of exon model per Million mapped reads), which was used to indicate the condition of transcriptional expression. The amount of expression was calculated for each read of the eight sequenced samples by Cuffdiff (17).

To determine the functional categories of differentially expressed genes (DEGs), all DEGs were subjected to GO and KEGG pathway analyses. GO enrichment analysis was used to map all DEGs to GO terms in the GO database.[2] The significance was calculated using a hypergeometric test by Yang (18).

To better understand the biological function of DEGs, all DEGs were annotated to KEGG (Kyoto encyclopedias of genes and genomes) pathways.[3]

Quantitative RT-PCR Validation

In order to verify the reproducibility and repeatability of gene expression data obtained by RNA-Seq, seven genes were selected for QRT PCR verification. In brief, cDNA was generated from total RNA using the PrimeScript II 1 st Strand cDNA Synthesis Kit (Takara, Dalian, China) following the manufacturer's instruction. Quantitative RT-PCR analysis was carried out with the cDNA using SYBR green on a Roche LightCycler 96 real-time PCR machine (Roche, Basel, Switzerland). The b-Actin was used as a reference gene for the standardization of the results. The relative expression levels were calculated as described previously (15). Three biological repeats were measured for each sample. The primers used were shown in **Table 2**.

TABLE 2 | Oligonucleotide primers used for quantitative real-time PCR.

Gene name	PrimerName	Sequence (5′-3′)
FABP3	FABP3-F	TGGAGTCGAGTTCGATGAG
	FABP3-R	TTTCCCGCACAAGTGATGTC
ACSL1	ACSL1-F	TACGAAGGCTACGGACAGAC
	ACSL1-R	CCTTGGCAGCCAGGTAATTC
SCD	SCD-F	AGCTGAGAAGCTGGTGATGT
	SCD-R	CAGCGTAACGGAGAAAGGTG
FAS	FAS-F	TGCTGTGCAACTATGCCCTA
	FAS-R	CAGGTGAGGAAGGTGACAGT
HSL	HSL-F	ATCTCCAGCGGACTGGTGTC
	HSL-R	GCACCTGGATCTCGGTGATA
ADPN	ADPN-F	TGGAGAAGGGTGACCAAGTC
	ADPN-R	AAGGAGGAGTCATGGACGTT
FoxO1	FoxO1-F	GTGACATCATGACGCCAGTC
	FoxO1-R	GATGTTGACTGAGCGTGTCC
Actin	Actin-F	TACAATGTGGCCGAGGACTT
	Actin-R	GAGAGAAGGAGGGTGGCTTT

FABP3, Fatty acid binding protein 3; ACSL1, Long-chain acyl-CoA synthetase 1; SCD, stearoyl-CoA desaturase; FAS, Fatty acid synthase; HSL, Hormone sensitive lipase; ADPN, Adiponectin; and FoxO1, Forkhead transcription factor 1.

Statistical Analyses

The serum biochemistry and hormone indexes (n = 6) were statistically analyzed by one-way ANOVA with SPSS statistical software (Ver.20 for windows, SPSS), and Tukey–Kramer's test was used to compare differences among the treatment groups. The muscle nutrients, fatty acid composition, and activity of the fatty metabolizing enzyme of muscle (n = 4) were statistically analyzed by T-test with SPSS statistical software (Ver.20 for windows, SPSS). All values were expressed as mean ± SE, P-value < 0.05 was considered to be significant and $0.05 \leq P < 0.10$ was considered as a tendency.

[1] ftp://ftp.ensembl.org/pub/release-75/fasta/bos_taurus/dna/

[2] http://geneontology.org

[3] https://www.genome.jp/kegg/pathway.html

RESULTS

Blood Biochemical Characteristics

As presented in **Table 3**, compared with the control group, dietary supplementation with *Puerarin* by 400 mg/kg and 800 mg/kg decreased the levels of LEP ($P < 0.001$), and the content of TC was reduced in the Pue200 group compared to control and Pue800 ($P = 0.048$). Moreover, the concentration of COR in the Pue400 group was decreased compared with the Pue200 group ($P = 0.056$). No difference was noticed on the contents of INS, T3, T4, ADPN, TG, HDL-C, and LDL-C among all groups.

Nutritional Components and Fatty Acid Composition of Muscle

As presented in **Table 4**, compared with the control group, the concentration of CP and CF were increased in the Pue800 group ($P = 0.039$ and $P = 0.025$, respectively). No difference was noticed about the contents of moisture, CA, Ca, and P among all groups. However, dietary supplementation with *Puerarin* by 400 mg/kg enhanced the contents of tetradecenoic acid (C14:1) and heptadecenoic acid (C17:1) compared with the control group ($P = 0.038$ and $P = 0.020$, respectively). Moreover, the Pue400 treatment tended to increase the contents of hexadecenoic acid (C16:1) compared with the control group in **Table 5** ($P = 0.079$).

TABLE 3 | Effects of puerarin on the blood biochemical characteristics in beef cattle under hot environment.

Item	Groups				*P*-value
	Control	Pue200	Pue400	Pue800	
INS (uIU/ml)	16.02 ± 1.32	16.57 ± 1.32	14.89 ± 2.59	12.69 ± 1.47	0.435
T3 (ng/ml)	1.36 ± 0.07	1.33 ± 0.10	1.20 ± 0.07	1.16 ± 0.16	0.507
T4 (ng/ml)	51.35 ± 3.42	50.69 ± 3.17	47.88 ± 1.09	48.51 ± 2.12	0.749
COR (ng/ml)	48.54 ± 1.72^{ab}	51.59 ± 2.59^{a}	45.83 ± 1.33^{b}	49.43 ± 1.39^{ab}	0.056
ADPN (mg/L)	14.92 ± 1.38	15.28 ± 1.68	14.51 ± 0.89	14.37 ± 1.03	0.561
LEP (ng/ml)	10.36 ± 0.28^{a}	9.46 ± 0.70^{a}	6.91 ± 0.36^{b}	5.64 ± 0.36^{b}	<0.001
TC(mmol/L)	4.44 ± 0.15^{a}	3.68 ± 0.28^{b}	4.26 ± 0.22^{ab}	4.54 ± 0.21^{a}	0.048
TG (mmol/L)	0.34 ± 0.05	0.34 ± 0.03	0.29 ± 0.03	0.35 ± 0.04	0.638
HDL-C(mmol/L)	2.46 ± 0.28	2.20 ± 0.24	2.67 ± 0.24	2.58 ± 0.19	0.545
LDL-C(mmol/L)	0.78 ± 0.13	0.70 ± 0.12	0.69 ± 0.10	0.87 ± 0.16	0.751

^{a,b}Means within a row with no common superscript differ significantly (P < 0.05). All traits in this table were analyzed with cattle as the experimental unit (n:6). INS, insulin; T3, triiodothyronine; T4, thyroxine; COR, cortisol; ADPN, adiponectin; LEP, leptin; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; and LDL-C, low density lipoprotein cholesterol.

TABLE 4 | Effect of Puerarin on the nutritional components of muscle in beef cattle under heat stress.

Item	Groups			*P*-value
	Control	Pue400	Pue800	
Moisture/%	71.55 ± 1.38	67.50 ± 2.74	70.68 ± 0.88	0.225
Crude protein/%	20.27 ± 0.43^{b}	19.54 ± 0.54^{b}	23.81 ± 0.15^{a}	0.039
Crude fat/%	4.31 ± 0.40^{b}	4.67 ± 0.52^{b}	5.20 ± 0.32^{a}	0.025
Crude ash/%	4.14 ± 0.16	3.87 ± 0.52	4.15 ± 0.23	0.234
Ca,mmol/g	0.58 ± 0.01	0.56 ± 0.006	0.57 ± 0.005	0.367
P,mmol/g	0.98 ± 0.13	0.87 ± 0.08	0.85 ± 0.01	0.554

^{a,b}Means within a row with no common superscript differ significantly (P < 0.05). All traits in this table were analyzed with cattle as the experimental unit (n:4).

TABLE 5 | Effects of Puerarin on fatty acid composition of *longissimus thoracis* muscle beef under heat stress (%).

Items	Groups			*P*-value
	Control	Pue400	Pue800	
C14:0	1.94 ± 0.16	2.63 ± 0.42	2.37 ± 0.34	0.370
C14:1	0.48 ± 0.02^{b}	0.62 ± 0.03^{a}	0.56 ± 0.06^{ab}	0.038
C15:0	0.25 ± 0.04	0.22 ± 0.01	0.26 ± 0.01	0.263
C16:0	20.92 ± 1.35	24.15 ± 1.17	23.93 ± 1.25	0.184
C16:1	2.62 ± 0.21	2.88 ± 0.12	2.49 ± 0.20	0.079
C17:0	0.62 ± 0.07	0.62 ± 0.02	0.69 ± 0.05	0.571
C17:1	0.41 ± 0.01^{b}	0.51 ± 0.06^{a}	0.39 ± 0.04^{b}	0.020
C18:0	17.00 ± 0.88	16.64 ± 0.72	18.21 ± 2.23	0.733
C18:1n9t	0.35 ± 0.05	0.37 ± 0.03	0.29 ± 0.02	0.278
C18:1n9c	38.81 ± 1.19	39.85 ± 1.25	36.75 ± 1.22	0.243
C18:2n6t	0.22 ± 0.02	0.19 ± 0.02	0.18 ± 0.00	0.412
C18:2n6c	2.95 ± 0.28	2.52 ± 0.34	2.84 ± 0.13	0.512
C20:0	0.18 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.126
C20:1	0.24 ± 0.07	0.18 ± 0.03	0.19 ± 0.03	0.610
C18:3n3	0.17 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	0.544
Other acids	11.28 ± 1.52	8.29 ± 1.34	10.00 ± 0.14	0.220

^{a,b}Means within a row with no common superscript differ significantly (P < 0.05). All traits in this table were analyzed with cattle as the experimental unit (n:4).

The Activity of the Fatty Metabolizing Enzyme

The results in **Figure 1** showed that diet supplemented with 400 mg/kg *Puerarin* improved the activity of FAS in LT (D) (P = 0.044), but the activity of HSL in the Pue800 was decreased compared with the control group (A) (P = 0.006). No difference was noticed on the activity of LPL and ACC among the three groups.

Overall Assessment for Mapping Statistics

The overall assessment for mapping statistics is shown in **Table 6**. The RNA-Seq of eight LT samples yielded around 4 billion raw reads. After quality filtering, the high-quality sequence data in each muscle sample was approximately 5.03 gigabases (Gb), ranging from 4.48 to 5.87 Gb. The correlation analysis based on the gene expression profiles found that the correlations between biological replicates were greater than 0.952 (**Figure 2**), the high reproductivity between samples indicated that the sequencing data could be used for further analyses.

Gene Differential Expression Analysis

As shown in **Figure 3**, there were 833 differentially expressed genes (DEGs) were found in LT muscles between the control and *Puerarin* groups, these DEGs were categorized into three gene ontology categories: molecular function, biological process, and cellular component (**Figure 4**). The top five cellular component categories of DEGs between the control and *Puerarin* groups included "binding," "catalytic activity," "molecular function regulator," "transport activity" and "molecular transducer activity." The top five biological processes of DEGs included "cellular process," "biological regulation," "metabolic process," "response stimulus" and "developmental process." The top five

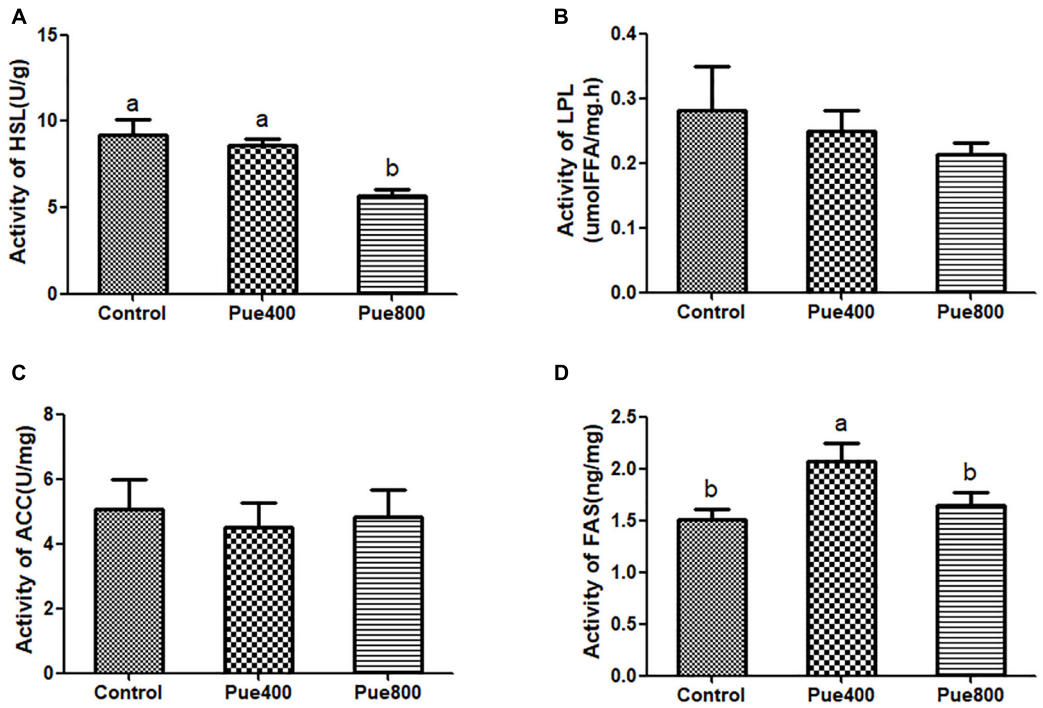

FIGURE 1 | Effect of *Puerarin* on the activity of fat metabolizing enzymes in heat stressed beef cattle (n = 4). Control: dietary supplementation with 0 mg/kg *Puerarin* in the feed concentrate; Pue400: dietary supplementation with 400 mg/kg *Puerarin* in the feed concentrate; Pue800: dietary supplementation with 800 mg/kg *Puerarin* in the feed concentrate. HSL, hormone sensitive lipase **(A)**; LPL, lipoprotein lipase **(B)**; ACC, acetyl CoA carboxylase **(C)**; FAS, fatty acid synthase **(D)**. ^{a,b}Means within a row with no common superscript differ significantly ($P < 0.05$).

TABLE 6 | Summary statistics for sequence quality and alignment information of eight *longissimus thoracis* muscle samples in two groups.

Items	Sample name							
	C1	C2	C3	C4	Pue1	Pue2	Pue3	Pue4
Raw reads	48652354	48175450	49644444	50765412	52708852	59764926	45526386	52692342
Clean reads	48120844	47648332	49077762	50105526	52105286	58726390	44767384	51755462
Valid Ratio%	98.91	98.91	98.86	98.70	98.85	98.26	98.33	98.22
Q30(%)	94.87	94.91	94.91	94.56	94.87	93.16	93.73	93.53
GC content(%)	54.16	53.19	53.73	54.00	53.97	53.93	53.88	54.10
Total reads	48120844	47648332	49077762	50105526	52105286	58726390	44767384	51755462
Reads Total mapped	46336007	45862232	47212767	48232193	50036325	55815890	42614063	49308979
Multiple mapped	3273916	4078776	3636977	3706831	4449377	4900461	3691312	4408835
Uniquely mapped	43062091	41783456	43575790	44525362	45586948	50915429	38922751	44900144
Mapping rate (%)	96.29	96.25	96.20	96.26	96.03	95.04	95.19	95.27

C1, C2, C3, C4 were four samples of the Control group (dietary supplementation with 0 mg/kg Puerarin in the feed concentrate), and Pue1,Pue2,Pue3,Pue4 were four samples of the Pue400 group (dietary supplementation with 400 mg/kg Puerarin in the feed concentrate).

cellular components of DEGs included "cell part," "organelle," "membrane part," "membrane" and "organelle part." Among the total 833 DEGs, 341 DEGs upregulated and 492 DEGs downregulated were identified in the *Puerarin* group compared with the control group.

There were 20 DEGs related to lipid metabolism, and *Puerarin* treatment enhanced the expression of 15 genes including FATP5, CD36, FABP3, FABP7, ACSL1, Gadd45G, SCD, IRS3, FAS (**Table 7**). To validate the reliability of the transcriptomic sequencing analyses, 7 differentially expressed genes were randomly selected for qRT-PCR verification (**Table 2**). As shown in **Figure 5**, the results from both methods were largely consistent, suggesting that the RNA-Seq results were credible.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the downregulated genes demonstrated. There were 36 significantly enriched signaling pathways ($P < 0.05$). As shown in **Table 8** in the top 20 significantly enriched signaling pathways, peroxisome

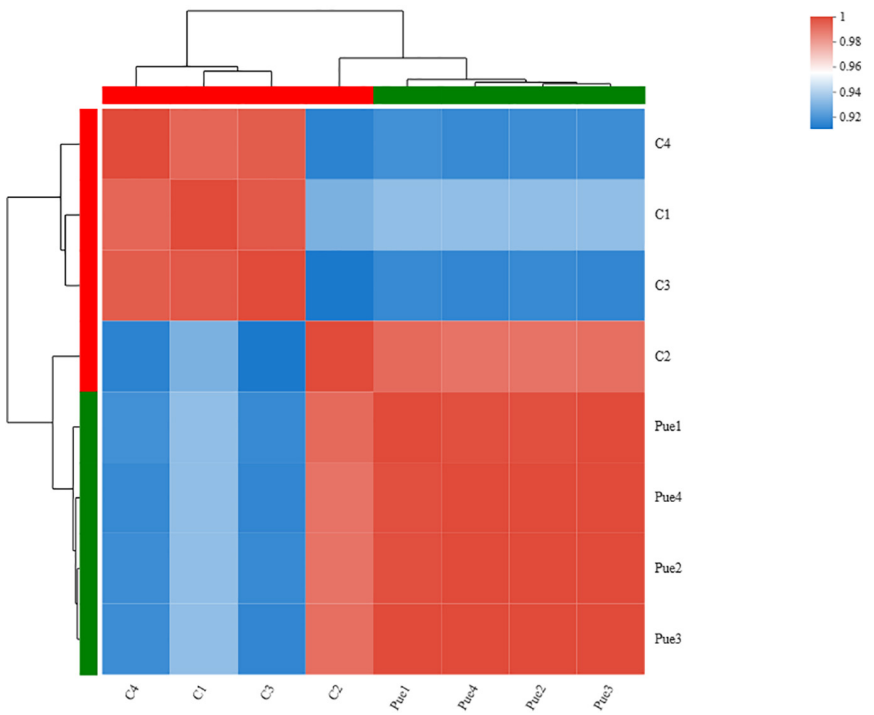

FIGURE 2 | Correlations of eight samples (*n* = 4). C1, C2, C3, C4 were four samples of the Control group (dietary supplementation with 0 mg/kg *Puerarin* in the feed concentrate), and Pue1, Pue2, Pue3, Pue4 were four samples of the Pue400 group (dietary supplementation with 400 mg/kg *Puerarin* in the feed concentrate). In the figure, the right and lower sides are the sample names, the left and upper sides are the sample clustering, and the different color squares represent the correlation of the two samples.

proliferator-activated receptor (PPAR) signaling pathway (P = 7.73E−07), adenylate activated protein kinase (AMPK) signaling pathway (P = 7.22E−05) and forkhead transcription factor (FoxO) signaling pathway (P = 1.84E−02) were closely related to lipid metabolism and meat quality in animals.

DISCUSSION

The present study was performed under high temperature and humidity during the summer months (the average THI = 81.81), which indicated that the experimental beef cattle were in a state of heat stress according to the report of Armstrong (19). Studies have shown that heat-stressed can activate the hypothalamic-pituitary-adrenal (HPA) axis, causing a series of complex physiological and metabolic changes, such as elevated level of corticosterone hormone and leptin, which can indirectly reflect the impact of heat-stressed on animals (20). Leptin, an adipokines secreted by adipose, plays an effective role in energy homeostasis, neuroendocrine function and metabolism (21). A study has shown that the concentration of leptin in the blood would increase under heat stress (22). The current results showed that *Puerarin* treatments declined the levels of leptin significantly compared with the control group, which indicates dietary supplementation with *Puerarin* may relieve the disordered endocrine function of beef cattle due to heat stress. *Puerarin* could block the increased levels of the adrenocortico-tropic hormone in the serum, which is induced by single prolonged stress (SPS) (23). Therefore, we can conclude that *Puerarin* can relieve the response of heat stress.

Puerarin has been shown to have a direct effect on lipid metabolism in our study. The concentrations of triglyceride (TG) and total cholesterol (TC) in serum can be used as an important index of lipid metabolism, and leptin can inhibit the expression of fatty acid synthase, which is negatively correlated with fat deposition. In this experiment, *Puerarin* treatment with 200 mg/kg decreased the levels of TC, and the levels of leptin in Pue400 and Pue800 groups were significantly lower than those in the control group, which confirmed that *Puerarin* had a direct role in promoting lipid metabolism. Some studies have mentioned that *Puerarin* has a negative effect on animal fat deposition (24, 25). While, other studies have shown that the addition of *Puerarin* enhanced preadipocyte differentiation as well as lipid accumulation (26, 27). The reason for different result may be the treatment concentration of puerarin and the species of laboratory animal.

The content and composition of fatty acids in muscle are closely related to muscle quality (28). Yang found that the content of monounsaturated fatty acids (such as oleic

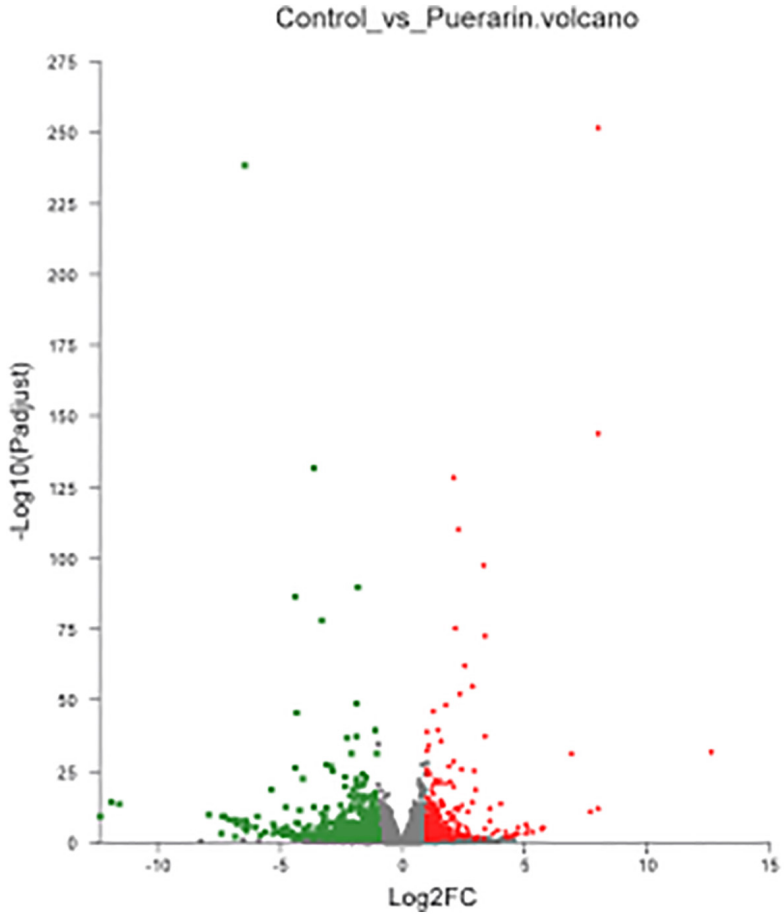

FIGURE 3 | Volcano plot of the differentially expressed genes between control and *Puerarin* groups of *longissimus thoracis* muscles (n = 4). Control: dietary supplementation with 0 mg/kg *Puerarin* in the feed concentrate; *Puerarin*: dietary supplementation with 400 mg/kg *Puerarin* in the feed concentrate. The red dots represent the up-regulated DEGs, the green dots represent the down-regulated DEGs and the blue dots represent non-DEGs.

acid and linolenic acid) was correlated with flavor positively, which can help prevent diseases and is beneficial to human health when absorbed unsaturated fatty acids appropriately (29). Tan found that adding isoflavones to the diet can reduce the level of saturated fatty acids in the muscle of the goat, increase the level of monounsaturated fatty acids, and increase the ratio of n-6 to n-3 fatty acids (30). Study has shown that grazing mutton has a better flavor than barn-fed sheep meat,another study found that C14:1 in grazing sheep meat was significantly higher than that in barn-fed sheep meat (31, 32). In this experiment, adding 400 mg/kg *Puerarin* significantly increased the content of C14:1 and C17:1 in muscle, which indicated that *Puerarin* can improve the flavor of postmortem beef.

Fatty acid synthase play an important catalytic role in the synthesis of long-chain fatty acids (33), and HSL is the key enzyme of regulating the rate of lipolysis. In this experiment, the activities of FAS in LT were significantly increased in the Pue400 group, while the activities of HSL were decreased in the Pue800 group, which suggested that *Puerarin* could regulate fat metabolism and promote fat synthesis. These results agree well with the findings of Zhao, which indicated that adding *Daidzein*, a similar structure with *Puerarin*, can affect the lipid metabolism and promote intramuscular fat deposition of Xiangzhong black cattle (34).

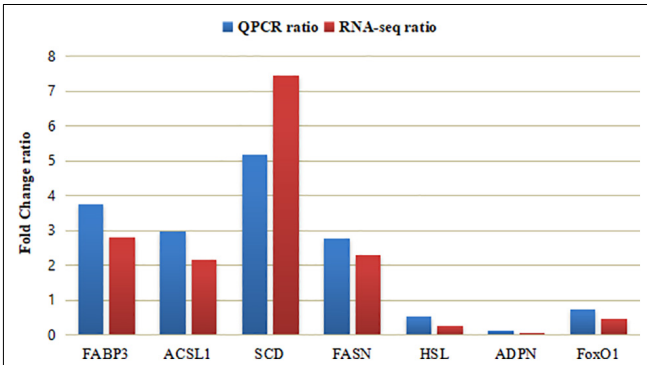

FIGURE 4 | Gene ontology (GO) annotation of differentially expressed genes.

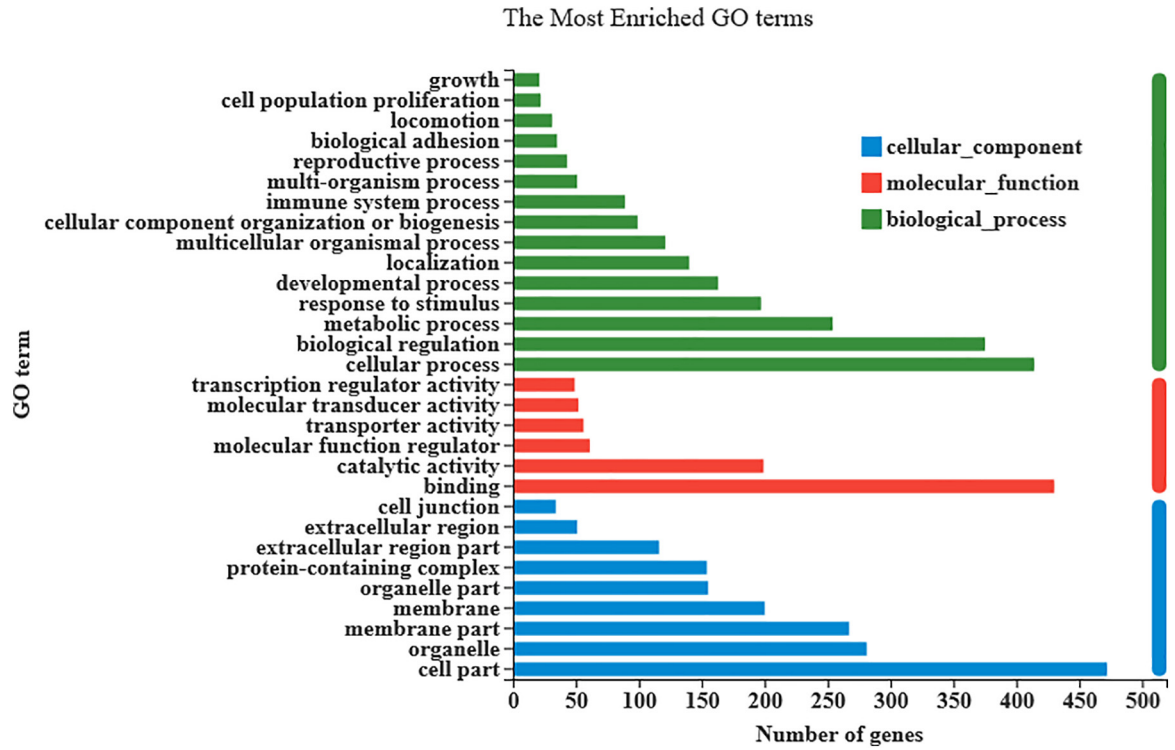

FIGURE 5 | Comparing analysis of relative gene expression in Control and *Puerarin* group (n = 4). Control: dietary supplementation with 0 mg/kg *Puerarin* in the feed concentrate; *Puerarin*: dietary supplementation with 400 mg/kg *Puerarin* in the feed concentrate. FABP3, Fatty acid binding protein 3; ACSL1, Long-chain acyl-CoA synthetase 1; SCD, stearoyl-CoA desaturase; FAS, Fatty acid synthase; HSL, Hormone sensitive lipase; ADPN, Adiponectin; and FoxO1, Forkhead transcription factor 1.

Further, to reveal the molecular mechanism of *Puerarin* promoting intramuscular fat deposition, high-throughput sequencing of mRNA technology (RNA-Seq) was performed. The current results showed that *Puerarin* treatment with 400 mg/kg up-regulated 341 DEGs and down-regulated 492 DEGs in LT muscle, and these DEGs are mainly enriched in the PPAR signaling pathway, AMPK signaling pathway, and FoxO signaling pathway. Among them, the PPAR signaling pathway and AMPK signaling pathway are correlated with lipid metabolism and meat quality, and the FoxO signaling pathway is associated with cell-lipid differentiation. The FoxO signaling pathway plays an important role in regulating preadipocyte differentiation (35). After differentiation of preadipocytes, the synthesis and deposition of triglycerides in adipose cells were accelerated, which increased the volume of fat cells. Lu found that FoxO could enhance glucose synthesis and lipolysis (36). Sakamoto suggested that the expression of the Gadd45 gene could be regulated directly by the FoxO signaling pathway, which could promote the differentiation of preadipocytes by participating in DNA methylation in cells (37). Chen found that activated FOXO1 binds to the PPARγ promoter and inhibits the transcriptional activity of PPARγ by competitively suppressing the formation of functional PPARγ/RXR/DNA complex, thereby inhibiting lipogenesis and adipocyte differentiation (38). Our results showed that the expression of FoxO1 genes in the FoxO signaling pathway in the *Puerarin* group was down-regulated significantly and the expression of the Gadd45 gene was up-regulating, which indicated *Puerarin* could promote the differentiation of fat cells in the heat-stressed beef cattle muscles.

Mammalian body contains four important fat depots, namely, visceral, subcutaneous, intermuscular, and intramuscular (IM) fat. But among them, the IM fat is considered one of the most important factors that determines carcass quality traits (39). PUFAs was decreased with the increasing IMF%, which may account for the reduce of PUFAs content in the *Puerarin* group (40). From the view of molecular, the deposition of adipose tissue is essentially the result of spatiotemporal specific expression regulation of many adipose synthesis genes (41). Studies have shown that the PPAR signaling pathway plays a leading role in the process of lipid synthesis, which can directly regulate the expression of SCD, FAS, FABP, GLUT4 and other genes (42, 43), and promote glucose absorption and fat synthesis of adipocytes when it was activated (44).

The PPAR signaling pathway regulates cellular differentiation, energy balance, and lipid metabolism (45). PPAR has three exists isoforms, α, β and γ (46). Furthermore, it was reported that activation of PPARγ is to be essential for deposition of intramuscular fat (47). PPARγ can increase lipid deposition

TABLE 7 | Differentially expressed genes related to lipid metabolism.

Gene ID	Gene name	Control	*Puerarin*	*P*-value	Expression trend
Gene18657	FATP5	0.85	1.83	2.14E−07	UP
Gene4568	CD36	0.44	0.97	1.36E−02	UP
Gene1326	FABP3	294.41	822.62	1.39E−03	UP
Gene9621	FABP7	0.69	1.83	1.15E−02	UP
Gene24921	ACSL1	52.49	113.48	2.03E−03	UP
Gene18229	Gadd45G/	1.84	4.09	3.73E−04	UP
Gene24505	SCD	3.67	27.35	1.98E−05	UP
Gene24080	IRS3	0.03	0.78	7.40E−06	UP
Gene19867	FAS	6.90	15.79	2.24E−06	UP
Gene17954	HSL	11.4	2.97	1.86E−03	DOWN
Gene7009	ACOX3	14.02	3.62	2.85E−04	DOWN
Gene470	ADPN	89.61	4.20	5.73E−07	DOWN
Gene4212	LEP	0.42	0.02	4.98E−07	DOWN
Gene14329	ADCY8	0.03	0.11	3.14E−02	UP
Gene16089	PIK3CD	0.45	0.94	2.79E−05	UP
Gene4517	PIK3CG	0.52	1.33	1.81E−03	UP
Gene6887	PRKG2	0.33	0.83	4.50E−04	UP
Gene12812	FoxO1	14.23	6.57	3.12E−03	DOWN
Gene6918	MAPK10	0.02	0.80	1.81E−02	UP
Gene19063	CAMKK1	0.21	0.50	2.38E−02	UP

Control: dietary supplementation with 0 mg/kg Puerarin in the feed concentrate; Puerarin: dietary supplementation with 400 mg/kg Puerarin in the feed concentrate. FATP5, Fatty acid transporter protein 5; CD36, Fatty acid transposase; FABP, Fatty acid binding protein; ACSL1, Long-chain acyl-CoA synthetase 1; Gadd45G/, Growth arrest and DNA Damage-inducible Protein GADD45 gamma; SCD, stearoyl-CoA desaturase; IRS3, Insulin receptor substrate 3; FAS, Fatty acid synthase; HSL, Hormone sensitive lipase; ACOX3, Acyl-CoA oxidase 3; ADPN, Adiponectin; LEP, Leptin; ADCY8, Adenylate cyclase 8; PIK3CD, Phosphoinositide-3-kinase catalytic delta polypeptide; PIK3CG, Phosphoinositide-3-kinase catalytic gamma polypeptide; PRKG2, Protein kinase cGMP-dependent type II; FoxO1, Forkhead transcription factor 1; MAPK10, Mitogen activated protein kinase 10; and CAMKK1, Calcium/calmodulin dependent protein kin 1.

TABLE 8 | Classification of DEG according to the KEGG pathways enrichment analysis.

Pathway name	Input number	Background number	*P*-value
PPAR signaling pathway	17	93	7.73E−07
AMPK signaling pathway	18	150	7.22E−05
Regulation of lipolysis in adipocytes	12	65	7.57E−05
Insulin resistance	15	125	4.48E−04
Natural killer cell-mediated cytotoxicity	20	221	8.69E−04
Adipocytokine signaling pathway	11	79	1.26E−03
Phagosome	19	225	2.61E−03
Neuroactive ligand-receptor interaction	25	364	5.19E−03
Type II diabetes mellitus	8	53	5.36E−03
Kaposi sarcoma-associated herpesvirus infection	20	263	5.54E−03
Breast cancer	15	176	8.64E−03
Cellular senescence	17	217	8.85E−03
Human T-cell leukemia virus 1 infection	24	389	1.63E−02
Estrogen signaling pathway	13	154	1.69E−02
Viral carcinogenesis	20	298	1.77E−02
FoxO signaling pathway	13	153	1.84E−02
Influenza A	16	228	2.64E−02
Hepatitis C	16	226	2.69E−02
C-type lectin receptor signaling pathway	10	108	2.71E−02
Endocrine resistance	10	110	2.80E−02

in adipocytes by regulating the levels of expression of HSL, LEP, ADPN and other cytokines produced by adipose tissue (48), and regulating the transcription of a variety of genes involved in fat synthesis, such as FATP, FABP, CD36 (49, 50). After the activation of PPARγ, the levels of expression of FABP3/FABP7, ACSL1, CD36, IRS3, SCD, FAS and other genes in the pathway were significantly up-regulated, and the levels of expression of HSL, LEP, ADPN and other genes were significantly down-regulated. Among the up-regulated genes, FABP is a member of the fatty acid-binding protein family and plays a very important role in the uptake of long-chain fatty acids. ACSL1 can prolong the long-chain fatty acids in cells (51), IRS3 is the receptor of short-chain fatty acids on cell membrane (52), SCD is the main enzyme for *de novo* synthesis of monounsaturated fatty acids (53), FAS is the key enzyme in the process of fatty acid synthesis. Among the down-regulated genes, HSL, LEP and ADPN are the key factors affecting lipolysis. ACSL1 is elevated by PPARγ agonists in the adipose tissue, and ACSL1 overexpression can promote triglyceride accumulation in adipocytes (54, 55). A previous study showed that the higher ACSL1 expression in the F line than the C line coincided with the greater IMF deposition found in the former (56), which was in line with our result. In bovine mammary glands, mRNA abundance at 60 days postpartum of FABP3 and ACSL1 were 80- and 7-fold greater relative to 15 days antenatal, respectively, which are significantly associated with milk fat synthesis (57). Kae found that isoflavone daidzein and its metabolite equol enhance adipocyte differentiation through activating PPARγ (58). Genistein, a main soy isoflavone, can directly bind to and activate peroxisome proliferators-activated receptor a (PPARa) or PPARc (59). Therefore, the activation of PPAR and the expression of its downstream regulatory genes are the most fundamental reason for promoting fat deposition, especially intramuscular fat deposition.

CONCLUSION

In a word, *Puerarin* can activate the PPARγ signaling pathway, up-regulate the levels of expression of genes related to fat synthesis, and down-regulated genes expression promoting muscle fatty acid oxidation, so as to regulate lipid metabolism, improve the beef flavor of Jinjiang cattle and enhance intramuscular fat deposition in LT muscle of heat-stressed beef cattle.

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 reviewed and approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-20190015).

AUTHOR CONTRIBUTIONS

XSo, HC, and TP designed the overall study. HS, XSh, XZ, and MQ performed the animal feeding experiment and sample analysis. XSo and HC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Quality More Than Quantity: The Use of Carbohydrates in High-Fat Diets to Tackle Obesity in Growing Rats

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Childhood obesity prevention is important to avoid obesity and its comorbidities into adulthood. Although the energy density of food has been considered a main obesogenic factor, a focus on food quality rather that the quantity of the different macronutrients is needed. Therefore, this study investigates the effects of changing the quality of carbohydrates from rapidly to slowly digestible carbohydrates on metabolic abnormalities and its impact on obesity in growing rats fed a high-fat diet (HFD). Growing rats were fed on HFD containing carbohydrates with different digestion rates: a HFD containing rapid-digesting carbohydrates (OBE group) or slow-digesting carbohydrates (ISR group), for 4 weeks and the effect on the metabolism and signaling pathways were analyzed in different tissues. Animals from OBE group presented an overweight/obese phenotype with a higher body weight gain and greater accumulation of fat in adipose tissue and liver. This state was associated with an increase of HOMA index, serum diacylglycerols and triacylglycerides, insulin, leptin, and pro-inflammatory cytokines. In contrast, the change of carbohydrate profile in the diet to one based on slow digestible prevented the obesity-related adverse effects. In adipose tissue, GLUT4 was increased and UCPs and PPARγ were decreased in ISR group respect to OBE group. In liver, GLUT2, FAS, and SRBP1 were lower in ISR group than OBE group. In muscle, an increase of glycogen, GLUT4, AMPK, and Akt were observed in comparison to OBE group. In conclusion, this study demonstrates that the replacement of rapidly digestible carbohydrates for slowly digestible carbohydrates within a high-fat diet promoted a protective effect against the development of obesity and its associated comorbidities.

Keywords: obesity, slow digestive carbohydrates, metabolism, lipidomic analysis, growing rats

INTRODUCTION

The global prevalence of childhood obesity is increasing in developed and developing countries; it is considered the biggest public health challenge of the 21st Century. The World Health Organization reports that over 340 million children and adolescents aged 5–19 presented overweight or obesity in 2016. The increase was similar in boys and girls: in 2016, 18% of girls and 19% of boys were overweight (1). The rapid transition from underweight to overweight and obesity children has been identified as a relevant public health problem with social consequences (2), especially in developing countries.

Overweight and obesity in children are linked to serious short- and long-term complications. Studies have shown that overweight and obesity children are likely to retain their weight into adult life, increasing the incidence of non-communicable diseases and the risk of morbidity and premature death in adulthood (1, 3). Furthermore, children with obesity could have future risks: breathing problems, increased risk of fractures, hypertension, and early markers of cardiovascular disease, insulin resistance, and psychological effects (4).

Obesity is a complex multifactorial disease that affects all the organs in the body. It is characterized by irregular or excessive fat accumulation, as a consequence of an imbalance between energy intake and energy expenditure combined with a genetic predisposition for weight gain (2). Epidemiological studies have identified a correlation between dietary fat intake and obesity and its related complications (5). In addition, studies in children aged 5–7 years, and followed up to 15 years, found that the dietary pattern consisting of a diet high in energy density, fats, and sugars and scarce in fiber, fruits, and vegetables was associated with higher percentage of body fat and an excess of adiposity in childhood and adolescence (6, 7). Other factors in the lifestyle of children, such as insufficient physical activity and excessive sedentarism, and food consumption of high-calorie sweetened beverages, have also been related to the increase of childhood obesity (8, 9).

The high energy density of food has been considered an obesogenic factor. However, several studies using energy-dense foods have failed to establish a relationship with weight gain, leading to the suggestion that the obesogenic capacity of foods may be due to their composition rather than their energy density. In addition to reducing calorie intake, an alternative would be to identify the role of certain nutrients and how they should be distributed in diets. For this, diets have been proposed for the prevention or treatment of obesity that uses different sources of carbohydrates (CHO), proteins, and fats combined in turn in different proportions. However, preventing obesity may need to focus on the quality rather than the quantity of the different macronutrients.

Therefore, behavioral strategies to decrease caloric intake, decrease sedentary lifestyle, and increase physical activity are the key for pediatric weight management. The standard approach in treating obesity involves reducing dietary fat, the most energy-dense nutrient. However, the weight loss characteristics of a reduced-fat diet are moderate and transient (10). Recently, dietary CHO have been proposed as a target for the treatment of obesity.

Carbohydrates are essential for growth and development and one of the main sources of energy in infancy and childhood. As indicated previously (8), an increase in the consumption of added sugars is associated with increased obesity in children. Concerning the quality of CHO, epidemiological studies have suggested different measures for their evaluation, the main criteria being the consumption of whole grains, the intake of dietary fiber, the solid or liquid form of CHO, and the glycemic index (GI) (11). GI classifies CHO-containing foods based on the postprandial glucose response and represents the quality of CHO. A higher GI produces a faster rise in the postprandial serum glucose and rapid insulin response. The fast response to insulin leads to rapid hypoglycemia, combined with hunger and therefore, a higher caloric intake. On the contrary, a low GI diet translates into a slower absorption of CHO and less blood glucose fluctuations reflecting a better glycemic control (12, 13). An adult study shows how the consumption of slow-digesting sugars such as isomaltulose in comparison to sucrose can help with weight management by reducing fat. In contrast, studies of pre-adolescent children have revealed that when high-glycemic CHO are consumed for breakfast, the amount of food consumed during lunch is greater. In summary, these previous studies indicate that the intake of different types of CHO [i.e., rapid-digested versus slow-digested CHO (ISR)] not only affects the blood sugar response but also affects subsequent energy intake (14). In terms of animals, previous studies have shown that the mother's consumption of ISR induce a greater metabolic flexibility later on in the growth and development of the offspring, regulating lipid metabolism, improving muscle functionality, and reducing predisposition to liver disease (15–17). However, studies conducted in humans have reported conflicting results in the relationship between GI and obesity, suggesting the importance of not only quantity but also the CHO profile of the diet (18).

In the present study, we investigate the effects of changing the quality of CHO from rapidly digestible CHO to slowly digestible CHO on metabolic abnormalities and its impact on overweight and obesity in growing rats upon high-fat diet (HFD) feeding. Our working hypothesis is that the quality of CHO that have a low GI and sustained glucose release (such as isomaltulose and Sucromalt®) and prebiotic effects (such as resistant maltodextrin, inulin, and fructooligosaccharides) might prevent these metabolic alterations. In our study, the main results clearly showed that the replacement of rapid digestible CHO for slowly digestible CHO (ISR) in a HFD prevented the HFD-obesity-related adverse effects and improve lipid metabolism and glucose control through different mechanisms including regulation of hormones, such as insulin and leptin, and incretins (GLP-1), as well as the management of cellular metabolic pathways related to lipogenesis and protein homeostasis.

MATERIALS AND METHODS

Housing

Thirty weanling male Wistar Han International Genetic Standard (IGS) rats (21–25 days old) were provided by Charles Rivers (Orleans Cedex, France). Animals were individually housed in cages and kept under 12 h light-12 h dark cycles. The room temperature was maintained at 21°C.

All experimental procedures (approval code 29/10/2018/152) were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985) as well as to the ethical guidelines for animal experimentation provided by the Spanish National Research Council (RD 1201/2005 October 10).

Experimental Design

An HFD-induced animal model has been considered as an appropriate model to study dietary obesity. This animal model is adequate to study the mechanisms by which dietary fat influences the regulation of energy balance (14). Post-weaning rats in a HFD also presented a phenotype of metabolic syndrome and increased hepatic steatosis (11).

Rats (n = 10/group) were randomly assigned to three nutritional groups based on the diet received:

(i) NOB, a lean group. Rats were fed on a standard rodent diet (AIN93G) (19).

(ii) OBE, an obese group. Rats were fed on an HFD (20).

(iii) OBE-ISR, an obese group. Rats were fed on an HFD formulated with ISR.

All diets were formulated to have the same amount of total fiber. In addition, both HFD were designed to be isoenergetic. All information regarding the diets is listed in **Table 1**.

The nutritional intervention was conducted for 4 weeks. All rats had free access to food and water, and animal weight and food consumption were determined weekly. Body composition, body fat mass, and lean body mass were measured at baseline and the end of the nutritional treatment by quantitative nuclear magnetic resonance imaging (EchoMRI 700 system; Echo Medical Systems, Houston, TX, United States). After the feeding period, rats were introduced to a Phenomaster Indirect Calorimetry System (TSE System, Bad Homburg, Germany) for analyzing energy expenditure.

At the end of the study, animals were euthanized in post-absorptive conditions [1 h after an oral meal tolerance challenge with their corresponding experimental diets; 10 kcal diet/kg of body weight (BW)]. Blood samples were taken before (12 h fasting) and after oral gavage. Blood was collected either into serum tubes or in tubes containing anticoagulant EDTA (for isolating plasma). Tissues were immediately isolated, weighed, and snap-frozen in liquid nitrogen and kept at −80°C for posterior analysis.

Biochemical Parameters

A hemogram was analyzed using the hematology analyzer Pentra 80 (Horiba ABX, Montpellier, France). Serum/plasma was collected and parameters such as glucose, triacylglycerols (TG), cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, and non-esterified fatty acids (NEFA) were analyzed using a clinical chemistry analyzer Pentra 400 (Horiba ABX, Montpellier, France). Insulin and leptin concentration in serum, as well as serum interleukin-(IL)-6, IL-1β, monocyte attractant proteins 1 (MCP-1), and tumor necrosis factor α (TNF-α) were measured by Bio-Plex 200 system (Bio-Rad, Hercules, CA, United States). The assays were performed on a 96-well plate according to product instructions. Glucagon-like peptide-1 (GLP-1) secreted was measured using an ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. The Homeostatic Model Assessment index of insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5 (21).

Lipidomics Analysis

The concentration of serum TG was measured using a TG-LQ kit (Spinreact, Barcelona, Spain). Metabolite profiles were analyzed as previously described (22, 23). Concisely, the semi-quantification of lipid species was performed using two separate UHPLC-time-of-flight (TOF)–MS-based platforms (Agilent Technologies, Santa Clara, CA, United States) and, afterward, analyzing the combination of methanol and chloroform-methanol liver extracts. Non-esterified fatty acids, bile acids, and lysoglycerophospholipids were evaluated in the methanol extract platform. The chloroform-methanol extract platform allowed the analysis of glycerolipids, glycerophospholipids, sterol lipids, and sphingolipids. This combined analysis was established for rodent serum by OWL Metabolomics (Derio, Spain). Data obtained with the UHPLC–MS were processed with

TABLE 1 | Composition of diets.

Macronutrients	AIN93G	HF	HF-ISR
CHO (g/100 g diet)	64.59	49.42	49.42
Sucrose (g/100 g CHO)	14.37	36.07	
Isomaltulose (g/100 g CHO)			26.40
Sucromalt (g/100 g CHO)			22.10
Cornstarch (g/100 g CHO)	50.66	23.50	
Maltodextrins (g/100 g CHO)	18.97	24.43	34.50
Resistant starch (g/100 g CHO)			10.00
Inulin:FOS (g/100 g CHO)			7.00
Cellulose (g/100 g CHO)	16.00	16.00	
Total sugar (g/100 g CHO)	*15.00*	*36.85*	*39.10*
Total fiber (g/100 g CHO)	*16.00*	*16.00*	*16.00*
Glycemic load	*726*	*687*	*338*
Protein (g/100 g diet)	17.00	24.19	24.19
Fat (g/100 g diet)	7.00	20.00	20.00
Energy (calories/100 g diet)	372.13	458.63	458.63

HF, high-fat diet; RDC, diet with rapidly digestible CHO; ISR, diet with slowly digestible CHO; CHO, carbohydrates; Inulin:FOS, 1:1 mixture of fructooligosaccharides (FOS).

Glycemic load estimates the glycemic index of the carbohydrate blend resulting from the sum of each component glycemic index multiplied by its amount in the diet.

the TargetLynx application manager for MassLynx 4.1 (Waters Corp., Milford, MA, United States). Intra- and inter-batch normalization followed the procedure published by Martinez-Herranz et al. (24). All the calculations were performed with R v3.2.0 (R Development Core Team, Vienna, Austria, 2010).

Western Blot Analysis

Tissue samples were lysed using a 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 20 mM NaPPi, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM β-glycerophosphate, 2 mM sodium orthovanadate, 2 mM EDTA, 2 mM PMSF, 4 μg/l leupeptin buffer. Tissues were homogenized for 15 s in Polytron at setting #4. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4°C. The supernatant was transferred to a new Eppendorf tube (1.5 mL) and sonicated for 15 s (cycle 0.5, amplitude 70%). Rat gastrocnemius muscle was pulverized using a mortar and liquid nitrogen before being subjected to the above homogenization protocol. The protein concentration of the samples was measured using the bicinchoninic acid method (25). Specific antibodies against GLUT4 (Biogenesis Ltd., Poole, United Kingdom); total and phospho (Ser473)-PKB/Akt, total and Phospho-AMPKα 1/2 (Thr172), and MEF2D (Cell Signaling, Beverly, MA, United States); creatine kinase, carbohydrate-responsive element-binding protein (ChREBP), fatty acid synthase (FAS), glucose transporter 2 (GLUT2), sterol regulatory element-binding protein-1 (SREBP1), Pyruvate Kinase (PKM1/2), Pyruvate dehydrogenase kinase (PDK4), ATPase5B, uncoupling protein (UCP)-1, and UCP-2 (Santa Cruz Biotechnology, Dallas, TX, United States) were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) was used as load control. Data were normalized using the values of the reference animals as 100%.

Glycogen Quantification

Hepatic and muscle glycogen was isolated as described (26). Tissue homogenates (10%) were prepared in 0.03 N HCl and spread evenly on pieces of filter paper (Whatman 3M chromatography paper, 2.0 × 2.0 cm) in duplicate. The papers were gently stirred in a beaker containing 66% EtOH. Then, papers were washed three times for 40 min in 66% EtOH, rinsed with acetone, and dried. The dried filter papers were cut into four pieces and placed in a tube containing 0.4 mL of 0.2 M acetate buffer, pH 4.8; 0.2 mg of amylo-α-1,4-α-1,6-glucosidase; and H_2O to a final volume of 2 mL. samples were incubated for 90 min at 37°C with gentle shaking. Controls were prepared by incubating aliquots of homogenate in acetate buffer minus amyloglucosidase. Glucose content in the incubated samples was determined by the glucose oxidase method.

Statistical Analysis

Results were expressed as means ± SEM. The statistical significance of variations was evaluated using one- or two-way ANOVA or the corresponding non-parametric test depending on the homoscedasticity test (Bartlett's test). *Post hoc* paired comparisons, using Tukey's test or Dunn's test, were performed to check for significantly different effects between all pairs of diets using the GraphPad Prism 8.0 software. A p-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

The present preclinical study aimed to evaluate the effects of a specialized CHO diet that differs in quality rather than in the quantity of CHO (rapid digestible vs. slowly digestible CHO) to prevent excessive fat deposition in a well-established HFD rodent model of childhood obesity induced by feeding a HFD. Since metabolic syndrome in children and adolescents has increased dramatically in recent years, animal studies using younger rats may be of interest, to apply the experimental results in the pediatric population. Thus, the use of rats after weaning could allow mimicking the childhood condition. Rats fed an HFD after weaning developed the main characteristics of the metabolic syndrome: central obesity, systolic and diastolic hypertension, altered fasting glucose, hypertriglyceridemia, and decreased HDL cholesterol levels (27). The nutritional intervention was carried out for 4 weeks from weaning to infancy. A non-obese control group was termed NOB in which the animals were fed *ad libitum* with a standard growth diet AIN93G for rodents. The model used has proven to be valid to decide the time of nutritional intervention in rodents to adapt their metabolism and to avoid obese syndrome.

After 4 weeks, consumption of HFD promoted a significantly higher weight gain than rats in the lean group from the third week of nutritional intervention (**Figure 1**). In contrast, rats fed on the OBE-ISR diet showed a similar pattern to the lean group, with significantly less weight gain compared to the OBE group (**Figure 1A**). As previously mentioned, obesity is the consequence of a maintained positive energy balance over time. It has been reported in humans that a very small deviation from energy balance, on the order of 1–2% of daily energy, can increase body weight by approximately 20 kg (28). In our study, rats fed on the OBE diet had a higher daily intake than the other groups (**Figure 1B**), while energy expenditure was also greater than the lean group (**Figure 1C**). Therefore, the energy balance of the OBE rats was approximately 15 calories/day, more than 5 calories/day compared to lean. In contrast, the OBE-ISR group showed an energy balance similar to the lean group (**Figure 1D**).

A positive energy balance drives excess lipid accumulation in adipose tissue (29). The OBE group showed significant differences regarding the percentage of total body fat compared to the NOB and OBE-ISR groups (**Table 2**). The OBE group presented an increase of up to 20% in fat mass. The increase in fat tissue was mainly due to a higher accumulation in visceral and subcutaneous depots. However, there were no differences in the lean body mass among dietary groups, and hind-leg muscle weights were also similar (**Table 2**). Hence, the results obtained from the OBE group indicated that the increase in body weight gain during the experimental period rendered an increase in fat tissue without gain of lean body mass and, in contrast, this effect was not observed in the OBE-ISR group.

Obesity-related complications are associated with alterations in TG storage from adipocytes and the release of fatty acids

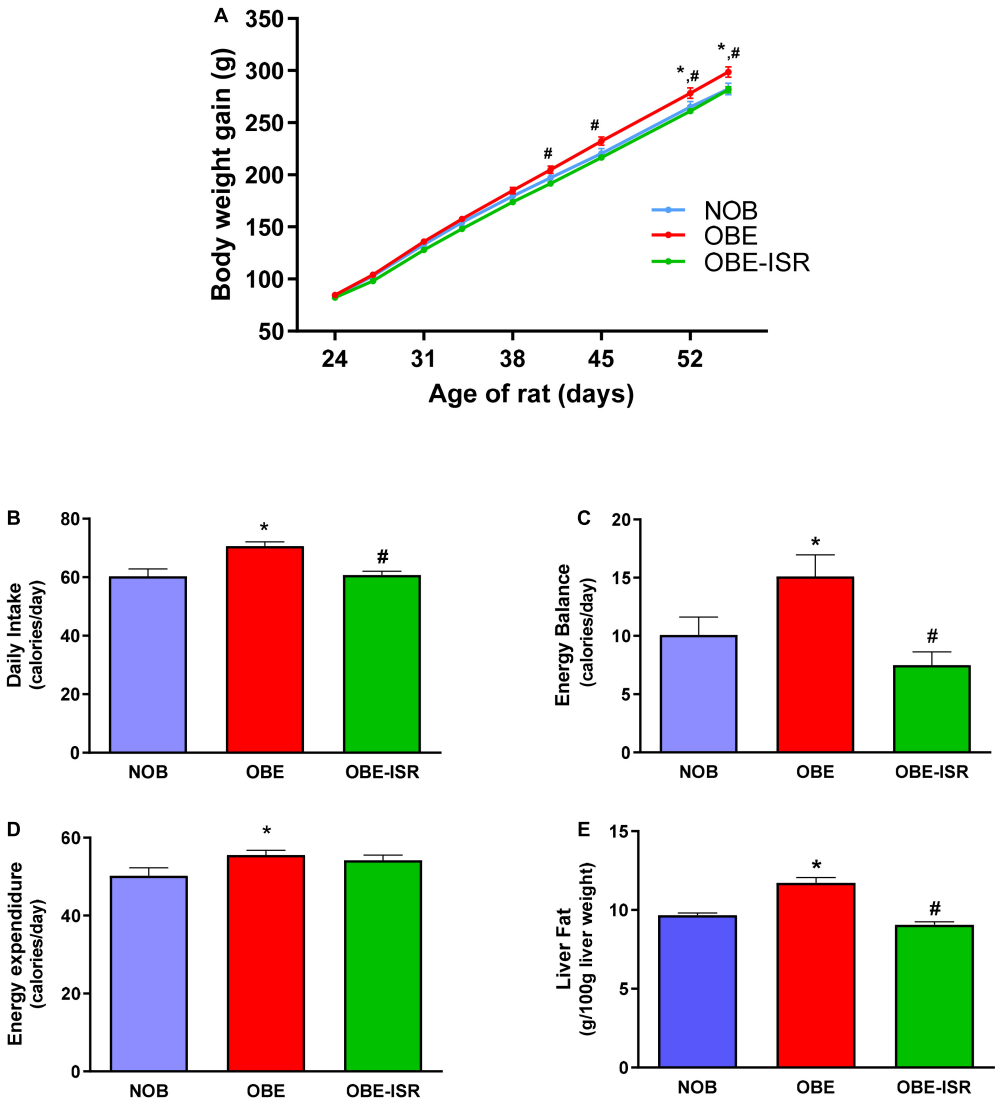

FIGURE 1 | Growth and energy expenditure in the experimental model. **(A)** Body Weight evolution. **(B)** Daily intake. **(C)** Energy balance. **(D)** Energy Expenditure. **(E)** Liver fat. Rats were fed a normal rodent diet (NOB), or a high-fat diet (HFD) formulated with rapidly (OBE) or with slowly digestible carbohydrates (OBE-ISR). Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$.

(FA), leading to dysfunctional adipose tissue and triggering lipodystrophy (30). These individuals show a poor ability to recruit adipocytes to healthy subcutaneous locations in response to increased energy intake. Instead, they store excess fat in ectopic deposits, such as liver or visceral fat, which are directly associated with the metabolic dysregulation of obesity. In the liver, this metabolic imbalance can lead to a condition known as non-alcoholic fatty liver disease (22), which represents the most common cause of chronic liver disease in children and adolescents (31). Our results showed that 4 weeks of feeding with the OBE diet significantly increased the presence of fat in the liver compared to the NOB group. In contrast, the group that was fed an OBE-ISR diet did not increase liver fat accumulation, maintaining normal values (**Figure 1E**).

In addition, other obesity-related biomarkers such as serum TG, cholesterol, LDL, HDL, and NEFA were analyzed in fasting (**Table 2**). HFD produced an increase in serum TG compared to the other groups, while no differences in cholesterol, LDL-c, HDL-c or NEFA were found in the dietary groups. High levels of circulating TG are a feature in obese children and adolescents, and at the same time, hypertriglyceridemia is a known biomarker of cardiometabolic risk. Observational studies suggest that cardiovascular disease is caused by high levels of circulating TG and TG-rich lipoproteins. Recently, the TG/HDL-cholesterol ratio has been proposed as a marker of structural vascular changes in obese young people (30) better than the TG or cholesterol levels. In our experimental setting, rats from the OBE-ISR showed the lowest ratio compared to the other two groups

TABLE 2 | Body composition and serum biochemistry of the different experimental groups fed on the different experimental diets for 4 weeks to induce obesity.

	NOB	OBE	OBE-ISR
Final fat body mass (g)	25.85 ± 2.12	31.79 ± 2.27*	25.75 ± 1.02#
Final lean body mass (g)	233.6 ± 5.1	245.5 ± 4.8	233.5 ± 3.4
Visceral WAT depots (g)	10.18 ± 2.78	14.31 ± 0.61*	10.72 ± 0.84#
Subcutaneous WAT depots (g)	6.32 ± 032	8.12 ± 0.56*	5.99 ± 0.30#
Hind-leg muscle (g)	2.19 ± 0.08	2.38 ± 0.08	2.38 ± 0.05
Fasting serum TG (mg/dL)	62.59 ± 10.90	81.39 ± 6.12	48.49 ± 5.92#
Fasting serum cholesterol (mg/dL)	68.15 ± 5.05	75.56 ± 4.72	75.39 ± 6.55
Fasting serum LDL-cholesterol (mg/dL)	6.23 ± 0.64	6.93 ± 0.48	7.73 ± 0.82
Fasting serum HDL-cholesterol (mg/dL)	24.56 ± 1.32	25.96 ± 1.23	27.53 ± 1.93
Fasting serum NEFA (mmol/L)	0.83 ± 0.08	0.71 ± 0.08	0.59 ± 0.05
TG/HDL ratio	2.20 ± 0.40	2.96 ± 0.37	1.44 ± 0.14#
Fasting serum glucose (mg/dL)	109.5 ± 9.3	130.0 ± 8.8	130.0 ± 8.1
Fasting serum insulin (pg/mL)	615.3 ± 143.7	788.0 ± 166.2	556.3 ± 90.8
HOMA-IR index	3.63 ± 1.11	7.28 ± 1.41*	4.56 ± 0.79#
Fasting serum leptin (mg/dL)	2175 ± 429	3667 ± 474*	1597 ± 275#
Postprandial serum GLP-1 (pmol/L)	2.294 ± 0.127	2.778 ± 0.280	4.514 ± 0.267*#

Data expressed as mean ± SEM (n = 10).
**Significant difference with NOB group, $p < 0.05$.*
#Significant difference with OBE group, $p < 0.05$.
WAT, white adipose tissue; TG, triacylglycerols; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NEFA, non-esterified fatty acids; GLP-1, glucagon-like peptide-1.

(**Table 2**), indicating that changing the CHO profile could reduce the risk of developing cardiovascular disease in adulthood.

To further explore the metabolic changes induced by the diets during the growing period, a global lipidomics approach was applied in serum. A total of 361 metabolic features were individually semi quantified in fasting serum. Both OBE and OBE-ISR experimental groups led to several changes in the serum metabolic profile when compared to the lean group. Results were plotted as a heatmap in **Figure 2A**. The OBE group showed high levels of monounsaturated fatty acids, diacylglycerols (DG), TG, and phosphoethanolamine (PE), while the sphingomyelin (SM) group was decreased (**Figures 2B–E**). These results observed in the OBE group would be associated with an obesogenic profile as they would be in line with the changes described in obese individuals (32). In obesity, levels of lysophosphatidylcholines, ceramides, SM and total fatty acids are increased, whereas ethanolamine and lysoPE are decreased (32–35). In contrast to OBE group, when the OBE-ISR group was analyzed, the lipid profile was not so affected, showing a metabolic profile close to the lean group (**Figures 2B–E**).

Both body fat mass and lipid profile have been considered to significantly increase the risk of later development of diabetes (32). Children with obesity exhibit increased lipid deposition in the visceral and intra-myocellular compartments, which causes severe insulin resistance (36). In our study, fasting glucose and insulin levels were measured (**Table 2**). Although there were no changes in blood glucose between the groups, the animals fed the OBE diet tended to show hyperinsulinemia compared to the OBE-ISR group ($p = 0.10$). Next, the HOMA index was calculated as a measure of insulin resistance and the highest value was observed in the HFD group, with significant differences to NOB and OBE-ISR groups (**Table 2**). These results are consistent with different randomized controlled trials in children and adolescents, which described the positive effect of a low GI/glycemic load (GL) diet on insulin resistance compared to a high GI/GL dietary approach (37).

In addition to insulin, peripheral hormones, such as leptin and GLP-1, play central roles in the central control of energy metabolism. Leptin is considered an antiobesity hormone that controls body weight and fat accumulation through its interaction with hypothalamic receptors, and its level is related to body fat mass. Among its main actions are appetite inhibition, metabolic rate stimulation and thermogenesis. In fasting conditions, a decrease in leptin levels is observed while they increase after feeding, following a mechanism that helps to regulate energy balance in humans. However, several studies indicate the existence of an endogenous mechanism of leptin resistance in obesity (38, 39). Our results in the OBE group agree with the hyperleptinemia described in obese individuals, reaching statistical differences to the NOB group. In contrast, the HFD supplemented with ISR in the OBE-ISR group led to leptin levels similar to those obtained in the lean control group (**Table 2**), indicating an improvement of the putative leptin resistance in the rats.

Glucagon-like peptide-1 is an incretin synthesized and released by intestinal L-cells. Nutrient intake is the primary stimulus for GLP-1 secretion, which increases its levels two-threefold after a meal. It has been shown that GLP-1 suppresses appetite in both normal and obese individuals, playing a major role in energy metabolism regulation. Additionally, GLP-1 improves glucose control, increases insulin sensitivity and stimulates insulin secretion. Therefore, the role of GLP-1 in the regulation of the metabolic disorders induced by obesity is crucial. However, some studies suggest that GLP-1 secretion

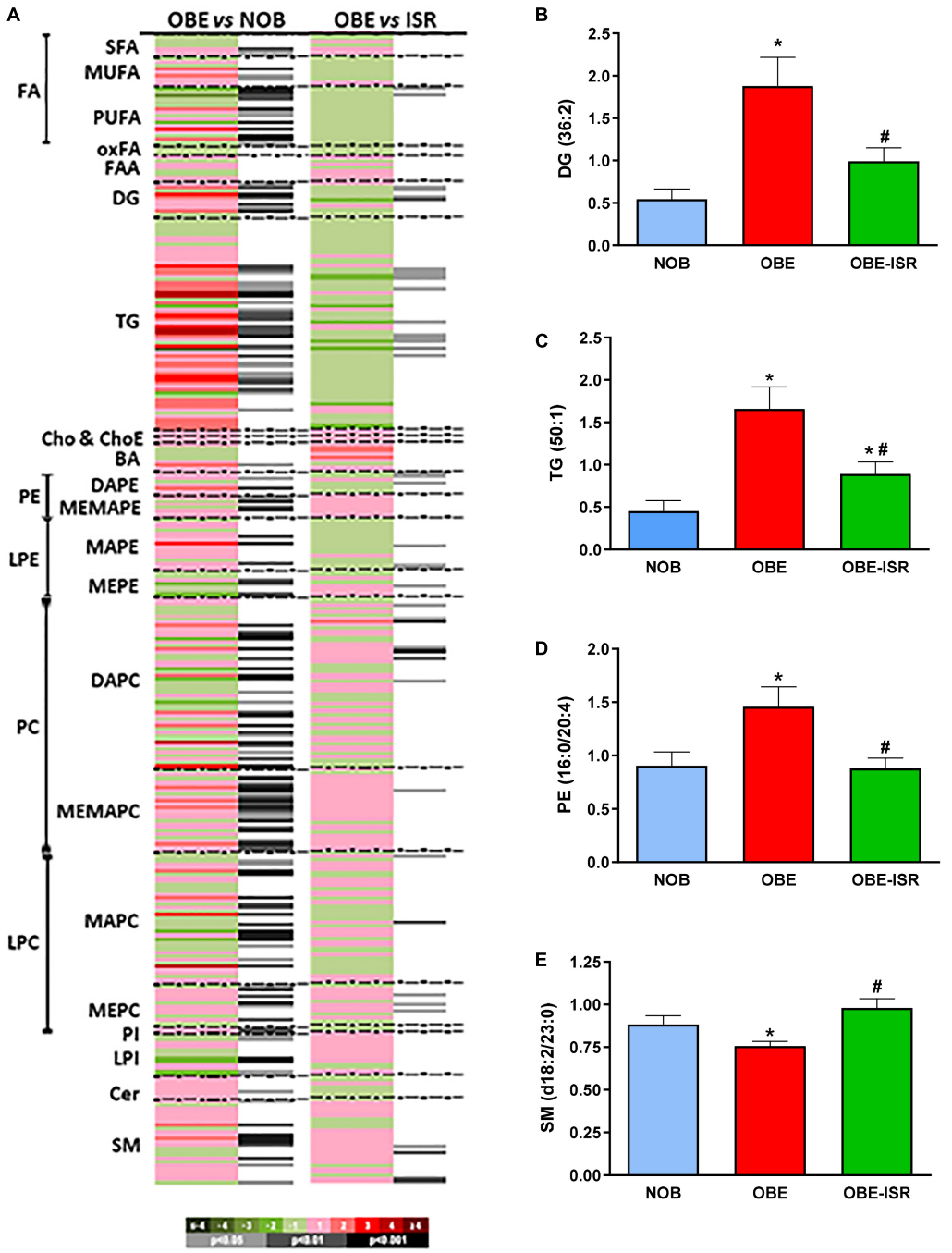

FIGURE 2 | Lipidomic analysis of the plasma in the experimental groups. **(A)** Heat map showing relevant changes in plasma. Each metabolite is shown as a line whose color is defined by the sign and magnitude of the change. Adjacent column to each comparison shows the results of the *t*-test. Relevant lipid species of **(B)** Diacylglycerols, **(C)** Triglycerides, **(D)** Phosphatidylethanolamines, **(E)** Sphingomyelins of the different experimental groups fed on the different experimental diets for 4 weeks to induce obesity. Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$. Cer, ceramides; ChoE, cholesterol esters; DAG, diacylglycerols; FFAox, oxidized free fatty acids; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SM, sphingomyelins; TG, triacylglycerols.

and signaling are reduced in obese adolescents (40). In our experimental design, postprandial GLP-1 secretion was not affected by the obesogenic diet, showing similar levels in NOB and OBE groups (**Table 2**). Nonetheless, postprandial GLP-1 levels in the OBE-ISR group were significantly higher than in the other two groups. This effect could be related to the CHO

profile of the diet since it has been described that GLP-1 secretion is affected by the GI. Thus, CHOs that are more slowly digested are likely to be exposed to more distal intestinal regions, with correspondingly later, but potentially more substantial, GLP-1 secretion (40).

Overall, the OBE-ISR diet generated an increase in GLP-1 and a decrease in leptin with respect to the HF diet, which indicates its positive effect on both appetite control and satiety and other metabolic processes such as glucose control.

In addition to the above, adipose tissue expansion in obesity could drive adipocyte dysfunction, low-grade systemic inflammation, and ultimately insulin resistance. Indeed, adipose tissue is not only a site of energy storage but also an active metabolic/endocrine organ involved in the secretion of adipokines, chemokines and pro-inflammatory cytokines. The association between childhood overweight and low-grade systemic inflammation was based on a large cross-sectional analysis (NHANES III) (41). In this study, serum inflammatory markers and white blood cell counts were significantly higher in overweight than in lean children, indicative of an inflammation state. In this way, a significant increase in leukocytes, especially in monocytes, was observed when rats were fed the OBE diet in comparison with the NOB group (**Table 3**). Along with this, an increase in the levels of pro-inflammatory cytokines in postprandial serum was observed, such as IL-6, IL-1β, TNF-α, and MCP-1 (**Table 3**). These results would be in agreement with those described in the process of inflammation in obesity, where an infiltration of macrophages into adipose tissue can be due to the migration of monocytes from the bloodstream by the action of a chemoattractant agent. The increase of macrophages in adipose tissue would increase the secretion of inflammatory cytokines, which is negative feedback would increase the state of inflammation (42, 43). When data for OBE-ISR groups were analyzed, there were no differences in comparison to the lean control group (**Table 3**). These results would indicate the prevention of HFD-associate adipocyte metabolic dysfunction by the CHO profile in this diet.

The results obtained up to this point show that the animals fed a HFD and rapidly digestible CHO presented an overweight/obese phenotype with a higher body weight gain and greater accumulation of fat in adipose tissue and liver. This state was associated with a lipid imbalance and insulin resistance, as well as a low-grade inflammatory state. In contrast, the change of CHO profile in the diet to one based on ISR prevented metabolic imbalance and the animals, even with a HFD and the same quantity of CHO, presented a phenotype close to the lean group fed a standard growth diet. Considering these results, we analyze the different cellular pathways involved in these metabolic processes in adipose tissue, liver and muscle to determine the mechanisms of action underlying this effect.

TABLE 3 | Serum immunological markers of the different experimental groups fed on the different experimental diets for 4 weeks to induce obesity.

	NOB	OBE	OBE-ISR
Blood leukocytes (10^3/mm^3)	9.88 ± 0.775	13.10 ± 1.46*	11.18 ± 0.50#
Blood lymphocytes (%)	72.58 ± 0.89	73.31 ± 3.07	73.92 ± 2.08
Blood monocytes (10^3/mm^3)	0.224 ± 0.026	0.331 ± 0.030*	0.221 ± 0.025#
Serum IL-6 (pg/mL)	243.6 ± 70.9	2203.0 ± 1121.0*	550.2 ± 51.0*#
Serum IL-1β (pg/mL)	12.64 ± 2.37	20.30 ± 3.33*	15.20 ± 1.24#
Serum TNF-α (pg/mL)	4.69 ± 0.96	5.60 ± 0.76	5.30 ± 0.92
Serum MCP-1 (pg/mL)	703.2 ± 157.1	980.6 ± 185.8	767.8 ± 75.63

Data expressed as mean ± SEM (n = 10).
**Significant difference with NOB group, $p < 0.05$.*
#Significant difference with OBE group, $p < 0.05$.
IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemoattractant protein 1.

Metabolically, adipose tissue is characterized by the storage of TG and the withdrawal of glucose from the blood in the postprandial period in a process dependent on the insulin-dependent GLUT4 transporter. Insulin resistance states, such as diabetes and obesity, are associated with lower expression of GLUT4 in adipose tissue (44–46). Here, GLUT4 protein levels were significantly higher in the OBE-ISR group than in the OBE and NOB groups (**Figure 3A**), which would indicate an improvement of insulin resistance in the OBE-ISR group compared to the OBE group, according with HOMA data (**Table 2**). In addition, insulin resistance provokes changes in the flow of adipocyte substrates, releasing of FA and glycerol (47). We observed higher levels of blood FA in the NOB (31.20 ± 2.24) and OBE (30.92 ± 2.94) compared with OBE-ISR (23.9 ± 1.02) group. In terms of improvement of insulin sensitivity due to the observed effects on GLUT4 in adipose tissue and HOMA, the glycolytic use of the glucose was measured as the expression of PKM1/2 and we did not find differences between groups (**Figure 3B**). On the other hand, the uncoupling proteins (UCP1 and UCP2) catalyze proton leakage across the mitochondrial membrane, causing uncoupling of the electron transport chain and ATP synthesis (48). UCP1, which was first discovered in brown adipose tissue, has a relevant role in thermogenesis and body weight control. Furthermore, an increase in the expression of UCP1 due to low temperatures or overfeeding has been described. UCP2 is a mitochondrial transporter that plays a key role in the regulation of glucose/lipid metabolism and energy homeostasis. UCP2 downregulation increases insulin resistance in white adipose tissue (49). The OBE-ISR rats showed no changes in the expression of the ATP synthase (data not shown), although the levels of UCP1 and UCP2 were significantly lower in the OBE-ISR group compared to the OBE rats (**Figures 3C,D**), which could be interpreted in terms of better functionality of these mitochondria for the aerobic oxidation of substrates.

The OBE group presented an increase in visceral adiposity compared to the OBE-ISR, and it was evidenced by the analysis of enzymes and transcription factors related to lipogenesis. The expression of fatty acid synthase (FAS), a key enzyme implicated in *the novo* synthesis of FA in adipose tissue, was significantly higher in the OBE than in the other groups (**Figure 3E**). Furthermore, in adipose tissue, adipogenesis is regulated by transcription factors such as peroxisome proliferator-activated receptors (PPARs). In adipose tissue, insulin sensitivity and adipogenesis are controlled by PPARs, transcription factors whose expression is also regulated by alterations in glucose and lipid homeostasis (50). Among the different PPARs, PPARγ

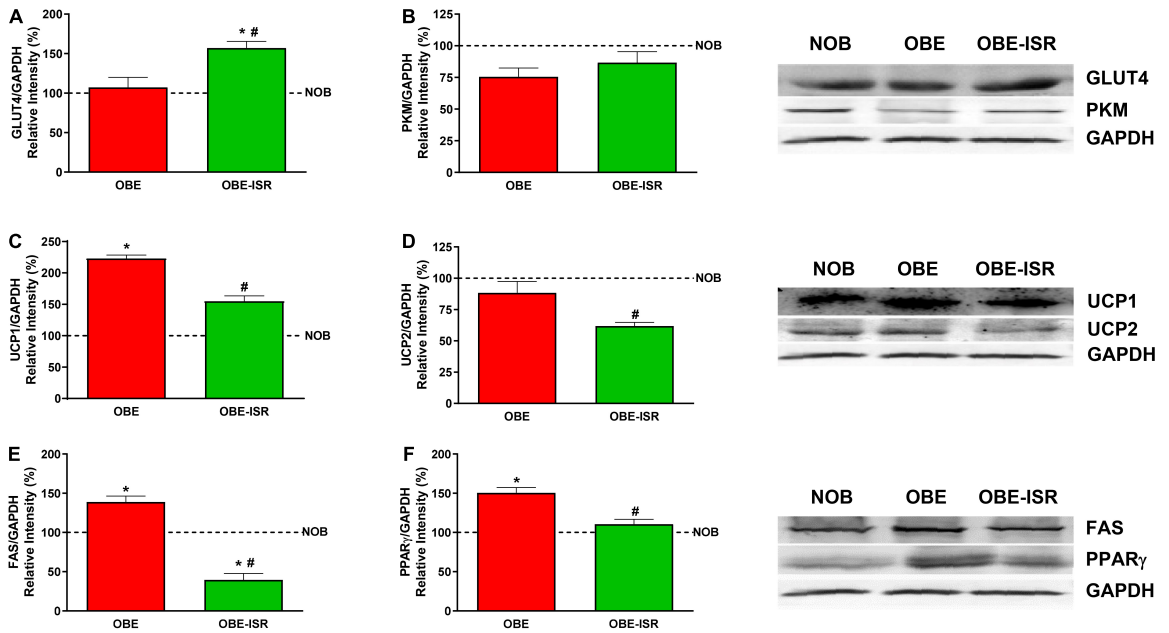

FIGURE 3 | Adipose tissue expression of GLUT4, PKM, uncoupling proteins and lipid synthesis related proteins in the experimental groups. **(A)** GLUT4. **(B)** PKM. **(C)** UCP1. **(D)** UCP2. **(E)** FAS. **(F)** PPARγ. Rats were fed a normal rodent diet (NOB) or a high-fat diet (HFD) formulated with rapidly (OBE) or with slowly digestible carbohydrates (OBE-ISR). Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$.

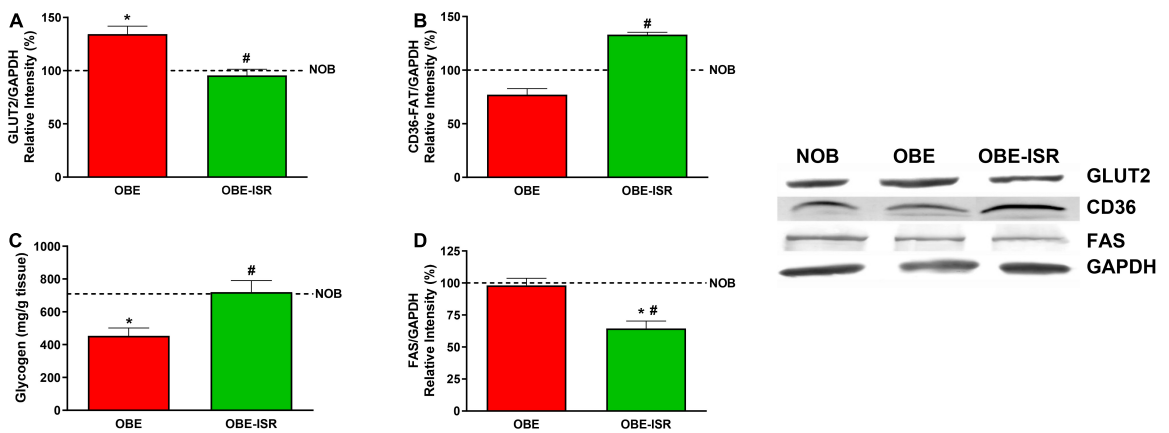

FIGURE 4 | Uptake and storage of metabolic substrates in liver. **(A)** GLUT2. **(B)** CD36-FAT. **(C)** Glycogen. **(D)** FAS. Rats were fed a normal rodent diet (NOB) or a high-fat diet (HFD) formulated with rapidly (OBE) or with slowly digestible carbohydrates (OBE-ISR). Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$.

regulates the expression of genes involved in the endogenous synthesis of cholesterol, fatty acids, and triacylglycerols (51). Our results indicate that the OBE diet stimulated the expression of PPARγ and would be an additional factor in the increased adiposity observed in these rats. In contrast, the ISR diet decreased PPARγ expression to levels comparable to the lean control group (**Figure 3F**).

Overall, in adipose tissue, the OBE-ISR diet increased GLUT4 levels, which would be associated with better insulin sensitivity. However, the higher insulin sensitivity was not associated with greater adiposity since our data indicate that FA synthesis and the expression of lipogenesis-related transcription factors (PPARγ) are decreased in these rats.

The liver provides energy and substrates to other tissues, being responsible for the homeostasis of glucose and other metabolic fuels (52). Thus, it participates in the processing of dietary fats, proteins and CHO. It also plays an important role in glucose metabolism either to obtain energy in the form of ATP or metabolic intermediates that will be substrates for the synthesis of glycogen or TG (53, 54).

The diet OBE is associated with an increase in the levels of serum TG (**Table 2**) and also with higher liver fat (**Figure 1E**).

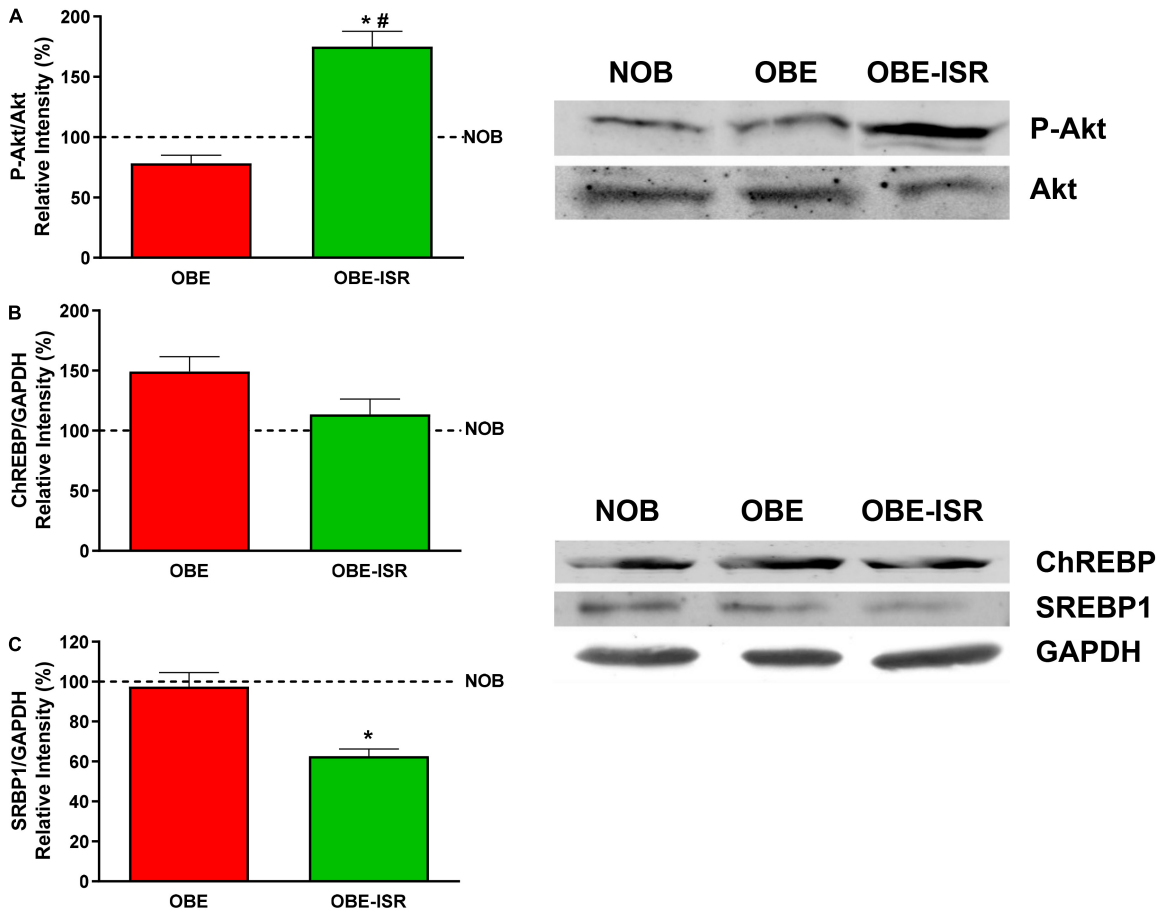

FIGURE 5 | Key signaling elements related to insulin sensitivity and lipid synthesis in the liver. **(A)** p-Akt. **(B)** ChREBP. **(C)** SRBP1. Rats were fed a normal rodent diet (NOB) or a high-fat diet (HFD) formulated with rapidly (OBE) or with slowly digestible carbohydrates (OBE-ISR). Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$.

These circulating TG come from hepatic synthesis. Here, we analyzed the uptake of glucose and fatty acids into the liver through the expression of specific transporters, the flow of these substrates toward their storage as glycogen or TG, as well as the regulation of these processes were analyzed. To do this, the phosphorylative state of Akt, as the kinase responsible for insulin sensitivity in liver cells and transcription factors such as ChREBP and SRBP1, whose expression is associated with higher lipid synthesis, were measured.

The incorporation of CHO with a high GI to a HFD led to higher protein levels of the glucose transporter, GLUT2, and a decrease of the fatty acid transporter expression, CD36, in the liver of animals that received this diet (**Figures 4A,B**). Concomitantly, the greater amount of glucose that has entered the liver of OBE animals does not translate into an increase in glycogen synthesis (**Figure 4C**), being this group the one with the lowest levels of glycogen. As suggested by Irimia et al. (55), we can speculate that inability to synthesize liver glycogen is caused by CHO with a high GI induced lipid synthesis and liver fat over-accumulation, accompanied by impairment in hepatic insulin sensitivity. Both the reference (AIN93G) and the HFD are rich in fast-digesting CHO. These CHO can be converted into fatty acids, as indicated by the expression of FAS, which is higher in these two groups than in the OBE-ISR one (**Figure 4D**). The changes in FAS expression in adipose tissue and liver were associated with circulating FA and TG levels in fasted animals and also with higher lipid storage in the own liver. In states of insulin resistance, the increased flow of FA to the liver is the result of a failure of insulin to suppress TG lipolysis in adipose tissue.

Fatty acids are packaged as TG into VLDL particles and transported to adipose and other extrahepatic tissues. The presence of hepatic fat in the OBE group could indicate that the hepatic synthesis of FA might be greater than its capacity to secrete VLDL; therefore, they remain stored. It has been described that in obese individuals or those with insulin resistance, there is an increase in lipid synthesis. Furthermore, many obese subjects have normal plasma VLDL-TG levels, whereas those with high blood VLDL-TG levels have an increased VLDL secretion rate. Although plasma VLDL-TG levels could be higher in obese than in lean individuals, hepatic TG accumulation is mainly caused by an imbalance between hepatic TG synthesis and TG export *via* VLDL (56–58).

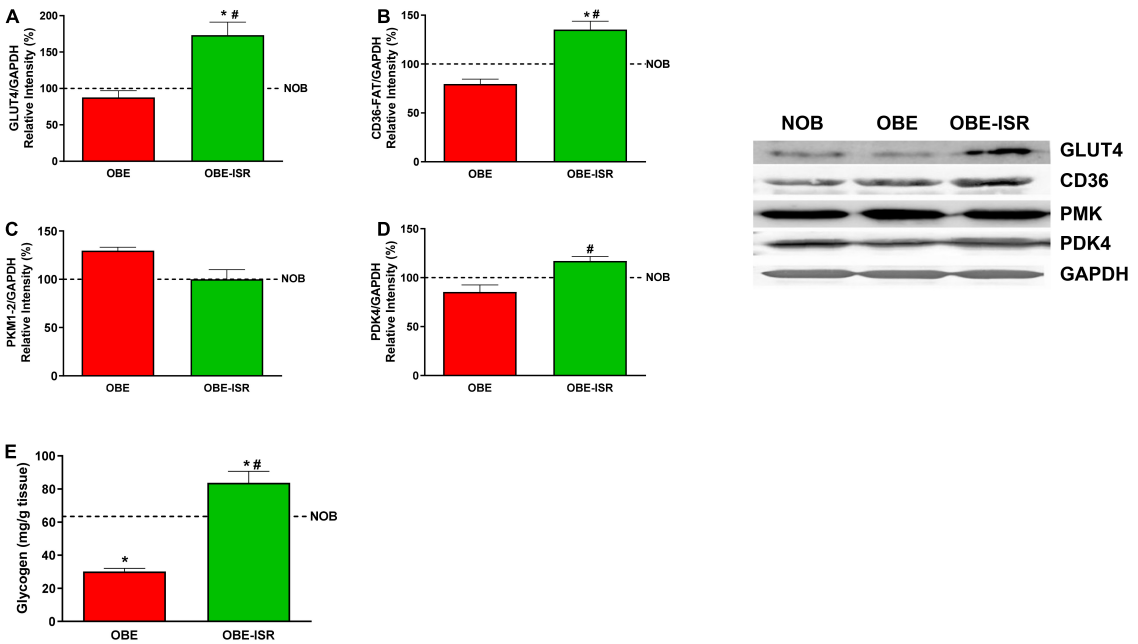

FIGURE 6 | Uptake and metabolism of glucose and fatty acids in the muscle. **(A)** GLUT4. **(B)** CD36-FAT. **(C)** PKM1/2. **(D)** PDK4. **(E)** Glycogen. Rats were fed a normal rodent diet (NOB) or a high-fat diet (HFD) formulated with rapidly (OBE) or with slowly digestible carbohydrates (OBE-ISR). Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$.

In the liver, the glycogen content of the OBE-ISR group is higher than in the other two experimental groups, indicating a relevant role for the liver in glycemic control in response to this complex CHO-enriched diet (**Figure 4C**). Another positive effect of the ISR diet is that hepatic FAS showed a significant decrease compared to the OBE and lean groups (**Figure 4D**). These results indicated a shift in liver CHO metabolism toward glycogen synthesis in the OBE-ISR group rather than the use of glucose as a lipogenic substrate.

The key signaling elements measured in the liver confirmed this behavior. Animals that were fed the OBE-ISR showed a significant increase in phosphorylated Akt/PKB kinase (**Figure 5A**), indicating again that the composition of CHO can reverse the impact of a HFD on the sensibility to insulin. Furthermore, the regulation of lipogenesis in the liver is mainly dependent on the expression of ChREBP and SREBP1 proteins (59). SREBP1c regulates most of the genes involved in the synthesis of FA and TG: acetyl-CoA carboxylase, FAS, elongases, and acyl-CoA desaturases. ChREBP is mainly regulated by glucose but plays a complementary role with SREBP1 in the regulation of hepatic lipogenesis. Our results indicate a decrease in these transcriptional regulators levels in the OBE-ISR group when compared to the OBE one preventing lipogenic metabolic programming (**Figures 5B,C**).

Finally, skeletal muscle contributes to maintaining a functional metabolism. Obesity and type 2 diabetes mellitus impaired glycogen synthesis in the skeletal muscle could be a side effect of skeletal muscle insulin resistance of glucose transport. The insulin-dependent glucose transporter, GLUT4, was overexpressed in the OBE-ISR group compared to the NOB and OBE control groups (**Figure 6A**). The higher expression of GLUT4 transporter in the OBE-ISR group would be related to a better sensitivity to the action of insulin. The increase in glucose uptake in the OBE-ISR group was in parallel with an increase in the muscle glycogen content in this group while the glucose that entered the muscle of the OBE rats was utilized by the glycolytic pathway (**Figure 6E**). Furthermore, the FA transporter, CD-36, was also overexpressed in the OBE-ISR group compared to the NOB and OBE control groups (**Figure 6B**) indicating higher oxidation of the FA that constitutes the preferred fuel of the muscle at rest (60). Another interesting feature of muscle function in rats that received the OBE-ISR diet can be obtained from the measurement of proteins that regulate the oxidation of fuels or the differentiation of muscle cells, as well as the activation of related signaling pathways. The pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) is a protein responsible for controlling fuel use in muscles. An increase in the PDK4 expression is associated with phosphorylation/inhibition of the pyruvate dehydrogenase complex. In this situation, the pyruvate is not converted to acetyl-CoA and is not used for fatty acid synthesis. The OBE-ISR group showed a higher PDK4 expression than the OBE one (**Figure 6D**). Therefore, the OBE-ISR could oxidize the FA that came through the CD36 transport to obtain energy. In addition, the inhibition of the pyruvate dehydrogenase complex by PDK4 observed in this group would lead to an inhibition of the entry of pyruvate into the Krebs cycle, it would block glucose oxidation (52), which would be in agreement with the lower expression of PKM in the OBE-ISR group (**Figure 6C**).

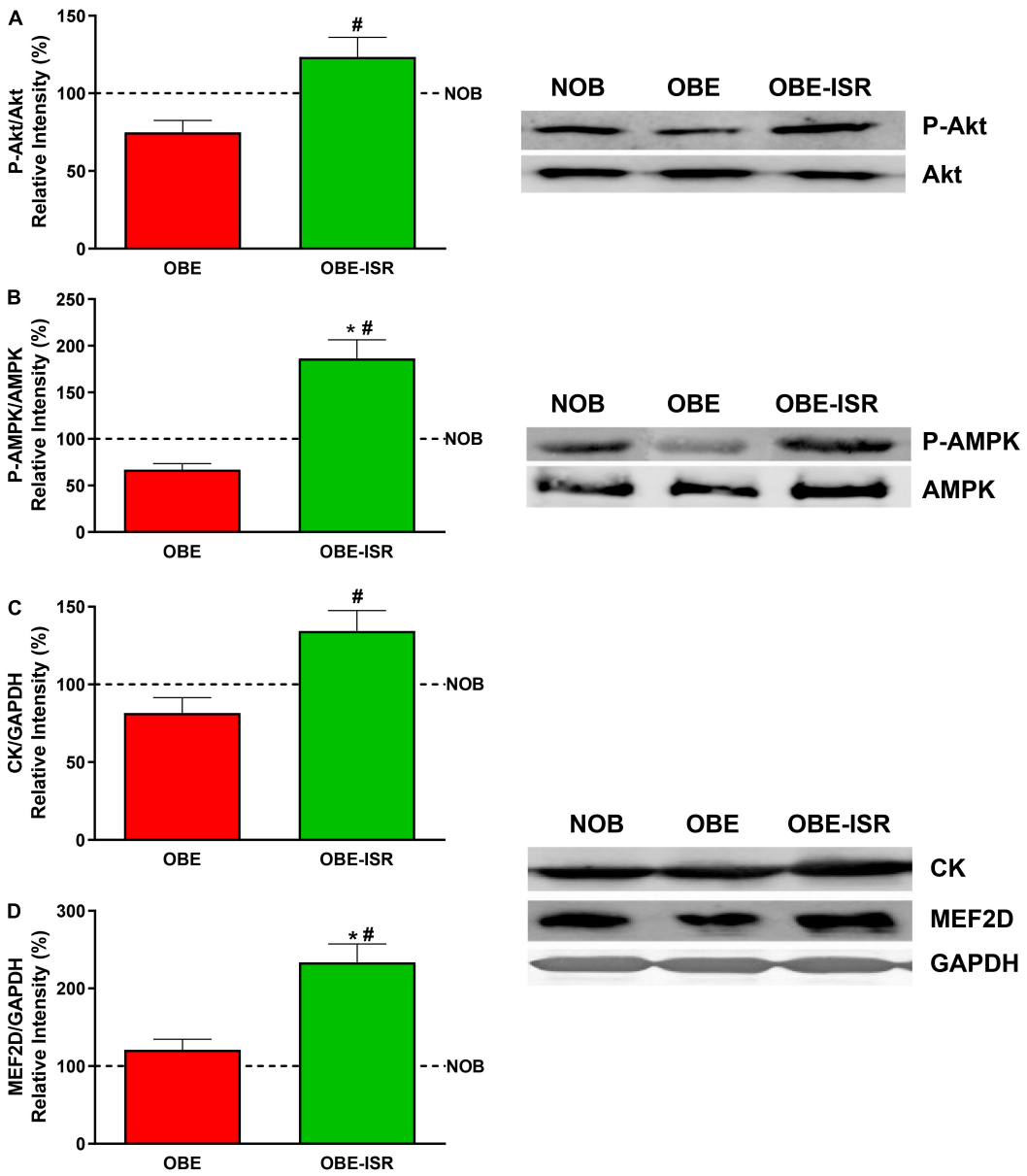

FIGURE 7 | Activation of key signaling elements and expression of differentiation markers in the muscle. **(A)** p-Akt. **(B)** p-AMPK. **(C)** CK. **(D)** MEF2D. Rats were fed a normal rodent diet (NOB) or a high-fat diet (HFD) formulated with rapidly (OBE) or with slowly digestible carbohydrates (OBE-ISR). Data expressed as mean ± SEM. *Significant difference with NOB group, $p < 0.05$. #Significant difference with OBE group, $p < 0.05$.

Our results also revealed that the phosphorylation of Akt and AMPK is higher in the OBE-ISR rats than in the other groups (**Figures 7A,B**). The activation of Akt pointed out to an increase in the sensibility to insulin and that of AMPK is related to increased glucose uptake in muscle and FA oxidation in muscles at rest (61).

Finally, despite the fact that the lean body mass was not increased in the OBE-ISR group, the muscles of these animals had increased expression of creatine kinase and MEF2D (**Figures 7C,D**), which translated into better muscle functionality and differentiation compared to the other groups (62).

CONCLUSION

In this work focused on evaluating the effect of CHO on the development of obesity in childhood, replacement of rapidly digestible CHO by ISR in an obesogenic HF diet promotes a significant protective effect against the development of obesity and its associated comorbidities. The quality of the diet exerted its effect on energy balance through complex hormonal and cellular pathways such as lipogenesis, cellular energy, insulin sensitivity and inflammation. Overall, the presence of these CHO in the diet in the childhood stage would preserve the lean phenotype in the

individual and prevent the appearance of obesity and its associated comorbidities in adulthood.

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 animal study was reviewed and approved by the Consejería de Agricultura, Ganaderia, Pesca y Desarrollo Sostenible, Junta de Andalucia, Spain.

AUTHOR CONTRIBUTIONS

MM, MDG, RR, and JL-P: conceptualization and supervision. MM, RS, MDG, JV, FR-P, EC, and AL-P: data curation and investigation. RR and JL-P: funding acquisition. RS, MDG, JL-P, and MM: writing—original draft. RS, MDG, MM, JL-P, JP-D, FR-O, and AG: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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The Role of Oxidative Stress and Inflammation in X-Link Adrenoleukodystrophy

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X-linked adrenoleukodystrophy (X-ALD) is an inherited disease caused by a mutation in the ABCD1 gene encoding a peroxisomal transmembrane protein. It is characterized by the accumulation of very-long-chain fatty acids (VLCFAs) in body fluids and tissues, leading to progressive demyelination and adrenal insufficiency. ALD has various phenotypes, among which the most common and severe is childhood cerebral adrenoleukodystrophy (CCALD). The pathophysiological mechanisms of ALD remain unclear, but some *in vitro/in vivo* research showed that VLCFA could induce oxidative stress and inflammation, leading to damage. In addition, the evidence that oxidative stress and inflammation are increased in patients with X-ALD also proves that it is a potential mechanism of brain and adrenal damage. Therefore, normalizing the redox balance becomes a critical therapeutic target. This study focuses on the possible predictors of the severity and progression of X-ALD, the potential mechanisms of pathogenesis, and the promising targeted drugs involved in oxidative stress and inflammation.

Keywords: X-ALD, oxidative stress, inflammation, biomarker, pathogenesis, treatment

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INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder with an estimated incidence ratio of 1:14,700 in neonates (1), caused by mutations in the ABCD1 gene. Until present, over 2,700 types of mutations have been identified (2). ABCD1 codes the protein of adenosine 5′-triphosphate (ATP) binding cassette subfamily D member 1, which is located in the peroxisomal membrane to transport the very-long-chain fatty acids (VLCFAs) into peroxisomes for β-oxidation (3). The dysfunctional ABCD1 causes the failure of VLCFA degradation (4, 5), leading to the accumulation of VLCFA, especially C:26 and C:24 in tissues and plasma, and damage to organs, particularly in the cerebral white matter, spinal cord, and adrenal cortex (6, 7). Thus, VLCFA constitutes pathognomonic biomarkers for X-ALD diagnosis (8).

Various phenotypes are presented in X-ALD, including cerebral ALD (CALD), adrenomyeloneuropathy (AMN), Addison-only (AO), and presymptomatic ALD, among which the most severe is CALD (9, 10). Approximately one-third of boys with X-ALD develop

CALD under the age of 12 years (11), characterized by initial learning and behavioral problems, followed by a rapid and severe progressive inflammatory demyelination resulting in a severe cognitive and physical disorder with a total disability that develops within 6 months to 2 years and dies within 5–10 years of the diagnosis (12–14). Besides, AMN, which is another prominent clinical phenotype of X-ALD that mainly manifests in adults, develops progressive stiffness and weakness of the legs, an impaired sense of vibration, and sphincter disturbances. The risk of developing CALD secondary to AMN is estimated to be at least 20% over 10 years (15). While other phenotypes are also likely to develop to cerebral form, the exact risk is unclear, and the occurrence of CALD remains unpredictable. Therefore, the identification of reliable biomarkers is of utmost importance, and it is critically required to predict the occurrence of disease and progression. Several countries have approved the newborn screening program of X-ALD in addition to routine screening (16–18).

However, the pathogenesis mechanism of X-ALD remains obscure. Oxidation stress and inflammation are the most typical features in most neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (19–21). Therefore, it is believed that oxidation damage and inflammation are the main pathogenesis of neuropathy, which are also found in patients with X-ALD (22–24).

Oxidative stress is "a transient or long-term increase in steady-state reactive oxygen species (ROS) levels, disturbing cellular metabolic and signaling pathways, particularly ROS-based ones, and leading to oxidative modifications of an organism's macromolecules that, if not counterbalanced, may culminate in cell death *via* necrosis or apoptosis" (25). ROS includes both free radicals such as O^{2-}, OH^{-}, and more stable peroxide molecules such as H_2O_2 (26, 27). Because of the very short half-life and rapid reactivity of ROS, currently, there is no reliable method or technology to measure its level. Consequently, redox levels are mostly indirectly reflected by measuring antioxidant capacity (28).

Antioxidant defense systems include enzymatic and non-enzymatic types. The enzymatic antioxidant system predominately comprises superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), and the non-enzymatic antioxidant system is classified as endogenous and exogenous antioxidants, the former including GSH, uric acid, transferrin, and the latter encompassing parts of vitamins, α-tocopherol, α-lipoic acid, and others (22). NRF2 is the master regulator of the endogenous antioxidant system, and ROS activate the NRF2 pathway to promote the transcription of downstream genes including heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL), and NAD(P)H: quinone oxidoreductase-1 (NQO1), subsequently regulating the metabolism of endogenous antioxidants to defense oxidative stress (29, 30). The antioxidant system maintains redox balance *in vivo*, but oxidative stress occurs if it is insufficient to resist excessive ROS. Moreover, ROS directly stimulates the release of pro-inflammatory cytokines and activates the nuclear factor κB (NF-κB) pathway to cause inflammation (31, 32).

In the three-hit hypothesis of X-ALD, oxidative stress is described as the first hit to cause axonal injury of AMN, or subsequently induce inflammatory demyelination and cell death of CALD, triggered by stochastic factors such as lipid peroxides, environmental factors, and genetics (33). The exact cause of the onset and damage caused by oxidative stress and inflammation in X-ALD was unknown. However multiple *in vivo*/*in vitro* studies have demonstrated that drugs targeting redox imbalance and inflammation effectively improve symptoms and prevent disease progression (8, 34–37).

This study introduces the relationship between the X-ALD and oxidative stress and inflammation. Moreover, we will discuss the possible predictors for the severity and progression of X-ALD, the potential mechanisms of pathogenesis, and the promising targeted drugs involved in oxidative stress and inflammation.

INCREASED OXIDATIVE STRESS AND INFLAMMATION IN THE BRAIN AND ADRENAL GLAND

ROS in cells is mainly produced by mitochondria. The brain white matter and adrenal gland, which are involved in X-ALD, are high metabolic tissues with active mitochondria (38). In addition, both the brain and adrenal glands are lipid-rich organs that are highly susceptible to oxidative stress and have active cholesterol metabolism (39). Cholesterol metabolism is closely related to redox balance because mitochondrial ROS is produced during cholesterol generation; conversely, oxidative stress can obstruct steroidogenesis (40). Thus, oxidative stress may be the underlying influencing factor that contributes to predisposed X-ALD sites.

As the vital organ in our body, the brain consumes approximately 20% of total oxygen (41). High oxygen consumption tends to be associated with high utilization of mitochondria in the brain (30, 42, 43), leading to ROS production. In addition, axons and myelin in the white matter have an extremely high energy demand, which is mostly met by mitochondria to support the function of neural conduction. However, the brain's antioxidant capacity is frail (41, 44, 45). As a result, hyperactive mitochondrial metabolism and a fragile antioxidant defense system may lead to the white matter susceptibility to oxidative stress.

Many oxidative markers such as manganese SOD (MnSOD), HO-1, lipid peroxidation 4-hydroxynonenal (4-HNE), malondialdehyde (MAL), and oxidized proteins (protein carbonyl) were found in the postmortem brains of patients with AMN and CALD (46–48), and the immunoreactivities of these indexes are related to the degree of inflammation and myelin destruction (46). Plasmalogen synthesized by peroxisome, an indicator of peroxisome disease diagnosis, is an important lipophilic myelin endogenous antioxidant that protects neural integrity and functions against oxidative stress (49). Researchers have found plasmalogens were reduced in the brain white matter of patients with CALD, and the reduction was associated with increased oxidative stress (48). Besides, recent studies also exhibit that the expression of various heat shock proteins (HSPs) increases in the brain tissue of X-ALD, while HSPs are

upregulated after oxidative stress, which triggers the release of pro-inflammatory factors (49, 50). Furthermore, this increase in HSPs was observed before inflammation and demyelination, suggesting that oxidative stress as an early injury may be involved in the pathogenesis of CALD.

Inflammation in the brain is an essential characteristic for distinguishing the severity of X-ALD. CALD is a neuroinflammatory disease with many pro-inflammatory macrophages/microglia in the area of the lesion (51, 52), which was not found in AMN. The expression of tumor necrosis factor α (TNF-α) and other pro-inflammatory mediators was found to be increased in the demyelinated regions of patients with CALD (24, 53–55). The different cytokine expressions inferred that the lesion mainly involved a proinflammatory T-helper type 1 (Th1) response (56–58). Interferon γ (IFN-γ) is a specific effector cytokine of the Th1 response that could lead to oxidative damage through the production of reactive species, which is high immunoactivity in affected areas of CLAD while almost non-reactive in the ALD heterozygote and patients with AMN (46).

However, unlike the brain, the adrenal gland has a robust antioxidant capacity (59), so oxidative stress in the adrenal gland may be related to a high turnover of lipids and ROS production in steroidogenesis. Although studies have found significant markers of oxidative stress in the adrenal cortex of X-ALD model mice, there was no exact change in the adrenal glands of patients with X-ALD (46, 60). As steroidogenesis is heavily influenced by mitochondrial function and redox homeostasis, a redox imbalance in the adrenal gland may suppress cortisol production, resulting in adrenal insufficiency.

REDOX IMBALANCE AND INFLAMMATORY MARKERS IN THE BLOOD REFLECT DISEASE PROGRESSION

There was no definite correlation between phenotypes and genotypes in X-ALD, and the symptoms of the disease varied considerably during the progression. Therefore, early diagnosis and progression prediction of the disease are necessary to improve the prognosis. Diagnosis of brain injury by magnetic resonance imaging (MRI) or clinical symptoms is extremely limited because most patients are already in an advanced stage of the disease; in some cases, inflammatory demyelination was found after autopsy. Some studies have used skin fibroblasts to predict disease severity by measuring levels of glycosphingolipid (GSL) species, reflecting more complex lipid metabolism (61). Researchers have even taken skin biopsies to extract and culture-induced pluripotent stem cells (iPSCs) to mimic brain tissue to get an early and accurate diagnosis of X-ALD (62). Compared with the high cost and hysteresis of MRI, and the invasiveness and harm of skin biopsy, blood-based biomarkers for X-ALD have gained prominence due to safety and convenience. Plasma VLCFA is a pathognomonic biomarker for X-ALD but independent of the severity of the disease. As important pathogenesis of X-ALD, oxidative stress and inflammation, the redox imbalance, and inflammatory indicators in blood could become biomarkers for disease prediction.

Changes in Antioxidant Capacity and Peroxides in Different Phenotypes

Oxidative stress in the body is usually reflected by antioxidant capacity. SOD is a critical enzymatic antioxidant *in vivo*, which effectively dismutases the active superoxide anion into less reactive H_2O_2, thus eliminating excessive ROS. A study has shown that SOD activity and level in plasma are significantly different in healthy controls, heterozygote carriers, patients with AMN, and CALD, and are decreased with the severity of symptoms. Besides, plasma SOD level was negatively correlated with MRI severity in patients with CALD (63). The results suggest that SOD in plasma likely serves as a potential biomarker for disease progression and as a predictor for the onset and prognosis of cerebral damage. Another major endogenous antioxidant is GSH, which is sensitive to changes in redox balance (64). Additionally, GSH blood concentration is an important indicator of whole-body GSH status, especially in some hard-to-reach tissues such as the brain (22, 65). GSH imbalance was observed in whole blood, including lymphocytes, erythrocytes, monocytes, and plasma of patients with X-ALD. One study has demonstrated that the level of GSH in lymphocytes of patients with AMN was significantly lower compared to patients with CALD (66), but another study showed GSH levels in CALD monocyte were markedly decreased, but not in patients with AMN (67), which may be an indicator to distinguish AMN from CLAD. However, previous research also demonstrated that GSH concentrations in plasma are all significantly decreased in patients with X-ALD having any phenotype (such as AMN, AO, or CALD) (68). Therefore, whether GSH is a promising biomarker remains to be determined.

Markers of oxidative damage to DNA, proteins, and lipids in the plasma can also reflect the degree of oxidative stress and disease severity *in vivo*, especially lipid peroxidation. The levels of lipid peroxide were increased in both symptomatic and asymptomatic patients compared with healthy subjects and were significantly higher in the patients with AMN than in patients with CALD and asymptomatic patients (8, 69). Furthermore, a remarkable reduction of oxidative damage markers was revealed after treatment with high-dose oxidants (8).

As to other non-enzymatic or enzymatic antioxidants, some investigations showed that both α-tocopherol and docosahexaenoic acid were shown to be non-differentially reduced in all phenotypes (68). In addition, one study found an increase in GPX activity in erythrocytes (47), whereas another observed no difference (70). Overall, none of the above indexes showed significance in assessing the severity, progression, and prognosis of X-ALD.

The antioxidant system *in vivo* is always in a state of flux, which could be increased in response to continuous oxidative stress or depleted in reaction to excessive oxidative stress. In addition, antioxidant capacity varies from the degree of oxidative imbalance in the body. Several studies have found more severe

oxidative stress and worse overall antioxidant capacity in AMN, which may be because oxidative stress as a direct injury factor leads to the occurrence of AMN. However, in the pathological mechanism of CALD, oxidative stress works as an early injury to trigger neuroinflammation under certain conditions, which lead to severe demyelination and brain damage, resulting in neurodegeneration at last. Hence, inflammation may be a marker of brain damage.

Inflammatory Factors in the Prediction of Disease Progression

Cytokine levels in the plasma are generally low, and a dynamic balance exists between pro-inflammatory and anti-inflammatory factors. However, the balance could be broken by inflammation, oxidative stress, or cell injury during the disease process (71, 72). The peripheral blood monocyte/macrophage cells from asymptomatic patients and patients with AMN and CLAD were all observed to have pro-inflammatory skewing (52, 55). Levels of pro-inflammatory cytokines, such as interleukin 1 β (IL-1β), IL-2, and IL-8, and TNF-α in plasma are significantly increased in asymptomatic patients compared to symptomatic patients, which are positively related to plasma C26:0. Besides, in AMN, the levels of anti-inflammatory IL-4 and IL-5 were mainly increased, which was negatively correlated with the VLCFA level (57). However, in CALD, both pro-inflammatory and anti-inflammatory factors are within the normal range. In a follow-up study, the previously asymptomatic patients, after progressing to the cerebral phenotype, show reduced levels of cytokines (57). Hence, there may be a time required for upregulation of inflammatory mediators in plasma in X-ALD: actively expressed in asymptomatic patients or patients with AMN, whereas turned normal when developed into CALD. These suggest inflammatory mediators might be an early biomarker of cerebral injury and demyelination. Inflammation might be the potential cause that asymptomatic or AMN developing into cerebral form. Therefore, plasmatic cytokines should be regularly tested in asymptomatic patients and patients with AMN to predict disease progression from asymptomatic or AMN to the CLAD.

In addition, antioxidant therapy can also improve inflammation. Various plasmatic cytokines and inflammatory markers in patients with AMN were reduced after multi-antioxidant combination therapy, among which inflammatory markers, such as monocyte chemoattractant protein-1 (MCP1) and 15-hydroxyeicosatetraenoic acid (15-HETE), were significantly downregulated to become the promising predictors of response to treatment (8).

In brief, these redox imbalances and inflammatory markers observed in the blood may be potential predictors of X-ALD, providing a reliable basis for disease severity, progression, as well as therapeutic response. However, as whole blood is a complex mix of blood cells and plasma, longitudinal comparisons should be conducted in plasma, lymphocytes, monocytes, and erythrocytes of antioxidant and inflammatory levels to evaluate better and predict disease.

THE UNDERLYING MECHANISMS OF ONSET AND DAMAGE INVOLVED IN OXIDATIVE STRESS AND INFLAMMATION OF X-LINKED ADRENOLEUKODYSTROPHY

Although oxidative stress and inflammation have been identified as important factors in the pathogenesis of X-ALD, neither the exact source of ROS and inflammatory factors nor the related signal pathway and molecular mechanism have been elucidated. Until present, excessive VLCFA has been considered the causative factor of oxidative stress and inflammation in X-ALD (73). Redox imbalance is caused by increased production of mitochondrial ROS due to excess of VLCFA and inadequate endogenous antioxidants; inflammation is a consequence of VLCFA directly or indirectly promoting the release of inflammatory factors (**Figure 1**).

In addition, various animal models with both advantages and drawbacks were used to study the mechanism of X-ALD (74, 75). ABCD1 knockout mice that exhibit delayed (20 months) onset axonopathy in the spinal cord without cerebral inflammatory demyelination (76), which are considered to mimic AMN, are often used to evaluate changes in biochemical markers. In addition, double mutant ABCD1/ABCD2 mice demonstrate

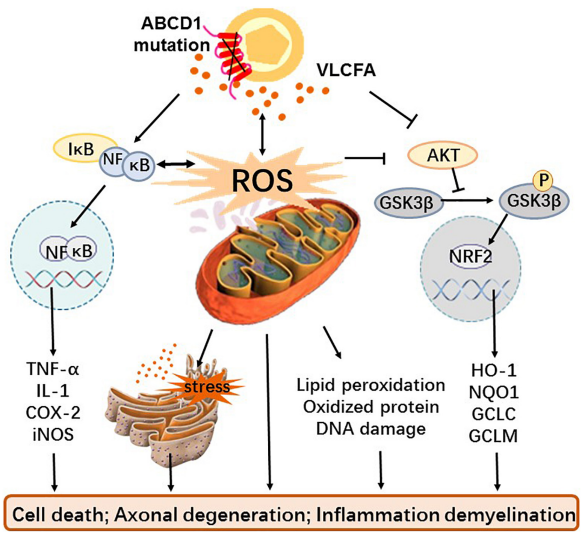

FIGURE 1 | The role and mechanism of oxidative stress and inflammation in X-ALD. Mutations in the transmembrane protein ABCD1 gene on the peroxisome prevents VLCFA from being transported to the peroxisome for β-oxidation, resulting in the accumulation of VLCFA *in vivo*. Dysfunctional peroxisome and cumulative VLCFA not only causes mitochondria that give rise to excessive ROS and oxidative stress but also directly interferes with the GSK3β-NRF2 antioxidant pathway leading to redox imbalance and activate the NF-κB pathway to induce inflammation. In addition, excessive ROS can also affect antioxidant systems as well as inflammatory pathways, cause endoplasmic reticulum stress with or without VLCFA, and generate macromolecular peroxidation damage. These alterations eventually cause cell death, axonal degeneration, and even inflammatory demyelination.

earlier and even worse symptoms that are suitable for therapeutic assays at 12 months but also in the absence of inflammatory demyelination (77). Similarly, studies have shown that ABCD1 knockout or ABCD1/2 double-knockout rabbit models still fail to recapitulate human CALD phenotypes (78).

The Cross-Talk Between Peroxisome and Mitochondria

Recently, the capacity of redox regulation of peroxisome has drawn great attention. Peroxisome plays a key role in cellular lipid metabolism and ROS, which not only generates ROS by itself (79) but also has interplay with mitochondria through messengers such as VLCFA, hydrogen peroxide, and PUFAs, leading to further oxidative stress (80, 81). Studies have revealed that inhibition of peroxisome activity or biogenesis rapidly results in mitochondrial oxidative damage and dysfunction (79). It is known that mitochondria are major regulators of redox. VLCFA may be the biological messengers that convey redox information between peroxisomes and mitochondria in X-ALD and then induce mitochondrial oxidative stress.

Toxicity of Very-Long-Chain Fatty Acids

Until present, excessive VLCFA has been considered as a causative factor of oxidative stress and inflammation in X-ALD (73), although the role that VLCFA plays in the mechanism is indeterminate. Studies have shown that 20 μM or a higher dose of VLCFA can induce mitochondrial inner membrane depolarization and permeability transformation, resulting in excessive ROS, which leads to oxidative stress characterized by lipid peroxidation, protein carbonylation, increased SOD activity, decreased CAT activity, and glutathione level, especially on microglia, astrocytes, and adrenocortical cells (73, 79–83). ABCD1 deficiency can induce the spontaneous production of ROS in low doses or even in the absence of VLCFA. Studies have shown that in the concentration range of 5–10 μM, C:26 could significantly increase the level of ROS and decrease the level of GSH in ABCD1 gene knockout oligodendrocytes and X-ALD fibroblasts, and the oxidative damage products were doubled at high dose C:26 (79, 84).

Then, VLFCA can also directly regulate inflammation-related signal pathways to promote the increased expression of NF-κB and AP-1 (85), but another study suggests that VLCFA influences the precursors of inflammation-resolving lipid mediators to regulate inflammation response and oxidative stress (86). In addition, studies have demonstrated that dysregulation of VLCFA induces endoplasmic reticulum (ER) stress and lysosomal and peroxisomal dysfunctions (87). In summary, VLCFA plays the role of cytotoxicity in various ways, leading to oxidative and inflammatory damage.

Silent Information Regulator 1/Coactivator-1/Peroxisome Proliferation-Activated Receptor Pathway and Mitochondrial Deletion

As previously mentioned, mitochondria are the main organelles that produce ROS in the brain; thus, mitochondrial dysfunction is the main cause of oxidative stress. Reduced levels of mitochondrial proteins such as NADH-ubiquinol oxidoreductase (NDUFB8) and voltage-dependent anion channel (VDAC) were observed in the affected white matter of patients with X-ALD (88), suggesting that mitochondrial content or function is impaired in X-ALD. Peroxisome proliferation-activated receptor-gamma (PPAR-γ) coactivator-1 (PGC-1) is the dominant regulator of mitochondrial biogenesis, regulated by upstream factor sirtuin 1 (SIRT1) partly, by changing the content and/or activity of transcription regulators such as PPARα/β/γ, estrogen-related receptor-α (ERR), nuclear respiratory factor 1 (NRF1), and the mitochondrial transcription factor (TFAM), which regulates the replication and transcription of mitochondrial DNA (88). The expression of SIRT1 in the affected cerebral white matter was lower than that in the unaffected white matter in the X-ALD human brain (89). Levels of SIRT1, PGC-1, and downstream transcription factors PPARα/β/γ, ERRα, and TFAM were all reported to decrease in the spinal cords of ABCD1-/- mice, further indicating impairment of mitochondrial biogenesis in the disease (88, 89).

In addition, the PPAR family, an important regulator, has been confirmed to demonstrate mitochondrial normalization and neuroprotection in various neurodegenerative diseases. Activation of PPARγ induces mitochondrial biogenesis and reverses energy depletion in X-ALD certainty. Erucic acid, an important ligand of PPARδ, has been used to treat X-ALD (90). The study showed that activation of PPARδ stimulated mitochondrial biogenesis and suppressed ROS production, protecting the brain from mitochondrial and oxidative damage (90). As for PPARα, it has been found that downregulation can affect antioxidant and anti-inflammatory responses in other degenerative diseases such as AD (91). However, in X-ALD, PPARα is not directly related to mitochondrial dysfunction and oxidative stress, but it may work by regulating the expression of ABCD1 homologous protein ABCD2 (77).

Nuclear Factor E2-Related Factor and Antioxidative Systems

Nuclear factor E2-related factor (NRF2) is recognized as the master regulator of cellular redox homeostasis. Generally, NRF2 binds to Kelch-like ECH-associated protein (KEAP1) in the cytoplasm but then dissociates from KEAP1 and translocates into the nucleus where it combines with the antioxidant response element (ARE) in DNA sequence to activate the transcription of various downstream genes coded endogenous antioxidants, including GSH, SOD, and CAT under oxidative stress conditions, consequently starting the antioxidant defense systems (92). Activation of the KEAP1/NRF2 pathway blocks the progression of degenerative diseases such as AD and amyotrophic lateral sclerosis (ALS) (93, 94). There is no difference in the expression of NRF2 protein in total skin-derived fibroblasts between patients with X-ALD and healthy people, but the level of NRF2 located in the nucleus and transcription of downstream genes are both reduced (29). However, no study shows the interaction between KEAP1 and NRF2, or the expression of KEAP1 was changed in X-ALD. Conversely, the KEAP1-independent regulation of

the AKT/GSK3β/NRF2 pathway may regulate the antioxidant system. Glycogen synthase kinase-3β (GSK3β), inhibited by AKT-mediated phosphorylation, is a protein kinase that can inhibit the NRF2 pathway by inducing NRF2 phosphorylation. Defective AKT phosphorylation and abnormal activation of GSK3β are both found in skin-derived fibroblasts from patients with X-ALD and in the spinal cord of ABCD1 knockout mice. Furthermore, when drugs inhibit GSK3β in X-ALD fibroblasts, NFR2 restores and activates transcription of downstream genes (29).

A variety of lipids produced by peroxisomes such as plasmalogen and DHA are also used as endogenous antioxidants to eliminate intercellular ROS to protect cells from oxidative damage (95). The genetic inactivation of PEX7 leads to defects in plasmalogen biosynthesis. It was found that PEX7:ABCD1 DKO mice showed more severe neuropathy with demyelination and axonal loss than ABCD1 KO mice (96), suggesting that plasmalogen plays an important role in the onset and development of X-ALD. The upregulation of NRF2 significantly reversed the decreased expression of plasmalogen in the AD model mouse brain to improve oxidative stress and neuroinflammation (97). Therefore, NRF2 may directly or indirectly regulate lipophilic antioxidants to play a role in redox regulation.

Nuclear Factor κB Pathway and Inflammation

NF-κB is a redox-regulated transcription factors involving various responses such as pro-inflammation, inflammation, and oxidative stress (98). Typically, it is held in resting-state thought association with inhibitor of κB (IκB) proteins. When stimulants, including ROS, cytokines, and VLCFA, bind to cell surface receptor toll-like receptors (TLRs), IκB is degraded, while NF-κB is activated and then migrated into nuclear, NF-κb combines with DNA responsive elements to promote transcription of genes of pro-inflammation and inflammation (92). Activation of cerebral inflammation in X-ALD disease may be associated with the NF-κB pathway. The increased expression of TLR2 was 1.3-fold, and the TLR4 coreceptor CD14 was 4.5-fold higher in postmortem brain tissues of patients with CALD than in the control group (24), indicating that the TLRs/NF-κB pathway is activated. NF-κB was observed to have a significantly increased activation and DNA binding activity in primary astrocytes after ABCD1/ABCD2 silencing (85). In addition, in spinal cords of ABCD1 KO mice, the massage RNA and protein products of NF-κB were also notably upregulated (24). The primary astrocytes and spinal cord mentioned in previous studies were detected increased levels of downstream pro-inflammatory mediators TNFα, IL-1β, C-C chemokine ligand 5 (CCL5), and cyclooxygenases-2 (COX-2), which could also confirm the activation of the NF-κB pathway (24, 85).

In addition, studies have shown that the blood–brain barrier (BBB) endothelial cell dysfunction in X-ALD is also related to the activation of the NF-κB signaling pathway (35). Because NF-κB combines and activates the transcription of adhesion molecules, leukocytes, and inflammatory cells can cross the BBB to the brain. The research found NF-κB expression increased in ABCD1 silenced human brain microvascular endothelial cells (HBMECs) (99). One of the characteristics of inflammatory demyelination in CALD is the increased BBB permeability to monocytes/macrophages (99). ABCD1-deficient primary monocytes from patients with X-ALD predispose for pro-inflammatory but unobtained to anti-inflammatory polarization activation, triggering an inflammatory response (55).

Peroxidation Induces Further Cell Damages

Excessive ROS can also indirectly affect the onset and progression of X-ALD by oxidizing modified macromolecules such as lipids, proteins, and nucleic acids in cells to change their structure and function. These peroxidation products could exacerbate functional impairment of mitochondria and peroxisomes and further induce oxidative stress and inflammation, leading to cellular damage and death (8, 34, 69, 84, 100, 101).

The brain has abundant lipids but low antioxidant defense, consequently, lipid peroxidation increases, notably when oxidative stress occurs. 7-Ketocholesterol (7-KC), one of the common oxidation products of cholesterol, has been found at increased levels in the plasma of different phenotypes of patients with X-ALD (68). *In vitro*, 7-KC was observed to inhibit the expression of ABCD1 and homologs, including ABCD2 and ABCD3 in microglia and oligodendrocytes, resulting in peroxisome dysfunction, the root of disease occurrence. Besides, 7-KC also promotes oxidative stress, mitochondrial dysfunction, and neuroinflammation, which are significant characteristics of X-ALD (68, 102). Another oxysterol, 25-hydroxycholesterol (25-HC), and the cholesterol 25-hydroxylase (H25CH) were found to be significantly upregulated in iPSCs and primary fibroblasts of patients with X-ALD. The increased level in CALD was more significant in AMN, suggesting that the expression of 25-HC and C25CH are closely associated with phenotypic severity to become a new biomarker. Moreover, the study showed that 25HC could induce inflammasome activation *via* stimulating mitochondrial ROS to cause microglial recruitment, IL-1β release, and oligodendrocyte death, and consequently severe neuroinflammation and demyelination (103). Lipid peroxidation, especially oxysterol, mainly accumulates in the inflammatory demyelinating lesions and adrenal cortex, which are the major affected areas in X-ALD. Thus, until present, cholesterol metabolism is considered to be strongly interrelated with the pathogenesis of X-ALD. Moreover, as active oxides in the body, lipid peroxidation can also damage other macromolecules.

Protein is the main functional molecule in cells, but the structure and function can be changed when it is oxidized and subsequently degraded by ubiquitin-proteasomes. Dysfunctions of the ubiquitin-proteasome system were found in both ABCD1 null mouse model spinal cords and X-ALD fibroblasts, which speculated that oxidative stress and inflammation stimulated an increase in oxidized proteins that needed to be ubiquitinated, consequently, resulting in the inhibition of proteasome activity because of massive accumulation of polyubiquitinated proteins (104). Mitochondria are the main participants of oxidative stress,

and their proteins are observed to be oxidized damaged in the X-ALD model, leading to the dysfunction of key enzymes in mitochondrial energy metabolism, such as α/β-ATP synthase, the trichloroacetic acid cycle enzymes malate dehydrogenase, and aconitase (105). In another example, cyclophilin D, an important component of mitochondrial permeability transition pore, was observed to be overexpressed and oxidatively modified when stimulated by oxidative stress in affected brain tissue and skin-derived fibroblasts from patients with X-ALD as well as the spinal cord of X-ALD mouse models. High level and/or increased carbonylation of cyclophilin D enhances the sensitivity of ABCD1-deficient cells to oxidative stress and then induce the opening of the mitochondrial permeability transition pore, leading to inner mitochondrial membrane depolarization and respiratory chain damage, and ultimately cell necrosis and apoptosis (106). Nevertheless, the inactivation of the proteasome leads to the failure in the elimination of oxidative mitochondrial proteins in a timely manner, then aggravating the toxicity of the damaged mitochondria and leading to more production and accumulation of ROS (104). Hence, the accumulation of oxidized proteins and the ubiquitin-proteasome inactivation further induces oxidative stress.

Excess ROS also leads to DNA oxidative damage, which, if not repaired, can lead to cell senescence and apoptosis. Significantly increased DNA damage was ascertained in leukocytes of symptomatic patients with X-ALD, and the researchers supposed that the damage might be caused by lipid peroxidation (105). Compared to nuclear DNA, mtDNA is more sensitive to oxidative stress because the mitochondrial genome is deficient in protection and closer to ROS. Increased mtDNA oxidation ratios were detected in the affected white matter areas, showing active demyelinating plaques of patients with X-ALD (107).

Endoplasmic Reticulum Stress Motivated by Oxidative Stress

Endoplasmic reticulum stress, or the unfolded protein response (UPR), has a complicated interaction with oxidative stress (108). It is currently believed to be closely associated with various peroxidase diseases and degenerative diseases such as AD, PD, and ALS. Although the accumulation of misfolded proteins is wildly considered to drive UPR, oxidative stress and lipid dyshomeostasis are perceived as the main cause of ER stress in X-ALD (108, 109). The evidence of UPR activation is detected in the X-ALD mouse model as well as skin-derived fibroblasts and brain tissues from patients with X-ALD (108–110). Furthermore, antioxidant treatment reversed ER stress and normalized the expression of related pathway molecules, which manifested that redox imbalance promoted UPR activation (109). Eventually, ER stress gives rise to cell apoptosis.

Other Potential Targets Regulate Redox and Inflammation in X-Linked Adrenozleukodystrophy

Bioenergy failure is one of the futures of X-ALD, and AMP-activated protein kinase (AMPK) plays an important role in controlling energy homeostasis. Deficiency of AMPK expression was found in white matter, fibroblasts, and lymphocytes from patients with X-ALD, especially cerebral form (37, 111). AMPK regulates mitochondrial biosynthesis and function as well as inflammatory response. In the mixed glial cells of ABCD1-knock-out mice, loss of AMPK induced mitochondrial dysfunction caused by reduced PGC-1 levels and spontaneous increased pro-inflammatory tendencies (111, 112). Inhibition of AMPK promotes pro-inflammatory gene expression, while stimulation with inflammatory cytokines contributes to dephosphorylation and inhibits AMPK (112). The vicious cycle of AMPK deletion and inflammation activation exacerbates inflammatory damage, which would be the potential mechanism of severe cerebral inflammatory demyelination because a greater reduction in AMPK was found in CALD than in AMN (112).

Receptor-interacting protein 140 (RIP140), a coregulator of nuclear receptors and other transcription factors, serves dual functions as a co-activator and a co-repressor. RIP140 interacts with PGC-1 to negatively regulate mitochondrial biogenesis and energy homeostasis and interacts with NF-κB to induce inflammatory activation. A study found that RIP140 was upregulated in specific tissues that affected cerebral white matter in patients with CALD and spinal cord in ABCD1 mouse models, which may be due to the redox imbalance and excessive ROS required for RIP140 induction. Genic-silenced RIP140 restored mitochondrial function and redox homeostasis and prevented inflammatory response in X-ALD mouse models (36). Thus, inhibition of RIP140 may be regarded as a potential therapeutic target for X-ALD.

In conclusion, although the exact mechanisms underlying oxidative stress and inflammation in X-ALD are ambiguous, several possible mechanisms have been proposed and gradually validated in various cells and animal models. These mechanisms can be identified as potential intervention targets for the treatment of X-ALD, and drugs aimed at the part of targets have been applied to preclinical research or even clinical research to explore more new directions for therapy.

NOVEL THERAPEUTIC STRATEGIES TARGETED OXIDATIVE STRESS AND INFLAMMATION

Current clinical treatments are only Lorenzo's oil (LO) and bone marrow transplant (BMT) or hematopoietic stem cell transplantation (HSCT), but the therapeutic effects have limitations and uncertainty. Yet the promising lentiviral hematopoietic stem cell (HSC) gene therapy was halted because it induced abnormal bone marrow cells even myelodysplastic syndrome (MDS) (113, 114). Consequently, more new therapeutic strategies need to be explored. Since oxidative stress and inflammation have been identified as key factors in the early stage of X-ALD, normalizing redox balance, mitochondrial function, and inflammation are key approaches in treatment.

Promising Clinical Trial of X-Linked Adrenoleukodystrophy

As previously noted, oxidative stress caused by excessive ROS and defective antioxidant defense is an essential pathological

mechanism of X-ALD, so antioxidants are naturally considered to be applied in the disease. N-Acetylcysteine (NAC), a strong thiol-containing antioxidant, can improve survival in advanced patients with CALD as adjunctive therapy to HSCT, which targets to increase HO-1 expression (115, 116). *In vitro* studies found that NAC alone could reduce VLCFA-induced oxidized damage, mitochondrial dysfunction, and inflammation (83, 100, 107), while dendrimer-NAC, which was revealed to travel the BBB and located specifically for better bioavailability in other cerebral injury models, increased GSH levels and minified proinflammatory cytokines (67). Furthermore, NAC still alleviated the oxidative damage of cyclophilin D in X-ALD mouse models and reduced its expression when combined with α-lipoic acid to suppress mitochondrial dysfunction. To improve the effectiveness of antioxidants, the therapy of combination of multiple antioxidants has entered the stage of clinical trials. NAC, α-tocopherol, and α-lipoic acid, which are well-known antioxidants proved to be able to cross BBB and to achieve a neuroprotective effect, were shown to restrain oxidative stress and damage and reverse the axonal degeneration and dyskinesia in combination in the preclinical study (34). Phase II trials showed the improvement of various biomarkers and athletic ability in patients with AMN for a year treatment with a high dose of NAC, α-tocopherol, and α-lipoic acid in combination. In addition, by comparing the expression of oxidative stress and inflammatory markers before and after treatment, the study provides a series of candidate biomarkers to predict antioxidants treatment response, patient stratification, and disease progressions such as MCP1 and 15-HETE (8). Unfortunately, this clinical study focused mainly on the curative effect in patients with AMN but neglected to evaluate in CALD.

As an agonist of PPARγ, pioglitazone reverses mitochondrial depletion and bioenergy failure *via* the activation of the PGC-1/PPARγ pathway and reduces oxidized damage of DNA as well as proteins through antioxidative action, preventing the progression of axonal degeneration and locomotor deficits (88). Besides, leriglitazone, a newly developed PPARγ agonist, has been revealed better therapeutic efficacy than pioglitazone because it possesses superior BBB penetration, bioavailability, and safety profile. Leriglitazone not only improves redox balance and mitochondrial biogenesis, more importantly, but it also inhibits the activation of NF-κB to control inflammation and effectively decreases pro-inflammatory biomarkers in plasma and cerebrospinal fluid in healthy volunteers, which is very important for the treatment of severe cerebral form (35). Hence, leriglitazone has been registered in phase II clinical trial for the treatment of CCALD and received orphan drug designation in Europe and the United States and is expected to become the first oral drug for the therapy of X-ALD.

Preclinical Studies of X-Linked Adrenoleukodystrophy for New Therapy

Studies on SIRT1 show evidence for its role in preventing oxidative stress and inflammation (117). Resveratrol, the most common SIRT1 activator, not only relieves the brain microvascular endothelial dysfunction and the permeability of the BBB to inflammatory cells caused by ABCD1 deletion through enhancing SIRT1 function to regulate NF-κB signal pathway (99) but also normalizes axonal degeneration and locomotor deficits *via* activation of SIRT1/PGC-1 pathway to promote mitochondrial biogenesis and function in ABCD1-/ABCD2-/- mice (89). Furthermore, resveratrol is a natural antioxidant that regulates antioxidant defense by eliminating excessive ROS in X-ALD fibroblasts (89).

Biotin is an essential cofactor for carboxylases to control energy metabolism and redox balance (118). In X-ALD mice models, treatment with a high dosage of biotin removes excessive ROS by inducing the endogenous antioxidant response of NRF2 and normalizes redox *in vivo via* recovering mitochondrial biogenesis and energy supplement to improve symptoms of dyskinesia and axon injury consequently. In addition, biotin could target lipid metabolism to cure X-ALD (119).

Targeting to NRF2 antioxidant pathway is a novel therapeutic strategy. Dimethyl-formamide (DMF), a classical activator of NRF2, has already been used to treat some neurodegenerative diseases (120), which has also expressed excellent antioxidant properties in X-ALD. DMF reactivated the expression of NRF2 and the transcription of classic target genes such as HO-1 and NQO1 to reverse oxidative damage. Besides, DMF can also prevent mitochondrial and bioenergy failure and an inflammatory imbalance to ultimately improve the clinical symptoms of X-ALD mice (29).

The ER stress inhibitor tauroursodeoxycholic acid (TUDCA) has been confirmed to effectively normalize several UPR responsive genes and ER stress sensors aberrantly regulated in ABCD1-mice and stop the progression of axonal and locomotor impairment in ABCD1/ABCD2 double knout out mice (109). Moreover, TUDCA, which has remarkably safe profiles and penetration ability through BBB that has been applied in clinical trials for degenerative diseases such as ALS and HD, is looking forward to being a potential candidate drug for X-ALD.

Metformin, the most common AMPK activator, was found to promote mitochondrial generation and function, increase ABCD2 expression, and lower VLCFA concentration in the fibroblasts derived from patients with AMN and CALD. Furthermore, a study has found metformin-induced AMPK, ABCD2, and mitochondrial complex subunit levels *in vivo* in ABCD1-KO mice (37). But the effect on symptoms of this disease was not explored.

Histone deacetylase (HDAC) inhibitor is proved to inhibit oxidative stress and inflammation in the brain to improve cerebral damage (121, 122) and interfere with pro-inflammatory skewing (51) as well as oxidative damage of proteins (123) in X-ALD. SAHA, the pan-HDAC inhibitor in clinical practice, has been found to restore mitochondrial integrity and function in ABCD1 silenced oligodendrocytes and astrocytes (124) and reduce the expression of proinflammatory cytokines, iNOS, as well as the activation of NF-κB in ABCD1/ABCD2-silenced mice primary astrocytes (125).

Current Clinical Treatment's Effects on Oxidative Stress and Inflammation

BMT, HSCT, and LO are currently the main clinical treatments for X-ALD. Lipid peroxidation and oxidized protein were significantly reduced in the plasma of patients with X-ALD treated with BMT or HSCT, suggesting that BMT can lower oxidative stress *in vivo* (126). In contrast, although erucic acid, the important component of LO, was revealed to have antioxidative and anti-neuroinflammatory effects in therapies of AD, HD, and MS (90, 127), the study showed that LO did not reduce oxidative stress and promote the antioxidant defense system in patients (70).

Studies have shown that lovastatin significantly inhibits the production of pro-inflammatory cytokines in astrocytes and microglia and normalizes the level of VLCFA in skin fibroblasts of X-ALD by increasing β-oxidation (128). Increased levels of antioxidant plasmalogens and decreased levels of ROS were detected in X-ALD mice treated with lovastatin (48). However, plasma VLCFA levels in patients treated with lovastatin decreased by only about 20%, which is still much higher than the normal level (129). Therefore, the clinical significance of lovastatin in the treatment of X-ALD needs to be further studied.

Most treatments are limited to spinal axonal lesions and early asymptomatic periods due to the lack of suitable animal models and are hardly effective for CALD or progressive conditions. Besides, the brain is the major affected site, so whether the drug penetrates the BBB also has to be considered. In conclusion, though the drugs mentioned above showed good efficacy in preclinical or clinical trials, many tests and a long time would be required to apply to clinical treatment.

CONCLUSION

There is increasing evidence of oxidative damage and inflammation in patients with X-ALD, and these would be correlated with the onset or severity of symptoms, making oxidative stress or inflammation indicators possible as biomarkers to predict disease progression. In particular, plasma inflammatory markers can be used as early biomarkers of brain injury (57) and predictors of therapeutic response and disease progression (8). Current studies on plasma cytokines have mostly included asymptomatic patients and patients with AMN and CALD but have not considered patients with only adrenal insufficiency. Especially adrenal insufficiency is often the first manifestation of X-ALD, frequently before the onset of neurologic symptoms (130), so the disease progression of AO patients is what we urgently want to know.

Although the exact molecular mechanism of the ROS and inflammation process is still unclear, continuous studies have found that mitochondrial dysfunction, redox imbalance, bioenergy failure, and inflammatory activation are the main features of the X-ALD, which may act synergistically with other underlying mechanisms involved in the pathophysiology of this disease, such as ER stress, leading to body damage. Ferroptosis, a newly identified regulatory form of cell death, is associated with the etiopathogenesis of various degenerative diseases, such as AD and PD, and has achieved positive effects in clinical treatments (131, 132). The typical characteristics of ferroptosis, including mitochondrial damage, GSH deficiency, and ROS accumulation, have also been found in X-ALD (69, 102). Malondialdehyde (MDA), an important ferroptosis marker and lipid peroxidation (133), increased significantly in plasma of patients with X-ALD and was markedly reduced after treatment with BMT (126). These results indicate that ferroptosis may provide new insights into the pathological mechanism and promising treatment of X-ALD.

Many promising therapeutic approaches targeted at oxidative stress and inflammation are being tested in preclinical or clinical trials with significant improvements in outcomes. Nevertheless, for the most severe phenotype of CALD, its molecular mechanisms and interventions are challenging to explore further due to the absence of appropriate experimental models. Hopefully, changes in oxidative stress and inflammation in CALD could be further explored and more promising treatments attempted.

AUTHOR CONTRIBUTIONS

JY drafted the manuscript. TC looked up the articles and revised this manuscript. XG, MIZ, HL, ZW, and JZ reviewed the manuscript structure and ideas during the development of the article. All authors contributed to the article and approved the submitted version.

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Prepubertal Children With Metabolically Healthy Obesity or Overweight Are More Active Than Their Metabolically Unhealthy Peers Irrespective of Weight Status: GENOBOX Study

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Background and Aim: The association of a metabolically healthy status with the practice of physical activity (PA) remains unclear. Sedentarism and low PA have been linked to increased cardiometabolic risk. The aim of this study was to evaluate the PA levels in metabolically healthy (MH) or unhealthy (MU) prepubertal children with or without overweight/obesity.

Methods: A total 275 children (144 boys) with 9 ± 2 years old were selected for the GENOBOX study. PA times and intensities were evaluated by accelerometry, and anthropometry, blood pressure, and blood biochemical markers were analyzed. Children were considered to have normal weight or obesity, and further classified as MH or MU upon fulfillment of the considered metabolic criteria.

Results: Classification resulted in 119 MH children (21% with overweight/obesity, referred to as MHO) and 156 MU children (47% with overweight/obesity, referred to as MUO). Regarding metabolic profile, MHO showed lower blood pressure levels, both systolic and diastolic and biochemical markers levels, such as glucose, Homeostatic Model Assessment of Insulin Resistance, triglycerides and higher HDL-c levels than MUO ($P < 0.001$). In addition, MHO children spent more time in PA of moderate intensity compared with MUO children. In relation to vigorous PA, MH normal weight (MHN)

children showed higher levels than MUO children. Considering sex, boys spent more time engaged in moderate, vigorous, and moderate–vigorous (MV) PA than girls, and the number of boys in the MH group was also higher.

Conclusion: Prepubertal MHO children are less sedentary, more active, and have better metabolic profiles than their MUO peers. However, all children, especially girls, should increase their PA engagement, both in terms of time and intensity because PA appears to be beneficial for metabolic health status itself.

Keywords: physical activity, childhood, body mass index, metabolism, body composition, health, metabolic syndrome

INTRODUCTION

Childhood obesity is a complex condition and can be associated with a number of cardiometabolic risk factors, including insulin resistance, dyslipidemia, and high blood pressure (1). To address this condition, the American Academy of Pediatrics recently recommended focusing on the clustering of metabolic and cardiovascular risk factors instead of defining metabolic syndrome (MetS) (2). The presence of comorbidities may vary depending on genetics, pubertal status, and ethnicity (3). Recently, criteria for the identification of children with obesity that are free from cardiometabolic risk, i.e., "metabolically healthy obesity", have been defined (3). These other criteria are high-density lipoprotein cholesterol (HDL-c) > 40 mg/dL (or >1.03 mmol/L), triglycerides (TGs) ≤ 150 mg/dL (or ≤1.7 mmol/L), systolic and diastolic blood pressure (SBP and DBP) ≤ 90th percentile, and a fasting glycemia <100 mg/dL (3). However, the diagnosis of metabolic risk in prepubertal children is still compromised by the absence of reference cut-off points for specific sex, age, pubertal stage, and ethnicity groups. Moreover, metabolically healthy obesity status is not a stable condition because it can crossover to the "metabolically unhealthy obesity" phenotype during puberty (4). Therefore, it is important to consider the prepubertal stage to try to elucidate the mechanisms protecting against the appearance of a cluster of cardiometabolic risk factors (5).

Sedentary behaviors and physical activity (PA) seem to play a role in the metabolic phenotype (6). There is evidence that supports the positive effects of high levels of PA, low levels of sedentarism, and high levels of cardiorespiratory fitness (CRF) on cardiometabolic health (7), although there are discrepancies between published studies (8, 9). With respect to sedentary behaviors, most studies have not found any differences between metabolically healthy obesity and non-metabolically healthy obesity (8, 9), while PA and CRF are proposed to play a protective role against metabolically unhealthy obesity (10). However, there is a lack of studies assessing the association between PA and metabolically healthy obesity in children, despite the increasing number of lifestyle interventions aiming to achieve a healthier metabolic profile and a lower risk of cardiovascular mortality/morbidity (10). Providing new insights concerning the role of PA in metabolically healthy and unhealthy prepubertal children may be useful to avoid the onset of cardiometabolic comorbidities.

Therefore, the aim of the present study was to assess PA, including time and intensity, and sedentarism using accelerometers and to determine their association with metabolically healthy or unhealthy profile in a sample of Spanish prepubertal children with and without overweight/obesity.

MATERIALS AND METHODS

Study Design

The present study was carried out in children recruited from three Spanish hospitals (Hospital Universitario Reina Sofía in Córdoba, Hospital Clínico Universitario in Santiago de Compostela, and Hospital Clínico Universitario Lozano Blesa in Zaragoza) under the framework of the GENOBOX study (11, 12). Participants were selected among those already attending these hospitals to confirm the diagnosis of overweight or obesity or for minor disorders that were not confirmed after clinical and laboratory investigations. For the current study, a subsample was selected using the following inclusion criteria: children >5 years of age and at the prepubertal stage (Tanner I), with the absence of endogenous obesity (caused by malfunction of the hormonal or metabolic system) or other metabolic or hormonal diseases, and with a minimum of valid accelerometer data (a minimum of 8 h of monitoring per day for at least 3 days, including at least 1 weekend day). Exclusion criteria were disease or malnutrition; the use of medications that alter blood pressure, glucose, or lipid metabolism; or not meeting the inclusion criteria.

Children and parents or guardians were informed about the purpose and procedures of the study and written consent was obtained from the parents/guardians, while children gave their assent to participate. The ethics committees of all of the participating institutions approved the study, which complied with the Declaration of Helsinki.

Physical Examination

Medical history and physical examination were carried out, including the evaluation of sexual maturity and puberty classification according to Tanner's five-stage scale (13), which was validated by plasma sex hormone concentrations. Body weight, height, and waist circumference (WC) were measured following previously published protocols (14). The body mass index (BMI) z-score was calculated based on the Spanish reference standards published in (15).

Children with normal weight, overweight, and obesity were defined using the International Obesity Task Force (IOTF)'s age- and sex-specific BMI cut-off points that are equivalent to the adult values of 25 kg/m^2 for overweight and 30 kg/m^2 for obesity (16).

SBP and DBP measurements were taken in triplicate by the same examiner using an electronic manometer (Omrom, M6 AC) following international recommendations (17), and the mean of the two closest values was used in further analyses.

Biochemical Analysis

Blood samples were drawn from the antecubital vein after an overnight fast. Routine blood tests were analyzed at the general laboratory of each participating hospital. Plasma glucose (CV = 1.0%), TGs (CV = 1.5%), total cholesterol (CV = 0.9%), HDL-c (CV = 0.8%), and low-density lipoprotein cholesterol (LDL-c) (CV = 1.5%) were measured using an automatic analyzer (Roche-Hitachi Modular P and D Autoanalyzer; Roche Laboratory Systems, Mannheim, Germany). Plasma insulin was analyzed by radioimmunoassay (CV = 2.6%) using an automatic microparticle analyzer (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Insulin resistance (IR) was assessed by means of the Homeostatic Model Assessment of IR (HOMA-IR) as insulin (μU/mL) × glucose (mmol/L)/22.5.

Physical Activity Assessment by Accelerometry

ActiGraph GT3X+ accelerometers (ActiGraph; Pensacola, FL) were used to assess PA, and were programmed to collect raw acceleration data at a frequency of 30 Hz over a dynamic range time of 15 s (epochs). Accelerometers were placed over the right iliac crest and held in place using an adjustable elastic belt for 24 h a day to measure the accelerations of the segment where the monitor was connected. They were removed only for showering or nocturnal rest if the instrument caused discomfort during sleep. Parents and children were instructed that the child should wear the ActiGraph 24 h/day for 7 days. A minimum of 8 h of monitoring per day for at least 3 days, including at least 1 weekend day, was considered acceptable for the evaluation of PA and sedentary time (18).

Two rules were used to exclude low-quality data: all negative counts were replaced by missing data code and periods of 20 min or more of consecutive zero counts were replaced by missing data code prior to the analysis. Unavailability of valid data, non-compliance with the minimum number of hours set, or not enough time on valid days during the week or weekend were exclusion criteria for this analysis. The output generated by the ActiGraph GT3X+ included the total volume of PA and the cut-off points of PA intensity recommended by Evenson et al. (18) of sedentary: ≤100 counts per min (CPM); light: >100–2,296 CPM; moderate: 2,296–4,012 CPM; vigorous PA: ≥4,012 CPM.

Metabolic Health Criteria

All children, independently of their BMI, where classified as metabolically healthy (MH) or unhealthy (MU) according to the published definition for metabolic syndrome in prepubertal children proposed by our group, based on previous authors' classifications (19, 20). This definition considers children as having MU status when fulfilling at least one of the following criteria: (1) SBP or DBP values higher or equal to the 90th percentile for age, sex, and height; (2) TG plasma concentrations higher than the 90th percentile for age, sex, and race; (3) HDL-c plasma concentration lower than the 10th percentile for age, sex, and race; (4) glucose plasma concentration higher or equal to 100 mg/dL; (5) HOMA-IR values higher than 2.5. Children that did not fulfill any of the mentioned criteria were considered to have an MH status. Finally, the sample was divided into the following four groups: metabolically healthy normal weight (MHN) group, metabolic unhealthy normal weight (MUN) group, metabolically healthy overweight/obesity (MHO) group, and metabolically unhealthy overweight/obesity (MUO) group.

Statistical Analysis

The sample size estimation for the GENOBOX study was calculated based on the principal metabolic risk factors for cardiovascular disease associated with obesity. The calculation of the sample size was carried out for a 95% degree of confidence (type I error alpha = 0.05) and a power of 80% (beta error = 0.20) according to the estimation equation of n by comparison with two proportions of one variable in two independent groups. Under these conditions, the sample size was increased to a total of 300 to ensure that significant differences between children with obesity and normal weight could be found with a minimal difference of 20% for each examined parameter. All continuous variables were tested for normality using the Shapiro–Wilk and Kolmogorov-Smirnov tests, and the variables following a non-normal distribution were logarithmically transformed: SBP, HOMA-IR, TGs, HDL-c, and sedentary and vigorous PA. The homogeneity of variances was estimated using Levene's test. Differences between MH and MU children were analyzed by two-independent-sample t-tests or Mann–Whitney U-tests. χ^2-tests were applied to categorical variables expressed as a percentage. Differences between the four groups based on MH/MU and weight status (MHN, MUN, MHO, and MUO) were analyzed using one-way ANOVA and Kruskal–Wallis test, and pairwise differences were assessed by *post-hoc* analyses, adjusted for age, to determine differences between experimental groups. Values in the descriptive tables and results are expressed as means and standard deviations. Differences were considered significant when $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics v.20 software.

RESULTS

A total of 275 children (144 boys) were included after excluding participants without valid accelerometer data or were missing information for metabolic risk and weight status or pubertal stage. Age, anthropometric measures, PA outcomes, and metabolically healthy and unhealthy status are shown in **Table 1**. The sample was homogeneously distributed in the MH and MU groups as well as by sex and age. Weight, BMI, and WC were higher in the MU group compared to the MH group, independently of age. Regarding moderate PA intensity, the MH group showed a higher engagement time (in minutes) than the

TABLE 1 | Demographic, anthropometric, and physical activity intensities compared between prepubertal children of metabolically healthy or unhealthy status.

	MH group ($N = 119$)		MU group ($N = 156$)		p
Age (years)	9.36 ± 1.87		8.91 ± 1.89		0.056
Weight (kg)	36.91 ± 10.71		45.77 ± 13.59		<0.001
Height (cm)	135 ± 10.22		137 ± 12.21		0.200
BMI (kg/m^2)	20 ± 4.49		23.94 ± 4.58		<0.001
BMI z-score	0.84 ± 0.2		2.20 ± 0.18		<0.001
WC (cm)	69.6 ± 12.83		79.3 ± 12.58		<0.001
Sedentary (min/day)	458.38 ± 86.13		461.12 ± 91.53		0.203
LPA (min/day)	266.15 ± 49.73		270.81 ± 55		0.899
MPA (min/day)	39.52 ± 13.99		36.80 ± 13.32		0.045
VPA (min/day)	14.26 ± 8.59		13.01 ± 9.31		0.131
MVPA (min/day)	53.78 ± 20.89		49.80 ± 20.97		0.074
	N	**%**	***N***	**%**	$p^{\dagger}$
Weight status	119	43.5	156	56.5	<0.001
Normal	58	21.2	26	9.1	
Overweight/obesity	61	22.3	130	47.4	
Sex					0.464
Boys	67	24.1	77	28.5	
Girls	52	19.3	79	28.1	
Boys					<0.001
Normal weight	31	21.5	13	9	
Overweight	12	8.3	19	13.2	
Obesity	24	16.7	45	31.2	
Girls					<0.001
Normal weight	28	21.4	12	9.2	
Overweight	13	9.9	14	10.7	
Obesity	11	8.4	53	40.5	

MH, metabolically healthy; MU, metabolic unhealthy; BMI, body max index; WC, waist circumference; LPA, low physical activity; MPA, moderate physical activity; VPA, vigorous physical activity; MVPA, moderate-to-vigorous physical activity.

Data are expressed as mean ± standard deviation. The p-values were obtained after two independent-samples t-tests or Mann–Whitney U-tests.

† *The p-values were obtained after chi-squared tests.*

MU group. MUN was the smallest group among both boys and girls.

The variables included for the definition of metabolic syndrome by Olza et al. (19) were compared between the MHN, MUN, MHO, and MUO groups and are shown in **Table 2**. The subgroup of MHN children showed lower SBP and DBP than for both the MUN and MUO groups as well as a lower HOMA-IR than the MUO group. We further observed a higher HDL-c in the MHN group ($p < 0.001$) than in the other groups, with the latter showing a linear decrease from MHN to MUO. Finally, the MHO group presented lower TG levels than the MU subgroups. Although differences were found between the MH and MU groups in relation to glucose, none of the values were considered pathological.

After adjusting for age, the results show that the MUO children spent less time (in min) engaged in moderate-intensity PA than the MHO children ($p = 0.039$), as well as less time (in min) engaged in vigorous-intensity PA than the MHN children ($p = 0.032$; **Figure 1**).

By contrast, when children with obesity were studied separately (i.e., without including overweight and normal weight) (**Supplementary Table 1**), the MH children with obesity spent more time engaged in moderate-intensity PA (min) (41.76 ± 11.85) than the MU children with obesity (36.42 ± 14.20; $p = 0.034$). In addition, the MHN children spent more time engaged in vigorous-intensity PA (15.42 ± 9.54) than the MU children with obesity (13.19 ± 10.27; $p = 0.050$). In relation to PA practice during the week and on weekends, the MHN children showed more time engaged in vigorous-intensity PA during the weekdays as well as moderate–vigorous PA (MVPA) during the weekend compared with the MUO children ($p < 0.05$; **Figure 1**).

After stratifying the analyses by sex, the results showed that boys engaged in more moderate PA than girls (41.23 ± 14.93 vs. 34.92 ± 11.73), vigorous PA (15.40 ± 10.58 vs. 11.96 ± 7.1), and MVPA (56.31 ± 22.49 vs. 46.91 ± 17.29) in the total sample ($p < 0.005$), as well as within MH/MU groups (**Supplementary Table 2**). Similar results were found for both the MHN and MHO groups. Only for MHN children was light PA also higher in boys than in girls ($p < 0.05$). When sex was studied

TABLE 2 | Measurements of metabolic risk markers in prepubertal children according to metabolically healthy or unhealthy status and normal or overweight/obesity.

Metabolic syndrome variables	MHN group ($N = 59$)	MUN group ($N = 25$)	MHO group ($N = 60$)	MUO group ($N = 131$)	p
SBP (mm Hg)	99 ± 7.5^{a}	106 ± 10.5^{b}	102 ± 8.8^{ab}	113 ± 12.2^{c}	<0.001
DBP (mm Hg)	59 ± 6.5^{a}	64 ± 10.4^{ab}	61 ± 6.7^{a}	68 ± 10.3^{b}	<0.001
Glucose (mg/dL)	83 ± 6.5^{a}	86 ± 8.2^{b}	81 ± 6.6^{a}	84 ± 8.6^{ab}	0.003
HOMA-IR	1.1 ± 0.5^{a}	1.7 ± 1.0^{a}	1.3 ± 0.6^{a}	2.6 ± 1.6^{b}	<0.001
TG (mg/dL)	52 ± 15.1^{a}	72 ± 35.1^{b}	50 ± 17.7^{a}	74 ± 31.5^{b}	<0.001
HDL-c (mg/dL)	66 ± 13.4^{c}	58 ± 15.3^{a}	53 ± 12.4^{ab}	47 ± 12.4^{b}	<0.001

MHN, metabolically healthy normal weight group; MUN, metabolic unhealthy normal weight group; MHO, metabolically healthy overweight/obesity group; MUO, metabolic unhealthy overweight/obesity group; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; TG, triglycerides.
Data are expressed as mean ± standard deviation. The p-values were obtained by two-way ANOVA or Kruskal–Wallis test. Non-matching superscript letters (^{a}, ^{b}, or ^{c}) indicate significant differences ($p < 0.05$) by pairwise post-hoc tests adjusted for age to determine differences between experimental groups.

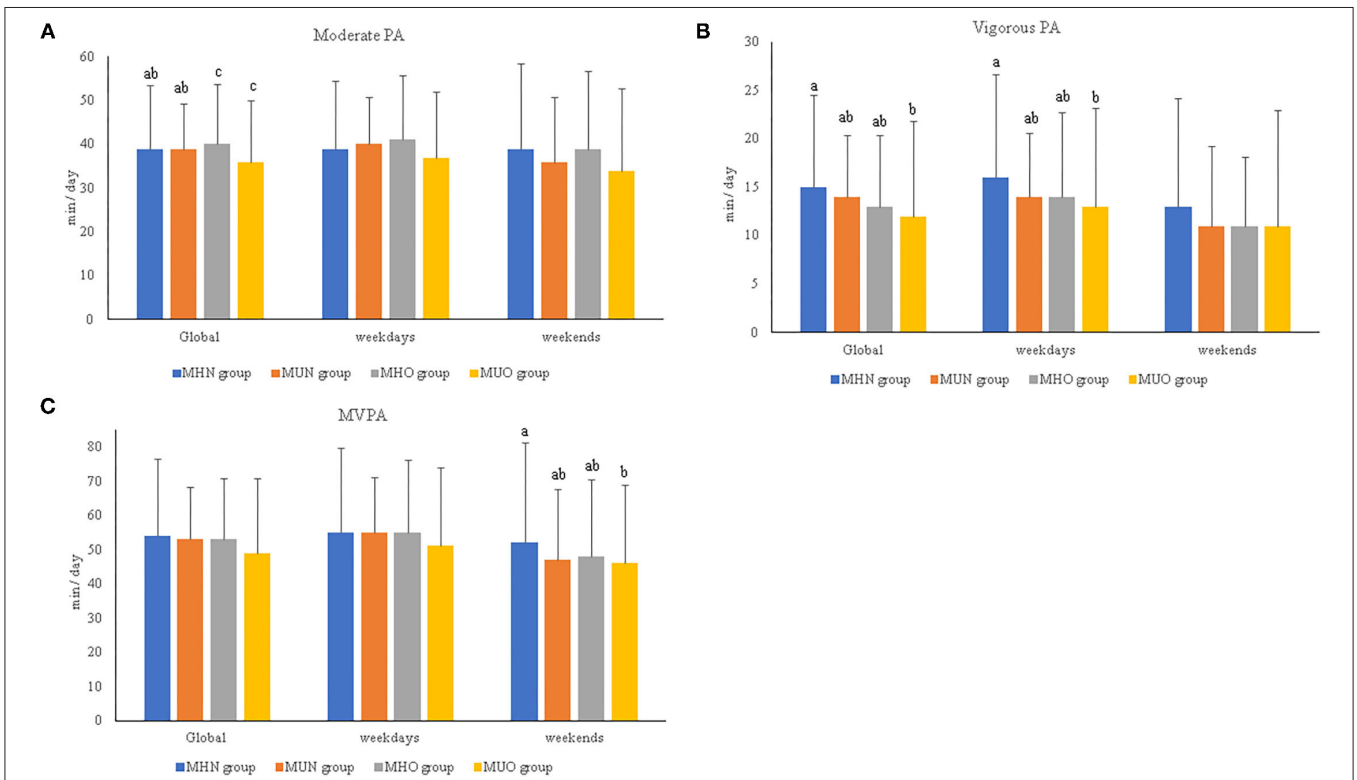

FIGURE 1 | (A–C) Relevant results of physical activity intensities and duration per day in prepubertal children according to metabolically healthy or unhealthy status and normal weight or overweight/obesity. MHN, metabolically healthy normal weight group; MUN, metabolic unhealthy normal weight group; MHO, metabolically healthy overweight/obesity group; MUO, metabolic unhealthy overweight/obesity group; PA, physical activity; MVPA, moderate-to-vigorous physical activity. The *p*-values were obtained two-way ANOVA or Kruskal–Wallis test. Non-matching superscript letters (^{a}, ^{b}, or ^{c}) indicate significant differences ($p < 0.05$) by pairwise *post-hoc* tests adjusted for age and sex to determine differences between experimental groups. The data are subdivided in global data, by weekend, or by weekdays.

independently, the MUO boys showed a lower time engaged in moderate-intensity PA ($p = 0.019$) than the MHO boys, as well as in vigorous-intensity PA and MVPA ($p = 0.032$ and $p = 0.031$, respectively) than the MHN boys (**Figure 2**). No differences were found between the groups of girls (**Supplementary Table 2**).

DISCUSSION

In the present study, MH children, even with obesity, were shown to spend more time engaged in objectively measured PA than children with at least one metabolic risk factor (i.e., the MU group). In the literature, there are inconsistent findings due to the information bias associated with MVPA and sedentary behavior being measured using questionnaires or pedometers (9, 21), which lack intensity estimates. Moreover, some previous studies have examined the association between PA and sedentary time and metabolic risk markers, but only a few have categorized children and adolescents as metabolically healthy/unhealthy across weight status categories (22, 23), and most lacked accelerometry measurements. The different protocols or methods

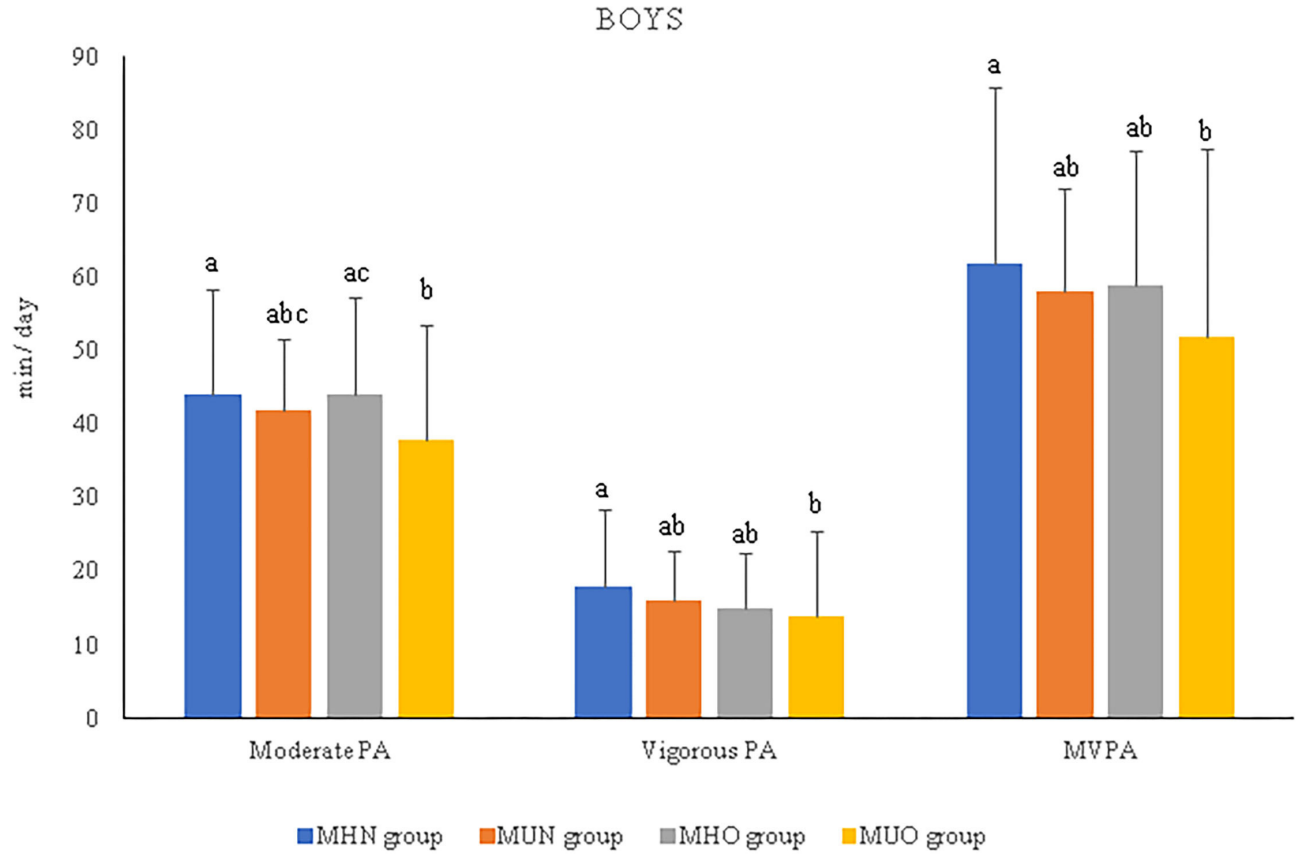

FIGURE 2 | Relevant results of physical activity intensities and duration per day in prepubertal boys according to metabolically healthy or unhealthy status, normal weight or overweight/obesity. MHN, metabolically healthy normal weight group; MUN, metabolic unhealthy normal weight group; MHO, metabolically healthy overweight/obesity group; MUO, metabolic unhealthy overweight/obesity group; PA, physical activity; MVPA, moderate-to-vigorous physical activity. The *p*-values were obtained two-way ANOVA or Kruskal–Wallis test. Non-matching superscript letters (^{a}, ^{b}, or ^{c}) indicate significant differences ($p < 0.05$) by pairwise *post-hoc* tests adjusted for age to determine differences between experimental groups.

used, as well as the other factors such as age, sex, or puberty, could have influenced the changes in metabolic status.

The homogeneous sample recruited by sex and age, considering only prepubertal children but with overweight and obese children represented, is a strength of the current study. Indeed, puberty can be a confounding factor since it is a crucial period for insulin resistance and the development of metabolic syndrome (4). An estimate of gluteofemoral fat mass, has now been established as an important determinant of MH in adults, independently of subcutaneous abdominal fat mass and visceral fat mass (24). One limitation in this study is the classification according to weight using BMI, since it was not possible to obtain data to estimate adiposity in all the children, nor the use of other techniques such as DXA. So, BMI cut off were used similarly as in the clinical practice. For pediatric population, especially in prepuberal, at this moment there are not waist or hip circumference reference values but it is known that body composition is modulated at puberty (4). There is an increase in fat mass in pubertal girls and alongside greater lean mass, the adolescent boys frequently exhibit less total fat mass but similar (or greater in some cases) central fat mass than do girls. In Genobox sample as published by our group, there are no relevant differences in total fat and lean mass between sex (25). Regardless of sex and its influence, an adequate cardiorespiratory fitness in childhood and adolescence has been associated with decreased fat mass over time (26) although is not strongly associated with MHO phenotype (21). So, different intensities and frequency of physical activity seems to influence a healthy fitness condition.

In the same way, there are not always age- or sex-specific reference values for some of these biochemical parameters in children, but there are differences in cardiometabolic risk factors and metabolic syndrome diagnosis along the pubertal stages that must be considered when evaluating the future risk and implicated factors (4, 27). In the present study, 47.4% of the children with overweight or obesity were MU, as well as 9% of the children of normal weight, compared to other studies also carried out in children (23, 28). The reason for these differences could be that cardiometabolic risk is very precocious in relation to age and prepubertal stage, as well as to the classification used to define MU status. Thus, future longitudinal research during childhood is needed to determine the true risk and cut-offs that are at least age- and sex-adjusted.

We also selected specific criteria (19) to identify prepubertal MUO children based on the highest percentile values for BP, TGs, and HDL-c for minimum age and sex, glucose plasma concentration higher or equal to 100 mg/dL, or HOMA-IR values higher than 2.5, which were used as the cut-offs for the prepubertal children in the present study and were based on what other authors have previously established (29).

Childhood obesity has been associated with a moderately increased risk of adult obesity-related morbidity, but BMI is not a good predictor of the incidence of these morbidities. Often, it occurs in adults who were of a healthy weight in childhood. Therefore, targeting obesity reduction solely in children with overweight or obesity may not substantially reduce the overall burden of obesity-related diseases in adulthood (30). In the IDEFICS study (Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS) performed with preadolescents, physical inactivity and sedentary lifestyle were also found to be associated with the development of insulin resistance, independently of weight status (31). In fact, in our study, MHN children had a better metabolic profile with lower SBP and HOMA-IR values and higher HDL-c compared with MUO children, also showing lower serum concentrations of glucose and triglycerides. Thus, perhaps we should also focus on other metabolic risk factors associated with low PA practice, and not only on obesity status. In a review by Kuzik et al. (23), 20 studies with 4,581 children and adolescents were included, and it was reported that each additional 60 min of sedentary time per day was associated with 8–11% higher odds of being classified as MU in the normal weight group, compared to being classified as MH. However, each additional 10 min of MVPA per day was associated with lower odds of MU classification in both the normal weight and overweight groups, as compared to being classified as MH. These results are complementary to ours, showing the principal role of time and intensity of PA in determining the status of metabolic health.

PA has been suggested to modulate fuel metabolism. The increased fat oxidation promoted by PA might be the basis for the prevention and restoration of insulin sensitivity and reduction of metabolic syndrome in children with obesity. Primarily, vigorous PA can decrease energy stores, improve body composition (by increasing lean mass as a substitute of fat mass lost), and restore fat distribution (by reducing visceral and intramuscular fat depots) (32). In another study, time spent engaging in PA for most children was lower than the 60 min of MVPA per day recommended by the WHO (33), being more alarming in girls, in which the time spent was even lower. In that study, MHO children spent more time engaged in moderate-intensity PA than the MUO children. In relation to vigorous activity, the MHN children showed higher levels than the MUO children. It is known that metabolic benefits are greater with MVPA practice than for lower-intensity PA, and decreasing sedentary time seems to be beneficial only for metabolic health (23). In the IDEFICS study, MVPA at baseline (upper two quartiles) showed a protective effect on the development of insulin resistance 2 years later also for children with normal weight at baseline, which indicates that the negative effect of low PA is not just mediated by obesity (31). In a study by Stabelini et al. (34), time spent engaging in MVPA was inversely associated with a continuous metabolic syndrome risk score (31) in both sexes. In most of the studies that have considered sex, boys display higher MVPA levels compared to girls (35, 36), as can be observed in our study, though in the MH subgroup of children with normal weight and obesity. In addition, MVPA time has been reported to be significantly higher in children on weekdays compared to at weekends (35). In the present study, the MHN children spent more time overall and also in greater intensity PA, i.e., in vigorous PA and MVPA, during the week and at the weekend compared with MUO children.

Although sedentary time did not indicate significant differences, it seems that the MHO children were less sedentary than the MHN and MUO children. In other recent article of our group on this subsample of children, a higher percentage of active children were reported to be members of a sport club or practiced collective sports when compared to sedentary children (12). The MHO children in this study, selected from hospitals, likely practice PA to control their weight and to be healthier. It has also been described that a decrease in MVPA and an increase in sedentary time after follow-up are significantly lower for children who participate in sports than for those who do not, both for boys and girls (36). Some authors have associated overweight with low levels of PA and sedentary behavior (37), but it seems that there is no association between sedentary time and metabolic health in people with obesity (8, 23). In a recent systematic review of 26 studies in children and adolescents, the MVPA levels in children with or without obesity were consistently below the recommendations, without marked differences in sedentary time between children with obesity and normal weight (38).

Engaging in PA during childhood can induce biomechanical, physiological, and psychological changes, resulting in beneficial adaptations that persist throughout adulthood (34). Therefore, it is important to identify the protective factors, avoiding the crossover of the MHO (considering adiposity) to MU phenotype. The principal limitation in this study is that there is no consensus about what traditional metabolic features use in children and how to estimate adiposity instead of using variables as BMIzscore. Metabolically unhealthy might be confounding especially when accompanied by obesity category. Research about metabolic health and its relationship with weight status and other factors related to PA should be included in individualized clinical interventions in children by determining specific lifestyle modifications providing the most health benefit for them. In conclusion, MHO children are more active than their MUO peers, and they have a better metabolic profile, so this active condition appears to be beneficial for metabolic health itself. However, all children, especially girls, should increase their PA engagement, both in terms of time and intensity.

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 human participants were reviewed and approved by Ethics Committees from Hospital Universitario Reina Sofía in Córdoba, Hospital Clínico Universitario in Santiago de Compostela, and Hospital Clínico Universitario Lozano Blesa in Zaragoza (Spain). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FL-C: conceptualization, data curation, formal analysis, methodology, and writing—original draft. RL: conceptualization, funding acquisition, investigation, methodology, project administration, resources, and writing—review and editing. AR: data curation, investigation, resources, and writing—review and editing. AA-R: data curation, formal analysis, software, and writing—review and editing. RV-C, KF-R, and EG-G: data curation, resources, and writing—review and editing. CA and LM: conceptualization, funding acquisition, investigation, project administration, and writing—review and editing. GB: conceptualization, funding acquisition, investigation, project administration, resources, and writing—review and editing. MG-C: conceptualization, investigation, methodology, project administration, resources, and writing—original draft. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.821548/full#supplementary-material

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Fitness Levels and Gender Are Related With the Response of Plasma Adipokines and Inflammatory Cytokines in Prepubertal Children

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Background and Aim: Changes in adipokines have been related with the development of metabolic syndrome, frequently associated with obesity, and other risk factors. Fitness seems to promote a healthy cardiovascular status and could be a protector factor, just from childhood. Therefore, the present study aimed to evaluate the relationship between fitness levels with plasma adipokines and inflammatory biomarkers in prepubertal children.

Methods: One hundred and thirty-seven healthy normal-weight prepubertal children were recruited from local schools and divided after performing the fitness tests, into two groups according to fitness level—low cardiovascular fitness group (LF) and equal or higher cardiovascular fitness group (HF). Anthropometric variables, blood pressure (BP) and plasma insulin, and leptin, resistin, adiponectin, tumor necrosis factor-alpha, hepatic growth factor, interleukin (IL)-8, monocyte chemoattractant protein-1, nerve growth factor (NGF), and plasminogen activator inhibitor-1 (PAI-1) were measured fasting in both groups to be compared. Univariate analysis of variance, comparative analysis, binary logistic regression, stepwise linear regression, and principal component analysis were conducted to evaluate the association between fitness, BMI, gender, and the biochemical parameters.

Results: Girls and boys with HF presented lower waist circumference Z-score, BMI Z-score, systolic BP (only boys) as well as lower levels of leptin and NGF compared with their respective LF group. Regarding the association between variables, fitness showed an inverse relationship with BMI Z-score, leptin, PAI-1, HOMA-IR, resistin, IL-8, and NGF.

Conclusion: An adequate level of fitness seems to protect against risk factors related to low-grade inflammation and altered adipokines that are related to the onset of obesity just from the prepubertal stage.

Keywords: inflammation, adipokines, fitness, exercise, childhood, cytokines

INTRODUCTION

Currently, it is known that adipocytes secrete a variety of proteins called adipokines that are involved in different biological functions (1). Proinflammatory cytokines—such as interleukin (IL)-8, tumor necrosis factor-alpha (TNF-α) and other proteins as C-reactive protein (CRP)—are increased in cardiovascular disease (CVD), type II diabetes and obesity (1). Moreover, several adipokines such as leptin and adiponectin play a role in glucose and lipid metabolism, and energy homeostasis, and alterations have been reported in them, related to a higher risk for the development of these pathologies, already in prepubertal children (2). To evaluate the significance of cytokines modulation in pathological conditions, it is necessary to establish the physiological ranges of these molecules in healthy subjects, mainly from early childhood; considering that obesity and metabolic syndrome risk factors are present in scholars (3). In addition, it should be considered that children are in continuous growth and will present changes at different stages of puberty; therefore it is important to study the low inflammatory status response at these early stages to evaluate the influence of all these factors (4).

On the other hand, low fitness levels have been associated with being more prone to develop metabolic risk, being able to act through different pathways, including changes in inflammatory status (5, 6). Infact, an adequate fitness level has been negatively associated with cardiovascular mortality (7), acting as a protector factor relatively static (7). During childhood, cardiorespiratory fitness (CRF) has been inversely correlated with traditional CVD risk factors (6) and low-grade inflammation (8). One of the mechanisms by which physical fitness might promote cardiovascular health is by supporting anti-inflammatory processes.

Moreover, differences between boys and girls have been observed mainly in those biomarkers related to adipose tissue metabolism (1). Although causality it is not well-established, some hypotheses attribute these differences to puberty changes (9). However, there is scarce knowledge about these differences and changes at the prepubertal stage.

As further research on boys and girls is necessary to clarify the role of fitness levels in adiposity, low-grade inflammation or other CVD risk factors (8, 10), this study aimed to evaluate the relationship between CRF levels, measured by alpha fitness battery (11), and adipokines and inflammatory biomarkers in healthy prepubertal boys and girls to provide more specific knowledge of the influence that both gender and fitness can have on a low state of inflammation and on the health status already in prepubertal children.

MATERIALS AND METHODS

Subjects

Healthy prepubertal children were recruited from different local elementary schools in Córdoba, Spain. Inclusion criteria were: prepubertal stage (Tanner I) and age between 7 and 12 years). Exclusion criteria were: pubertal stage, disease, a long period of rest due to illness, and the use of any medication or diet that alters blood pressure or metabolism.

Clinical Examination

A group of pediatricians examined children's medical histories and performed a physical examination to exclude any illness. Sexual maturity was assessed by physical examination using the Tanner five-stage scale. Weight, height, and waist circumference (WC) were measured using standardized techniques. Body mass index (BMI) was calculated as weight (kg)/height (m^2). The values obtained for these anthropometric measurements were compared with the Spanish references (12). Systolic and diastolic blood pressure (BP) were measured using a random-zero sphygmomanometer (Dinamap V-100).

Fitness Evaluation

Children were asked to perform the 20 m shuttle run test (20-mSRT) to evaluate their fitness level using a validated scale from Léger et al. (13). Subjects started running at an initial speed of 8 km/h, and the sprint was increased by 0.5 km/h at 1 min intervals (1 min = one stage), reaching 18.0 km/h by minute 20. Running speed cues were indicated by signals emitted by a commercially-available CD–ROM, and it is included in the ALPHA health-related fitness test battery (11).

Fitness level was considered to divide the sample in two groups. The value obtained after the 20-mSRT was the criteria to include the participants into one or another group: children with a score equaling or above the average of the reference values (14) were assigned to the group designated as "equal or higher cardiovascular fitness group" (HF), and those with a score under the average were assigned to the "low cardiovascular fitness" group (LF).

Cytokines Analysis

Blood samples were obtained from all the children using an indwelling venous line to draw a 3 ml sample after a 12-h overnight fasting. After centrifugation, aliquots of plasma were frozen immediately and stored at −80° until analyzed. C-reactive protein (CRP) was determined using a high-sensitivity, particle-enhanced turbidimetric immunoassay (PETIA) (Dade Behring Inc., IL). LINCOplex™ kits of human monoclonal antibodies (Linco Research, MO, USA) were analyzed on a Luminex® 200™ System (Luminex Corporation, Austin, TX, USA) to determine: adiponectin (CV: 9.2%) (Cat. #HCVD1-67AK), resistin (CV: 6.0%) (Cat. HADK1-61K-A), leptin (CV: 7.9%) (Cat. #HADK2-61K-B), plasma hepatic growth factor (HGF) (CV: 7.7%), interleukin (IL)-6 (CV: 7.8%), IL-8 (CV: 7.9%), monocyte chemoattractant protein-1 (MCP-1) (CV: 7.9%), nerve growth factor (NGF) (CV: 6%), plasminogen activator inhibitor-1 (PAI-1) (CV: 11.8%), and TNF-α (CV: 7.8%) levels, according to manufacturer's instructions (15).

TABLE 1 | Anthropometric and demographic variables as well as concentration of plasma adipokines and inflammatory cytokines classified by grouped by fitness levels and gender.

	LF N: 65	HF N: 72	Girls N: 53	Boys N: 84	G-LF N: 32	G-HF N: 21	B-LF N: 33	B-HF N: 51
Age (years)	9.67±1.09	9.58 ± 1.22§	8.79 ± 0.88	10.18 ± 0.98§	9.00 ± 0.67	8.48 ± 1.08	10.35 ± 1.02	10.04 ± 0.95
WC (cm)	70.97 ± 11.66	63.04 ± 7.56	63.33 ± 9.08	69.62 ± 11.20§	65.60 ± 10.25§	59.88 ± 5.56*	76.70 ± 10.40§	64.34 ± 7.93*
Z-score WC	0.98 ± 1.42	−0.10 ± 0.96§	0.36 ± 1.32	0.51 ± 1.36	0.59 ± 1.47*	0.01 ± 0.98	1.39 ± 1.26§	−0.14 ± 0.97
BMI (kg/m^2)	21.49 ± 4.04	18.62 ± 2.84§	18.98 ± 3.69	20.84 ± 3.80^{+}	19.93 ± 17.52§	17.51 ± 2.53^{+}	23.09 ± 3.42§	19.08 ± 2.87*
Z-score BMI	1.19 ± 1.20	0.32 ± 0.90§	0.47 ± 1.08	0.96 ± 1.19*	0.71 ± 1.17§	0.11 ± 0.81*	1.67 ± 1.05§	0.41 ± 0.93
SBP (mmHg)	123 ± 16.22	120.06 ± 10.49	115.19 ± 13.40	126.20 ± 13.08§	114.53 ± 15.57§	116.19 ± 9.45	131.74 ± 11.74§	121.68 ± 10.57*
DBP (mmHg)	67.14 ± 10.53	67 ± 9.26	63.77 ± 10.23	69.26 ± 9.12§	63.53 ± 64.14^{+}	64.14 ± 8.03	70.87 ± 7.90	68.20 ± 9.55
Adiponectin (nmg/l)	20.45 ± 16.71	21.09 ± 17.55	26.30 ± 24.93	17.55 ± 12.46*	24.62 ± 22.95	28.74 ± 27.98	16.42 ± 4.03	17.90 ± 9.33*
PAI-1 (μg/l)	19.03 ± 10.56	13.93 ± 9.53§	15.28 ± 6.83	17.63 ± 12.46	16.58 ± 6.36	13.31 ± 7.20	21.56 ± 13.26	14.18 ± 10.34
Resistin (μg/l)	12.85 ± 4.66	12.95 ± 7.19	13.40 ± 5.85	12.56 ± 6.15^{+}	13.06 ± 4.09*	13.93 ± 7.91	12.63 ± 5.24	12.54 ± 6.91
HGF (ng/l)	1861.35 ± 6115.71	1887.97 ± 919.99	1821.65 ± 703.67	1925.63 ± 829.72	1879.22 ± 502.19^{+}	1733.94 ± 939.74	1842.91 ± 722.61	1951.39 ± 913.53
IL-8 (ng/l)	3.42 ± 2.77	3.92 ± 3.01	3.12 ± 0.91	4.00 ± 3.52	3.13 ± 0.85	3.09 ± 1.02	3.71 ± 3.87	4.26 ± 3.47
Leptin (ng/l)	21927.41 ± 18794.85	8925.58 ± 8148.46*	12729.94 ± 10151.91	17467.79 ± 18713.56^{+}	15634.66 ± 10944.81*	8303.70 ± 6943.74	28423.14 ± 22812*	9186.78 ± 8656.69
MCP1 (ng/l)	147.50 ± 67.49	163.56 ± 96.86	154.42 ± 65.84	159.91 ± 98.59	153.93 ± 58.91	155.18 ± 76.74	140.87 ± 75.74	167.01 ± 104.52
NGF (ng/l)	11.22 ± 6.69	9.06 ± 5.80*	9.47 ± 7.60	10.26 ± 5.33^{+}	11.28 ± 7.96	6.73 ± 6.25^{+}	11.16 ± 5.19	10.01 ± 5.38^{+}
TNF-α (ng/l)	7.20 ± 2.72	7.04 ± 2.85	6.28 ± 1.83	7.63 ± 3.11	6.55 ± 2.04	5.87 ± 1.39	7.86 ± 3.18	7.52 ± 3.16

LF, low fitness group; HF, equal or higher fitness group; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HGF: hepatocyte growth factor; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; NGF, nerve growth factor; PAI 1, plasminogen activator inhibitor-1; TNF-α, tumour necrosis factor alpha.

Data are expressed as mean ± SD. $^{\S}P < 0.001$; $^{+}P < 0.010$; $^{*}P < 0.05$.

*Statistical significance between groups of fitness (LF vs. HF) and between gender (girls vs. boys) after application of ANCOVA (analysis of covariance) adjusting by sex, age, WC, and BMI.

The comparison among groups is the following:

Second column: LF vs. HF; Fourth column: Girls (G) vs. Boys (B); Fifth column: G-LF vs. B-HF; Sixth column: G-LF vs. G-HF; Seventh column: B-LF vs. B-HF; Eighth column: G-HF vs. B-HF.

Statistical Analysis

Data are expressed as mean ± SD. Normal data distribution was assessed by the Kolmogorov–Smirnov test. Homogeneity of variances was estimated using the Levene test. Inflammation data were log-transformed. Univariate Analysis of Variance was used to evaluate the effect of fitness and gender on cytokines and inflammatory biomarkers adjusted for age and BMI Z-score. Comparative analysis of two independent samples grouping by their levels of fitness or gender was performed using the Mann–Whitney test. General statistics analysis, binary logistic regression and stepwise linear regression were conducted to evaluate the association between fitness, gender and inflammation biomarkers. Principal component analysis (PCA) was performed to investigate the relationships among body mass index, peripheral tissue insulin resistance—as a risk feature of metabolic syndrome—fitness levels, and adipokines and inflammatory biomarkers in the 137 children. Extraction of the initial set of uncorrelated components was accomplished with the principal factor method, and then Varimax orthogonal rotation of components was used to facilitate interpretation. High loading values indicate a stronger relationship between a factor and an observed variable. Factor loadings lower than 0.359 (critical factor, $p < 0.001$) revealed marginal correlations. All statistical procedures were conducted using SPSS (IBM SPSS Statistics, Version 25.0. Armonk, NY, USA).

RESULTS

General Comparisons

A total of 137 children were recruited: 72 were included in the HF group and 65 in the LF group. **Table 1** shows the demographic and anthropometric characteristics and BP levels of this sample, as well as plasma cytokines, divided by gender and by fitness levels. No differences in age and BP were observed between groups classified by fitness. BMI was higher in the LF group. By gender, girls were younger and presented significantly lower BMI, SBP, and DBP values than boys. By level of fitness, girls and boys with HF (GHF and BHF, respectively) showed lower WC Z-score, BMI Z-score, and SBP (only boys) than girls and boys with LF (GLF and BLF, respectively). In addition, GLF presented lower values on all anthropometric variables than BLF. Lower WC, BMI, and SBP were observed in GHF compared with BHF.

Regarding the inflammation biomarkers, the LF group presented higher levels of leptin ($d = 0.536$) and NGF ($d = 0.615$) compared with the HF group. PAI-1 showed higher plasma levels in the LF group compared with the HF group ($p = <0.001$) before adjusting by BMI Z-score, but this significance disappeared after adjusting. When the fitness levels for boys and girls were considered, differences in adiponectin (d=0.506), resistin ($d = 0.819$), leptin ($d = 0.460$), HGF and NGF were observed. The GHF group presented the highest levels of anti-inflammatory parameters and the lowest in inflammatory markers after comparing with GLF, BHF, and BLF (**Table 1**).

Principal Component Analysis

From the 12 items included in the PCA (fitness, body composition, insulin resistance, adipokines and inflammatory biomarkers), four principal components were extracted (**Table 2**), which explained 59.04% of the total variance (25% of the variance was explained by the first factor, an additional 13% by the second factor, another 11% by the third factor and other 8% by the fourth factor; **Table 3**). The first principal component, termed "Fitness" showed a positive correlation between HOMA-IR, leptin, BMI, PAI-1, and negative with resistin, and cardiorespiratory fitness. The second component, termed "Cytokines," included correlations among PAI-1, IL-8, TNF- α, HGF and resistin. The third component named "Adiposity" included a positive correlation between BMI, Adiponectin, and MCP-1. The fourth component, termed "Growth Factors" included an inverse association between HGF and NGF.

TABLE 2 | Principal component analysis for the study extracted from fitness, body composition, insulin resistance, adipokines, and inflammatory biomarkers variables.

Variables	Component matrix[a]			
	Factor*			
	Fitness		Adiposity	Growth factors
Leptin	0.777			
CF olds	−0.745			
HOMA-IR	0.703			
PAI 1	0.590	0.449		
BMI	0.417		0.399	
IL8		0.852		
TNF-α		0.809		
HGF		0.601		0.406
Resistin	0.334	0.360		
Adiponectin			0.764	
MCP-1			0.613	
NGF				−0.895

CF olds, cardiorespiratory fitness classified by olds; HOMA-IR, homeostasis model assessment-insulin resistance; HGF, hepatocyte growth factor; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1; NGF: nerve growth factor; PAI 1: plasminogen activator inhibitor-1; TNF-α: tumour necrosis factor alpha.

[a]Extraction of the initial set of uncorrelated components was accomplished with the principal factor method, and then the Varimax orthogonal rotation of components was used to facilitate interpretation. The number of components retained was based on Scree plot analysis and eigenvalues >1 (with the components accounting for more of the total variance than any single variable).

[*]Factor loading is the product-moment correlation (a measure of linear association) between an observed variable and an underlying factor. A significant loading factor was defined as a value >0.359 ($p < 0.01$).

Correlations and Regression

Table 4 shows significant correlations between cytokines, fitness and demographic variables. There was a strong positive correlation between WC Z-score and BMI Z-score with PAI-1 and leptin; and a negative correlation with adiponectin and fitness. Fitness was also negatively correlated with leptin. Regarding the relationship between the cytokines, it was observed a strong positive association between HGF, IL-8, leptin, and TNF- α.

TABLE 3 | Eigen values and percentages of variance associated with each linear component (factor) before extraction, after extraction, and after rotation, in the principal component analysis for the study of children relating fitness, body composition, insulin resistance, adipokines, and inflammatory biomarkers variables.

Components	Total variance explained								
	Initial eigenvalues			Sums of loads squared from extraction			Sums of loads squared of rotation		
	Total	% of variance	% accumulated	Total	% of variance	% accumulated	Total	% of variance	% accumulated
1 (Fitness)	3.017	25.145	25.145	3.017	25.145	25.145	2.380	19.835	19.835
2 (Cytokines)	1.674	13.953	39.098	1.674	13.953	39.098	2.220	18.496	38.332
3 (Adiposity)	1.368	11.397	50.495	1.368	11.397	50.495	1.336	11.131	49.463
4 (Growth factors)	1.026	8.546	59.041	1.026	8.546	59.041	1.149	9.578	59.041

After a multivariate logistic regression analysis (**Table 5**), gender showed associations with CRF, resistin, age, Z-score BMI, and TNF-α and an independent relationship with adiponectin. Moreover, fitness showed associations with BMI Z-score, NGF, and leptin.

DISCUSSION

In the present study, changes in plasma cytokines were observed between prepubertal children with low or high fitness levels. In addition, adipokines and inflammatory biomarkers were affected by gender regardless of fitness levels, maybe due to a modulation of the adipose tissue on these biomarkers.

Preliminary evidences suggested that maintaining a healthy normo-weight during childhood and adolescence might be the most effective strategy to prevent chronic low-grade inflammation and cardiovascular and metabolic diseases in the future (16). It has also been demonstrated that an active lifestyle and an adequate fitness level may attenuate these adverse effects (17). In addition, genetic and early programming features have been associated with low-grade inflammation in young people (18). However, to date, the potential anti-inflammatory effects of fitness in children are not entirely clear. The results obtained in the present work add evidence of the positive effect of an adequate fitness status during childhood related with inflammatory status. So, those participants with HF levels, showed also lower BMI and WC, suggesting that fitness and gender might influence adipose tissue metabolism programming changing these biomarkers in this early stage of life, before puberty changes.

The association between fitness and fibrinolytic activity has been scarcely studied in prepubertal children, showing a high disparity among the results obtained in studies conducted on both variables. It has been described a strong correlation between fitness and PAI-1 levels in children after adjustment for age and fat mass (19) even though not all studies have observed this association (1). Most of these studies have been carried out on children with obesity. Although authors adjusted by fat mass, elevated adiposity might be associated with a higher physiological deposit of PAI-1 that might explain these differences. Here, although the participants were normal-weight, those in the LF group showed higher levels of PAI-1, similarly as reported Barbeau et al. (20) under the same conditions. Our differences might be due to a stricter criterion to classify the participants as high or low fitness levels. However, it seems to be an indirect effect, where an increase in the level of fitness would give as result a decrease in the BMI and consequently a reduction in PAI-1 plasma levels. Moreover, in the present study, PAI-1 was positively associated with other biomarkers such as resistin, leptin, IL-8, HGF, and TNF-α, and with WC (**Table 4**). Decreased resistin concentrations during fasting have been associated with a decrease in the percentage of body fat in adolescents (21). Therefore, an

TABLE 4 | Significant correlations between adipokines, fitness, and demographic variables in healthy prepubertal children.

Variables	*R*	*P*	Variables	*R*	*P*
Age/Z-score BMI	0.228	0.007	Adiponectin/Sex	−0.295	0.001
Age/Z-escore WC	0.240	0.005	Adiponectin/Leptin	−0.168	0.050
Age/Adiponectin	−0.227	0.009	Adiponectin/MCP 1	0.222	0.010
Age/Leptin	0.170	0.047	Adiponectin/NGF	−0.176	0.044
Age/MCP 1	−0.217	0.012	Adiponectin/TNF-α	−0.186	0.033
Age/TNF-α	0.179	0.036	PAI 1/Resistin	0.277	0.001
Z-score WC/Adiponectin	−0.272	0.002	PAI 1/HGF	0.437	<0.001
Z-score WC/PAI 1	0.506	<0.001	PAI 1/IL 8	0.383	<0.001
Z-score WC/Resistin	0.257	0.002	PAI 1/Leptin	0.535	<0.001
Z-score WC/HGF	0.275	0.001	PAI 1/TNF-α	0.315	<0.001
Z-score WC/Leptin	0.779	<0.001	Resistin/HGF	0.253	0.003
Z-score WC/TNF-α	0.209	0.014	Resistin/Leptin	0.234	0.006
Z-score BMI/Adiponectin	−0.236	0.002	HGF/IL 8	0.422	<0.001
Z-score BMI/PAI 1	0.464	<0.001	HGF/Leptin	0.343	<0.001
Z-score BMI/Resistin	0.181	0.034	HGF/TNF-α	0.411	<0.001
Z-score BMI/HGF	0.238	0.005			
Z-score BMI/TNF-α	0.173	0.043	IL 8/MCP 1	0.240	0.004
CRF/Sex	0.221	0.010	TNF-α/Sex	0.226	0.007
CRF/Z-score WC	−0.411	<0.001	TNF-α/Resistin	0.192	0.023
CRF/Z-score BMI	−0.381	<0.001	TNF-α/IL 8	0.445	<0.001
CRF/PAI 1	−0.289	0.001	TNF- α/Leptin	0.239	0.005
CRF/Leptin	−0.419	<0.001	TNF-α/NGF	0.204	0.016
CRF/NGF	−0.172	0.045			

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. HGF, hepatocyte growth factor; IL-8, interleukin-8; MCP-1, macrophage chemo attractant factor; PAI 1, plasminogen activator inhibitor-1; CRF, cardiorespiratory fitness; TNF-α, tumour necrosis factor alpha; r, Rho Spearman's correlation coefficient.

adequate fitness level may have an indirect effect on the inflammation status.

Leptin has been reported to be independently and inversely related to fitness (7). There is scarce literature regarding the association between fitness and leptin in prepubertal children. The current findings suggest that low levels of aerobic fitness, as well as elevated serum leptin, are major risk factors for the clustering of metabolic risk factors in obese (22) or those presenting a medium-healthy or unhealthy status (23). Our results support these findings showing higher levels of leptin in LF group even being younger children. On the other hand, fitness might stimulate insulin sensitivity and induce a decrease in insulin release, which in turn might reduce leptin levels. A reduction in levels of obesity seems to produce a reduction on TNF-α, which lead to a decline in leptin and an increase in adiponectin and insulin sensitivity (24). Therefore, it appears that the action of adiponectin depends on changes in fat mass (25). According to the previous hypothesis, our results might suggest an influence of fitness on leptin in healthy children and possibly on TNF-α, but not on adiponectin. In fact, this study supports the relationships among TNF-α with leptin, adiponectin and BMI (that includes fat mass).

The NGF is a well-known regulator of differentiation, plasticity, and phenotype of sensory and sympathetic neurons during the entire lifespan (26). Alterations in NGF tissue concentrations lead to altering nutritional factors in the muscle fibers after high-intensity exercises (27). Moreover, it has been demonstrated that inflammation can enhance the synthesis of NGF in the tissues (28) and give rise to an increase in other markers such as TNF-α. Therefore, the present study shows that the HF group could have a lower risk of inflammatory events and NGF-derived effects.

Parallel, the relationship between gender and inflammation biomarkers has not been clarified. According to some authors, girls have higher levels of leptin and resistin and lower levels of TNF-α in comparison with boys (1). As regards adiponectin levels in young people, it has been reported that prepubertal girls present higher adiponectin concentrations than boys (1, 9). It has been suggested that the effects of age on adiponectin levels in girls could be explained by their BMI and total fat mass (29). However, it is still difficult to explain the differences in inflammatory markers by sex, mainly in children because the changes among genders have usually been associated with their hormonal status. Therefore, considering our results, it seems that girls are low protected against inflammation during the prepubertal stage, although it might change once puberty is developed.

Finally, when we observed the effect of fitness levels on both gender separately, the girls with an elevated fitness were shown to be protected against the development of an inflammatory process by presenting

TABLE 5 | Logistic regression analysis for sex and fitness with different anthropometric variables and plams cytokines.

Variables	Variables	B	Exp (B)	*P*	95% confidence interval of B	
					Lower bound	Upper bound
Sex	Fitness	−2.378	0.093	<0.001	0.024	0.353
	Age	1.616	5.033	<0.001	2.586	9.797
	Z-score BMI	0.693	2.000	0.018	1.129	3.545
	Adiponectin	−0.028	0.973	0.199	0.933	1.015
	Resistin	−1.956	0.141	0.015	0.029	0.688
	IL-8	4.327	75.735	0.092	0.049	11694.26
	TNF- α	2.913	18.420	0.009	2.806	162.66
Fitness	Sex	−2.464	0.085	<0.001	0.024	0.300
	Age	−0.416	0.660	0.089	0.409	1.066
	Z-score BMI	−0.647	0.523	0.024	0.298	0.920
	Leptin	0.000	1.000	0.017	1.000	1.000
	NGF	−0.083	0.920	0.026	0.856	0.990
	IL-8	−2.555	0.078	0.098	0.004	1.602

BMI, body mass index; IL-8, interleukin-8; NGF, nerve growth factor; TNF-α, tumor necrosis factor alpha.

higher levels of adiponectin and lower plasma levels of leptin and NGF.

As limitations of the present study, it is important to emphasize the high difficulty to detect inflammatory molecules in the blood samples due to the low concentrations in children without pathology. Moreover, this study has carried out with a sample from a city in Spain, so it will be interesting to expand to other regions and different stages of life.

CONCLUSIONS, FUTURE SCOPE, AND LIMITATIONS

This study contributes to add new information about the effect of fitness and gender on plasma adipokines and inflammatory cytokines in healthy prepubertal children. An adequate or higher fitness level seems to be a protective factor against the development of obesity, metabolic risk and inflammation, decreasing leptin and NGF levels, so as contributing to control adiposity at the prepubertal stage. However, research focus in this and other stages of life should be developed to generate more accurate knowledge and relationships between a low grade of inflammation and cardiorespiratory fitness.

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 human participants were reviewed and approved by the Institutional Ethics Committee Reina Sofia University Hospital, Cordoba, Spain. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FL-C: conceptualization, data curation, formal analysis, methodology, writing—original draft, and review and editing. CA: conceptualization, funding acquisition, investigation, and writing—review and editing. JP-N and JB-S: conceptualization, methodology, and review and editing. AG: conceptualization, methodology, and writing—original draft. MG-C: conceptualization, investigation, methodology, project administration, resources, writing—original draft, and review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Changes in Human Milk Fat Globule Composition Throughout Lactation: A Review

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There has been a growing interest in understanding how the relative levels of human milk fat globule (MFG) components change over the course of lactation, how they differ between populations, and implications of these changes for the health of the infant. In this article, we describe studies published over the last 30 years which have investigated components of the MFG in term milk, focusing on changes over the course of lactation and highlighting infant and maternal factors that may influence these changes. We then consider how the potential health benefits of some of the milk fat globule membrane (MFGM) components and derived ingredients relate to compositional and functional aspects and how these change throughout lactation. The results show that the concentrations of phospholipids, gangliosides, cholesterol, fatty acids and proteins vary throughout lactation, and such changes are likely to reflect the changing requirements of the growing infant. There is a lack of consistent trends for changes in phospholipids and gangliosides across lactation which may reflect different methodological approaches. Other factors such as maternal diet and geographical location have been shown to influence human MFGM composition. The majority of research on the health benefits of MFGM have been conducted using MFGM ingredients derived from bovine milk, and using animal models which have clearly demonstrated the role of the MFGM in supporting cognitive and immune health of infants at different stages of growth and development.

Keywords: milk fat globule, lactation, human milk, maternal origin, phospholipids, fatty acids, gangliosides

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INTRODUCTION

Given the importance of breastfeeding, with the WHO recommendation that infants be exclusively breast fed for the first 6 months of life (1), there is a growing interest in the health benefits of specific components of human milk (HM). There is also interest in understanding how the relative levels of these components change over the course of lactation, how they differ between populations, and implications of these changes for the health of the infant. In addition, with the low prevalence of breastfeeding, especially in high-income countries ($\leq$40% at 6 months and $\leq$20% at 12 months)

(2), knowledge on the compositional variation of HM helps to tailor new infant formulations to better meet the nutritional requirements of a growing infant.

Fat is the component of HM that provides most of the energy and comprises a complex mixture of different lipid species (3). To enable this fat [including triglycerides, diglycerides, free fatty acids (FA), and cholesterol] to remain as a natural emulsion within milk, lipids produced within the secretory cells of the mammary gland are encapsulated by the milk fat globule membrane (MFGM) (**Figure 1**). As the milk fat globule (MFG) is synthesized in the rough endoplasmic reticulum, and transported through the cell cytoplasm, it is secreted through the apical membrane of the mammary epithelial cell. This results in the lipids being stabilized by a membrane with three distinct layers; an inner interfacial layer, the cytoplasm (enriched in protein), and finally a true bilayer membrane (4).

It has been proposed that the complex structure of the MFGM has arisen due to physiological constraints of the secretion process, and that it would not in itself be expected to contribute a significant health benefit to the offspring other than suppling the lipids necessary for growth and development (5). However, although some components of the MFGM are relatively minor within milk [for example, MFGM proteins contribute 1–4% of the total protein content in milk (6)], for others, such as phospholipids, gangliosides and cholesterol the MFGM represents the major source (7). Furthermore, although the fundamental physiological function of the MFGM is to allow for secretion of fat into milk, it is also clear that it communicates chemically important growth and immunological signals to the neonate (8). In addition, many different biological functions have been reported to be associated with MFGM proteins including protein synthesis/folding, signal transduction, transport, cell communication, as well as energy production metabolism, and immune function (6, 9).

In this article, we reviewed studies published from 1990 to 2020 that have investigated components of the MFGM in HM from mothers that delivered at term. The focuses were on those studies which have looked at changes over the course of lactation (at least two stages of lactation) and highlighted infant and maternal factors (including country of origin and diet) that may influence these changes. We then considered the functional and health effects of these compositional changes over the course of lactation. We also included a brief overview of the pre-clinical and clinical evidence of the health effects of MFGM components and we discussed whether MFGM is a necessary ingredient for infant formula products, to ensure that infants receive appropriate nutrition in the critical early years.

Abbreviations: HM, Human milk; MFG, milk fat globule; MFGM, milk fat globule membrane; FA, fatty acids; LA, linoleic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic; DHA, docosahexaenoic acid; ARA- arachidonic acid; PUFAs, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; MCFA, medium chain fatty acids; DPA, docosapentaenoic acid; OL, oleic acid; XO, xanthine oxidase; BTN, butyrophilin; ADPH, adipophilin; MEC, mammary epithelium cells; MUC, mucin; ER, endoplasmic reticulum; TAG, triacylglycerol; PC -phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; GD3, disialoganglioside; GM3, monosialodihexosylganglioside.

Search Strategy, Study Selection and Exclusion Criteria

We performed a search and retrieved over 2,000 studies from Ovid Medline and Scopus databases from 1990 to 15th July 2021, as shown in **Supplemental Material**. All articles resulting from the search were assessed for eligibility based on the titles and abstracts. Of the remaining articles, full text was screened to check for eligibility. Only observational studies were included in this review. Studies were included if a specific human milk fat globule membrane component (phospholipids, proteins, gangliosides, and cholesterol) was measured at two or more time points during lactation period. For each milk component, we defined lactation period as colostrum (1 to 7 days post-partum), transitional milk (8 to 15 days post-partum) and mature milk (from 15 days post-partum). Studies reporting data from mothers that delivered at term were included. The reference lists of identified studies, reviews, and textbooks were reviewed to avoid missing relevant publications. Only studies in English were included. We created worksheets to systematically manage study selection, collating relevant study details such as date of publication, country of origin, lactation stage, measurement points, results, and confounding factors (method of collection, full expression, pre- or post-feed). We excluded (1) duplicate publications, (2) multiple publications of the same trial, (3) conference abstracts, (4) study protocols, (5) nonhuman studies, (6) studies and trials where any intervention was administrated to the mothers, (6) studies where samples were not from individual participants (pooled samples), and (7) studies reporting preterm milk data only.

MILK FAT GLOBULE

Milk fat is the most dynamic macronutrient in HM, and its yield affects the MFG size distribution and the composition and profile of MFGM components (10). The largest portion of milk fat consist of triglycerides (98%) in the form of MFG and other minor components such as diacylglycerides (<2%) and free FA (11). Total fat concentration increases during lactation, especially during the transition from colostrum to transitional milk (12, 13) with smaller changes from transitional to mature milk (12, 14). Although colostrum is known to be rich in immune factors and proteins, mature milk, in contrast, is energy dense to support infant growth (15). Fat concentration in colostrum, transitional and mature milk shows a increase trend from 1.1–5.9 to 3.0–5.6 g/100 and 2.0–6.1 g/100 mL, respectively, despite individual variability, sample type (pooled milk, full breast expression, foremilk, hindmilk) or sampling time (morning, night) (**Supplementary Table 1**, provides a summary of studies regarding total fat concentration in human colostrum, transitional, and mature milks).

Total fat changes during nursing; foremilk has a lower fat concentration compared to hindmilk (16, 17) and fat also follows a circadian rhythm (morning milk has lower concentration of fat compared to evening milk) (12). The effects of circadian rhythms in human fat have been recently reviewed (18). The authors reported that 15 out of 19 reviewed studies described circadian

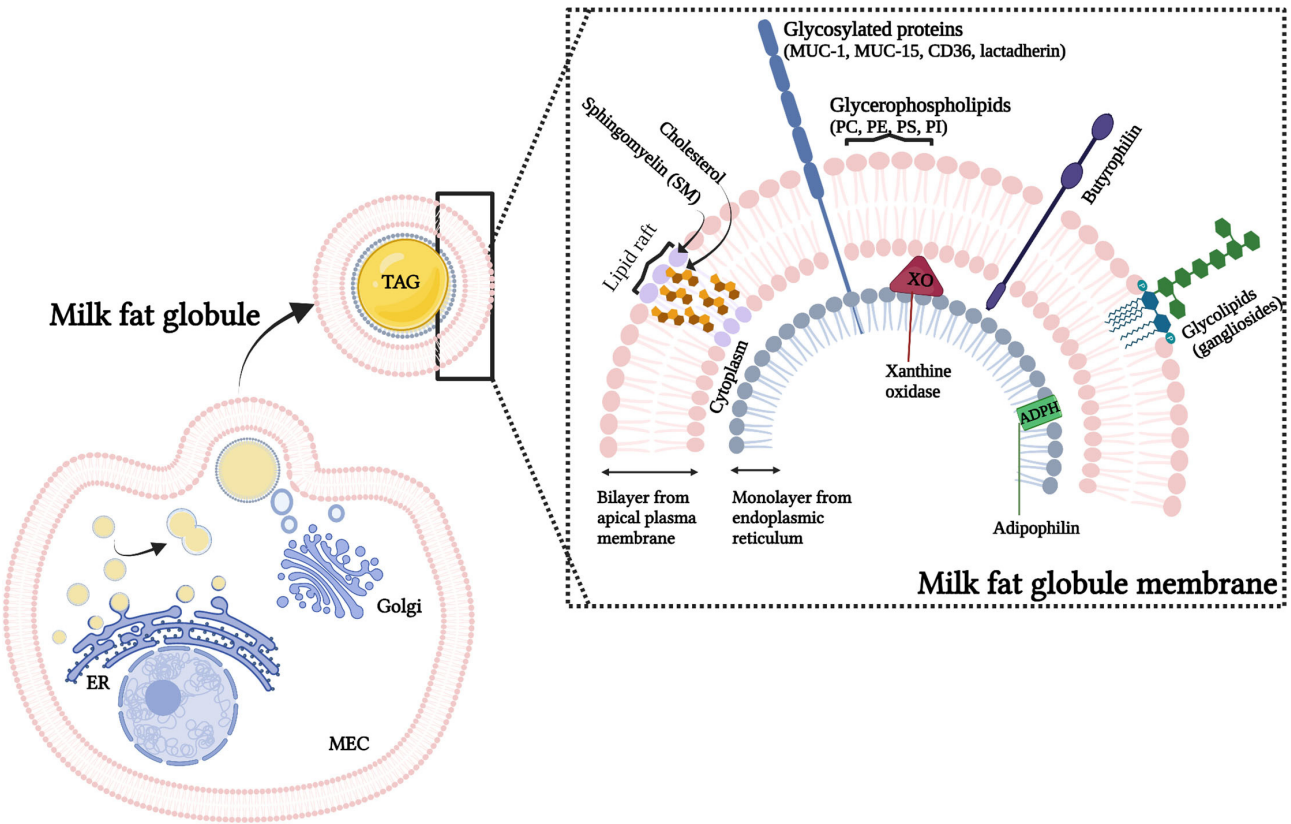

FIGURE 1 | Representation of the origin and structure of the milk fat globules (MFG). MFG are secreted from the mammary epithelium cell (MEC), with components from the endoplasmic reticulum (ER), cytoplasm and cellular membrane. The secreted MFG contain a triacylglycerol (TAG) core and a tri-layered structure, the milk fat globule membrane (MFGM). The MFGM, in detail, shows the lateral segregation of sphingomyelin and glycolipids surrounded by the glycerophospholipids. Illustrative pictures of some of the major MFGM proteins (which includes mucins (MUC-1, MUC15), lactadherin, adipophilin (ADPH), xanthine oxidase (XO) and butyrophilin) are also presented. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

variation with the peak in the evening, for total fat concentration. Challenging this current dogma, a recent study reported that the concentrations of human foremilk fat, collected daily, from both breasts for 21 consecutive days did not differ according to time of day, day of week or breast used for collection (19). This suggests that circadian effects on milk fat may be observed only in hind milk (where the concentration of fat is higher) and that other factors, such as infant feeding pattern, time since last feed, breast fullness and lactation period may also play an important role on HM fat content. These factors may explain the large variation in fat concentration observed between mothers, and the consequent wide standard deviation reported in most studies (**Supplementary Table 1**).

The average MFG size can vary during lactation, due to the changes in the total amounts of milk fat produced during lactation (20). For example, it has been reported (21) that fat content as well as MFG average size increased from the third day ($3.24 \pm 1.68\%$, $3.77 \pm 0.95\,\mu m$) until the 11 day of lactation ($4.96 \pm 2.13\%$, $5.09 \pm 0.88\,\mu m$) and remained stable until the thirtieth day. In contradiction, others reported that whereas fat increased during lactation, colostrum had a larger MFG average size than transitional and mature milk (22, 23). This may be explained by coalescence of small globules with incomplete membrane coating provided by the immature mammary gland (24). It has also been demonstrated that increase in fat content in milk leads primarily to the increase in the number of MFG rather than the size (25). Changes in MFG numbers, therefore, are also likely to influence the concentration of MFGM components in milk. Overall, the increase in milk fat observed from colostrum to mature milk, independent of individual variability, may lead to changes in the MFG numbers and or size, affecting the concentration of membrane components in milk.

Fatty Acids Composition

MFG (comprising a triacylglycerol core and the MFGM) are the main source of FAs in HM supplying not only energy but also essential and bioactive FAs for infant development. The HM FA profile is diverse with over 200 FA structures with different concentrations (26). Generally fatty acid compositional data from both the triacylglycerol core and the MFGM are reported as a total FAs profile.

Saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) represent 35–45%, 36–39 and ∼18% of the total fat content of HM whereas short-chain FA

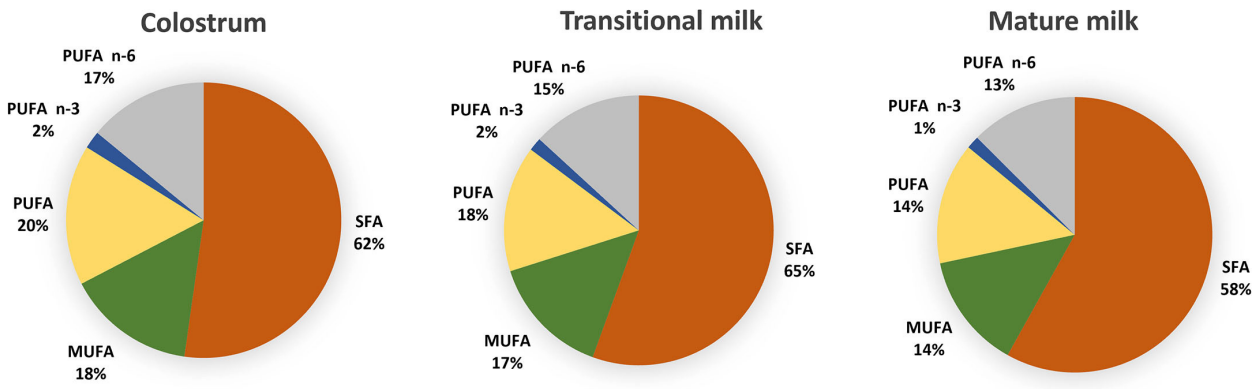

FIGURE 2 | Distribution of fatty acids categories in colostrum (1–7 days), transitional milk (8–15 days) and mature milk (15–60 days) after pooled data analysis of worldwide milk samples reported by Floris et al. (28). (PUFAs) polyunsaturated fatty acids; (n-3 PUFAs) n-3 polyunsaturated fatty acids; (n-6 PUFAs) n-6 polyunsaturated fatty acids; (MUFA) monounsaturated fatty acids; (SFA) saturated fatty acids and (MCFA) medium chain fatty acids.

(SCFA) and medium-chain FA (MCFAs) contribute relatively little (8%) (27, 28). **Figure 2** reports fatty acid composition of pooled data analysis reported by Floris et al. (28). Three main sources of lipids are utilized in the synthesis of milk lipids: maternal dietary lipid, FAs from adipose tissue and *de novo* synthesized lipids. The importance of these sources is FA-specific and may influence their concentration at different stages of lactation. For example, palmitic acid (C16:0) and the essential FAs linoleic acid (LA, C18:2 n−6) and α-linolenic acid (ALA; C18:3 n−3) are sourced predominantly from maternal fat storage (70%) and only some from maternal dietary intake (30%) (29). MCFAs, however, are only synthesized *de novo* in the mammary gland, and the concentration have been suggested to be linked to mammary gland maturation and therefore present at higher concentrations in mature milk (30).

A recent systematic review including data from 55 studies worldwide, and a total of 4,374 term milk samples reported analysis for the variation of 36 main FA across lactation (28). The most abundant SFA, palmitic (C16:0), stearic (C18:0) and myristic (C14:0) and MUFA oleic acid (C18:1 n-9) were shown to remain stable whereas gondoic (C20:1 n−9), erucic (C22:1 n−9) and nervonic (C24:1 n−9) acid were shown to decrease over the course of lactation (**Figure 3**) (28). The opposite pattern was observed for the MCFAs, specially the most abundant, lauric acid (C12:0), which was shown to almost double from colostrum to transitional milk. Among the long-chain PUFAs, n−6 PUFAs, and more specifically LA (C18:2 n−6, 16%), were the most abundant FA compared to n−3 PUFAs (3%). LA, eicosapentaenoic (EPA; C20:5 n−3) and ALA concentrations were shown to be relatively stable during lactation (**Figure 3**). However, a steady decrease of arachidonic acid (ARA; C20:4 n−6), docosahexaenoic acid (DHA, C22:6 n−3) and docosapentaenoic acid (DPA; C22:5 n−3) over the course of lactation has been consistently reported in other literature (28, 31–33) (**Figure 3**).

Factors Affecting Fatty Acid Profile

Numerous studies have indicated the influence of maternal origin, ethnicity (34), diet and cultural habits on the composition of milk FAs (35–41). Brenna et al. (42) in a meta-analysis of 65 studies worldwide found that DHA concentration varies greatly among countries with a mean (±SD) concentration of 0.32 ± 0.22 and a range of 0.06–1.4%. DHA levels were higher in countries with high consumption of fish, such as the Canadian Artic (1.4%) and the Philippines, Japan (43, 44), Chile (44, 45) and Taiwan (46) with levels above 0.4%. In contrast, countries such as Pakistan (0.06%), Canada and the United States, had very low DHA levels (below 0.2%). Geographical locations with the highest DHA levels, e.g., Philippines and Japan also had the highest EPA (C20:5n-3) levels ranging from 0.15–0.26% (44) (**Figure 4**). Compared to DHA, ARA levels were shown to be less variable among countries, with the mean level of all samples being 0.41% (44) (**Figure 4**). This may due to the poor conversion of dietary LA to ARA in milk (47). The mean ARA:DHA ratio found for most countries (Australia, Canada, UK, Mexico, China, Spain) was 1.6:1 whereas the ratio was lower for Japan (0.5:1) and higher for China (2:1 to 3:1) (35, 36, 48) and USA (3.2:1) (44) (**Figure 4**). Although optimal ARA:DHA ratios are not fully elucidated, the ratio of both LCPUFAs was suggested to impact immune response, cognitive and behavioral outcomes, competition for tissue incorporation and risk for atopic disease (49–51).

Human Milk LA levels were found to be relatively elevated in countries, such as Mexico and Chile (∼16%) that, typically consume a high-maize diet, compared to Australia, Canada, Iran and the UK (∼10%) (44, 45, 52, 53). High levels of HM LA (22%) and ARA (0.5%) were also reported in China compared to Sweden (10 and 0.3%, respectively) (36), which may be linked to the high consumption of SFA by the Chinese population (54). The Philippines presented an odd FA profile, with low LA levels (7%) and high levels of lauric (13%) and myristic acids (12%) (44) compared to other Asian countries (∼5 and 3–6%, respectively), which may indicate consumption of diets restricted in both total

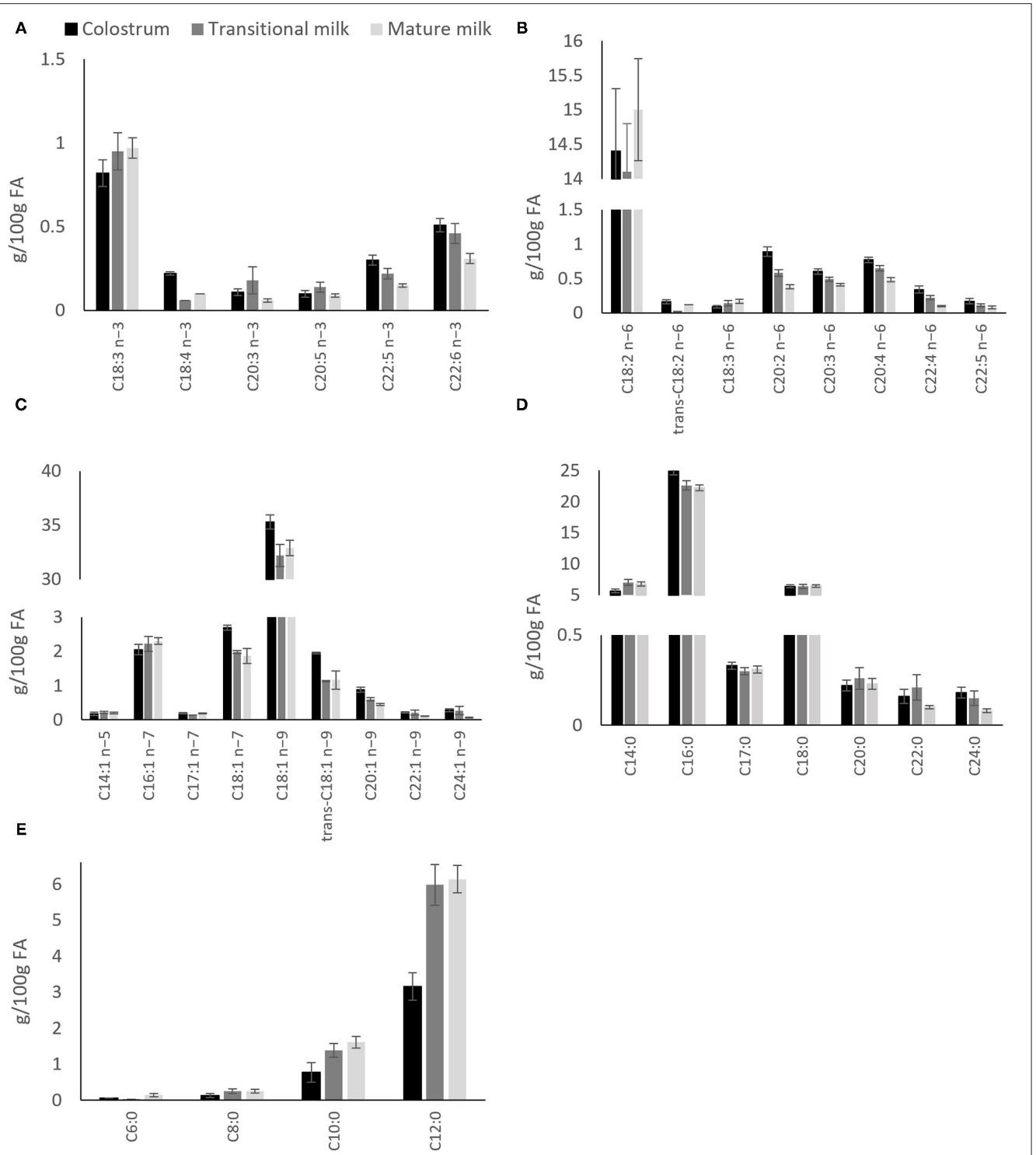

FIGURE 3 | Distribution of individual fatty acids in colostrum, transitional milk and mature milk after pooled data analysis of worldwide milk samples reported by Floris et al. (28). **(A)** n-3 polyunsaturated fatty acids; **(B)** n-6 polyunsaturated fatty acids; **(C)** Monounsaturated fatty acids; **(D)** Saturated fatty acids and; **(E)** Medium chain (C6-C12) saturated fatty acids.

fat and essential FA (55). The FA profile from Canada and China (∼35%) had the highest oleic acid content compared to UK, Australia, Japan, Chile and Mexico (26–32%) (44), most likely due to a relatively elevated intake of canola or rapeseed oils in these countries (56, 57). The ALA levels were constant, at ∼1% of FA for Australia, Canada, Chile, Japan, UK, USA, Iran and

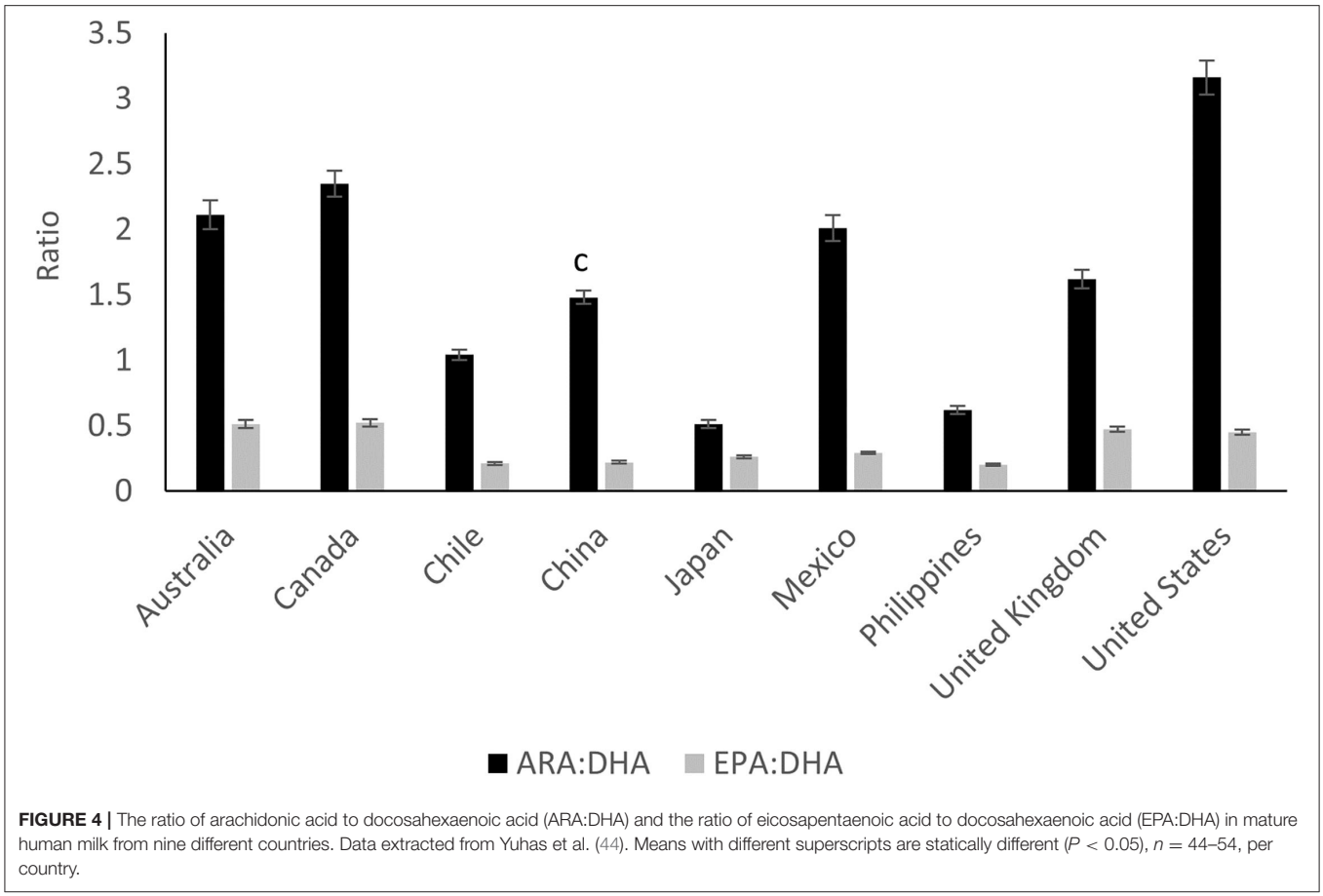

FIGURE 4 | The ratio of arachidonic acid to docosahexaenoic acid (ARA:DHA) and the ratio of eicosapentaenoic acid to docosahexaenoic acid (EPA:DHA) in mature human milk from nine different countries. Data extracted from Yuhas et al. (44). Means with different superscripts are statically different ($P < 0.05$), $n = 44$–54, per country.

Mexico, but lower for the Philippines (0.43%) and higher for China (2%) (44, 53). In general, higher percentages of PUFA have been reported in Chinese HM studies (22.4–30.0%) (41, 58–60) compared to developed Asian countries (17.25–21·50%) (44, 61) and those in the Western countries (11.5–21%) (23, 62).

It is important to note that studies generally report data from a specific country region, and interpretation of data must be related to that specific geographical area and not to the entire country. Previous studies have demonstrated that several countries, such as France (63) and China (64) have regional differences. It is also worth noting that factors other than diet and geographical location may affect the profile of FA in milk. LA, for example, was shown to be consistently more highly expressed in HM secreted for male infants (37% increase) compared to female (65). The effect of gestational age on FA profile has been reviewed and linked to changes in DHA concentration (increase in premature milk) in some studies (66) but not in others (28).

COMPOSITION OF THE MILK FAT GLOBULE MEMBRANE

The composition of the MFGM is more intricate than the MFG core, with a ratio of ∼1:1 of proteins and lipids (67). Other minor components, such as RNA, are also present (68). The major MFGM proteins (i.e., that are well-described, and are present in relatively high concentrations) are mucin 1 (MUC 1), xanthine oxidoreductase (XDH/XO or XOR), butyrophilin (BTN), lactadherin (PAS 6/7, MFG-E8), CD 36, adipophilin, and fatty acid-binding protein (FABP) (69). Proteomics studies have demonstrated that there are at least 200 (70, 71) and perhaps more than 400 (71, 72) proteins within the MFGM of HM, and their relevance for human health is an area of active research and commercial interest. In this review we focus on a number of studies in which the putative role of some of the major proteins are reported, and we also include more detail on some proteomics studies of the human MFGM, however it is beyond the scope of this review to consider the levels and function of these minor proteins in detail.

The key lipid species of the MFGM are phospholipids, with phosphatidylethanolamine (PE, 6–36%), phosphatidylcholine (PC, 14–38%), and sphingomyelin (SM, also a sphingolipid, 27–43%) being the major species, and phosphatidylserine (PS) and phosphatidylinositol (PI) are relatively minor components (**Table 2**) (58, 73). Other important lipid components of the MFGM are cholesterol, gangliosides and FA, which appear to play an integral role in cognitive development (58).

Phospholipids

Milk phospholipids have important functional properties influencing general lipid absorption (74, 75), brain development (76, 77) gut mucosal development (78), and immune maturation (79).

Table 1 provides a summary of studies of total phospholipid content of HM across lactation. Total phospholipids appear to vary through lactation, with some studies reporting a decrease over time (7, 58, 81, 83–86), whereas in one study the total milk phospholipids concentration was reported to not vary across lactation (82). Other studies have reported an increase in total phospholipids from colostrum to transitional milk followed by a decrease over the mature milk period (13, 23, 84). These results might be explained by the relationship between the phospholipids contents and the diameter of the MFG, where phospholipids are generally negatively correlated to the diameter of MFG (transitional milk < mature milk < colostrum). For a constant total fat content in milk, more phospholipids are required to cover the larger surface area of smaller MFG (see details in "Milk Fat Globule" section).

The effects of geographical location on total HM phospholipid composition were evaluated by Claumarchirant et al. (13), reporting a higher concentration of total phospholipids in transitional milk and at 6 months after delivery in a geographical coastal zone (Valencia, 33–53 mg 100 mL^{-1}) compared to the central zone (Madrid, 26–43 mg 100 mL^{-1}) of Spain. Similarly, the geographical differences in total phospholipid in colostrum among Chinese cities (Beijing, Suzhou and Guangzhou) has also been reported recently by Giuffrida et al. (58). This study showed higher concentrations of total phospholipid in mothers from Suzhou (38 mg 100 mL^{-1}) compared to the other cities (33 mg 100 mL^{-1}) (**Table 2**). The authors hypothesized that these differences could be due to the increased consumption of marine foods or rapeseed oil in this region.

Table 2 provides a summary of studies on the variation of HM phospholipid species across lactation. SM was found to be the most abundant phospholipid (27.4–43.4% of total phospholipid) from analysis using Phosphorus-31 nuclear magnetic resonance (^{31}P NMR), Thin-layer chromatography (TCL) (43.3 ± 2.6%) (82, 88) and by High-performance liquid chromatography with evaporative light-scattering detection (HPLC-ELSD) (23, 65, 84), with the exception of Giuffrida et al. (58) who reported PC as the most abundant phospholipid using ELSD. HM phospholipids analyses using liquid chromatography–mass spectrometry generally conclude that the major phospholipid is PE (7, 89). The difference in phospholipid composition may be explained by the response differences of the detectors, or by other factors, such as diet, geographical location, sampling time, and gestation age at birth (preterm vs. term), metabolic stage, and diurnal rhythm. **Supplementary Table 2**, summarizes the methodology used to collect and analyses the phospholipid composition in the studies reviewed.

Some differences in the distribution of HM phospholipid classes have also been reported in different geographical locations (**Figure 5**). A study conducted in a Chinese population showed higher proportions of PC (35%) and lower concentrations of PE (26%) in mature milk (58), compared to mothers from the Unites Arab Emirates [14 and 36%, respectively (85)], Spain [Madrid, Valencia and Murcia (15 and 31%, respectively) and Malaya (14 and 36%, respectively (86)]. The studies conducted in Spain showed that the distribution of mature milk phospholipids from Granada (84) had higher proportions of PC (38%) and lower proportions of PE (6%) compared to other parts of Spain (Madrid, Valencia and Murcia, 13 and 32% respectively) (13) (**Figure 5**). These discrepancies may be due to the type of sample collection, as one study collected hindmilk (84) and another did not report the type of sample collected (13) (**Supplementary Table 2**).

Variation in phospholipid classes during lactation is shown in **Table 2**. PC, PI and PS concentration was reported to be elevated in colostrum, and decreased to lower levels in transitional and mature milk (82) whereas, no significant differences were found in the concentration of PE (82, 85) or SM (82, 84, 85) during lactation. Other studies (7, 58, 81), however, found that all individual PL decreased from colostrum to mature milk. An increase in PE during lactation was observed by Sala-Vila et al. (84). These authors reported an increased ratio of PUFA to saturated fatty acid from colostrum and mature milk, suggesting that this variation is related to the evolution of the fatty acid content of total phospholipids. This increase may be due to the preferential pattern of distribution of FAs into the different classes of phospholipids. Although SM mainly esterifies mainly saturated and monounsaturated fatty acids (MUFAs), PUFA are mainly esterified in PE. PE exhibits an increase in LA (C18:2 n-6) as lactation progresses from the secretion of colostrum to transitional milk and then to mature milk (90).

Phospholipids Fatty Acids Composition

There are only a few studies that investigated the FAs composition associated with phospholipids in HM (23, 84, 88, 89, 91). Compared to a total milk FAs profile the MFGM had increased concentrations of SFAs at all lactation stages (23). The SFA (mainly C16:0 and C18:0) represent around 60–70% of FAs, followed by oleic acid (C18:1 n-9), LA (C18:2 n-6) and the LC-PUFA ARA (C20:4,n-6), adding up to 80% of total FAs (7, 82, 84, 91). Mature milk was reported to contain higher amounts of saturated MCFA and lower contents of C16:0 compared to colostrum and transitional milk, whereas the contents of total MUFAs and PUFAs were not different (23) (**Figure 6**). PUFAs (n-3) were found to increase from colostrum to mature milk, whereas no difference was found for n-6 PUFAs, especially for C18:2 n-6 (23). Another study found no difference among total SFAs, MUFAs and PUFAs at different lactation stages but an increase in C18:2 n-6 in mature milk (84). This is important as the degree of FAs unsaturation, together with cholesterol and SM, influence membrane fluidity. SFAs allow the phospholipids to pack more closely in the membrane decreasing fluidity, whereas unsaturated FAs increase fluidity (92), and affecting digestion and perhaps functionality (93). The high content of SFA and LCFAs (C22:0, C24:0 and C18:0) were shown to contribute to the structural role of SM, maintaining rigidity of the MFGM (94).

TABLE 1 | Total phospholipid concentration in human colostrum, transitional, and mature milks.

Mothers	Units	Colostrum	Transition milk	Mature milk					References
Country		(Day 1–7)	(Day 8–15)	1 month	2 months	3 months	4 months	Up to 8 months	
Singapore	mg/100 mL			23.0 ± 4.9	20.8 ± 8.5		24.2 ± 8.2		(65)
China (Beijing)	mg/100 mL	33.0 ± 11.2	24.4 ± 8.1					22.3 ± 9.9	(58)
China (Suzhou)	mg/100 mL	38.9 ± 18.8	34.9 ± 16.6					26.02 ± 11.3	(58)
China (Guangzhou)	mg/100 mL	33.2 ± 8.1	25.6 ± 11.1					25.3 ± 12.5	(58)
China (Shanghai, Huangpu)	mg/100 mL		40.7			22.9			(80)
China (Shanghai)*	mg/100 g	35.1 ± 10.8	35.1 ± 8.6		28.1 ± 7.8	–			(81)
China (Wuxi)	μmol/100 mL	25.8 ± 3.8	24.8 ± 3.5	23.7 ± 2.4	23.6 ± 3.6	22.6 ± 1.1			(82)
Ireland (Cork)	mg/100 mL	67.7 ± 14.5	48.7 ± 18.1					36.9 ± 16.4	(7)
France (Marseilles)	mg/100 mL	72 ± 51	55 ± 26	45 ± 26					(83)
Denmark	mg polar lipids/100 g total lipids	4.4 ± 0.4	5.9 ± 0.3	5.1 ± 0.4					(23)
Spain (Granada)	nmol/mL	202 ± 39	209 ± 38	147 ± 23					(84)
Spain (Madrid)	mg/100 mL	37.2 ± 1.0	43.7 ± 2.3	39.2 ± 2.8	35.9 ± 0.9			26.5 ± 0.7	(13)
Spain (Valencia)	mg/100 mL	31.5 ± 3.1	53.5 ± 2.5	42.2 ± 1.3		32.1 ± 1.8		33.4 ± 2.0	(13)
Spain (Murcia)	mg/100 mL			39.2 ± 2.5		34.9 ± 2.0		28.1 ± 1.4	(13)
United Arab Emirates (Sharjah, Dubai, and Ajman)	mg/L	NR	269.0 ± 89.2					219.6 ± 85.0	(85)
Malay	mg/L	352.4 ± 166.3	273.0 ± 58.4		147.1 ± 41.2			187.5 ± 110.0	(86)

Only measured PC, PE, and SM. NR, not reported.

The concentration is shown as mean ± standard deviation. Units differ according to each publication.

Although ARA and DHA are mainly found in a triglyceride structure within the core of the MFG, they are also found in MFGM phospholipids, principally in PE (7, 89). One study reported that around 10% of ARA is found in the phospholipid fraction for both transitional and mature milk, whereas ∼10 and 22% of the DHA was found in the phospholipid fraction in transitional and mature milk, respectively (83). This same study also reported that the DHA:ARA ratio was significantly higher in the phospholipid fraction compared to the triacylglycerol core, suggesting that HM with the higher phospholipid concentrations may be more efficient for brain and intestinal LC-PUFA accretion since phospholipids provides a best delivery system (95, 96).

Recent studies, using a lipidomic approach, described the distribution of HM glycerophospholipids molecular species across lactation (7, 97, 98). The major glycerophospholipids molecular species for PE, PC, PI and PS found in mature milk of a Chinese cohort was C36:2 (35–64%) followed by C36:1 [16–28%, with the exception of PC (4%)], with both phospholipids increasing during lactation (98). This may indicate that similar FAs moieties (34–36 carbons) across the range of glycerophospholipids may have a functional role in the MFGM. Similarly, high concentrations of C36:2 for PE were also found in the milk of Singaporean mothers (97), whereas C36:4 was the major molecular specie reported from Irish mothers (7). The major types of PC varied according to the study with C36:2 and C32:0 representing 31–41 and 10–13% of the molecular species, respectively, in some studies (97, 98), while in another, it was about 12 and 46%, respectively (7).

SM has a very distinct molecular profile in mature milk, with d40:1 (20%), d42:1 (16%), d36:1 (14%), d34:1 (13%), d42:2 and d38:1 (10% each), adding up to 85% of total SM molecules (98). Although these major SM molecules were identified in other studies (91, 97), their distribution were different. A very different profile of SM molecules, d38:0 (47%), d38:1 (13%), d40:1 (16%), d32:1 (7%), and d40:0 (13%), was reported in an Irish cohort (7). As previously discussed, FAs composition can be affected by maternal dietary factors and maternal geographical origin observed across different cohorts. To our knowledge, no study has addressed the effects of maternal geographical origin and diet on phospholipid FAs, only on the total fatty acids profile.

Gangliosides

Table 3 provides a summary of studies reporting total concentrations of HM gangliosides and GD3 (disialoganglioside) and GM3 (monosialodihexosylganglioside) molecular structures across lactation. Higher concentrations of total gangliosides were found in colostrum, followed by transitional milk with the lowest in mature milk, in most studies (99, 102, 105, 106). A few studies reported that the concentrations of gangliosides were relatively consistent during lactation (100, 101) or increased from colostrum to mature milk (58, 103) and continued to increase in mature milk (87, 106). Such

TABLE 2 | Concentration of phospholipid species in human colostrum, transitional, and mature milks.

Colostrum (Day 1–7)											
References	(81)	(82)	(84)	(23)	(7)	(58)	(58)	(58)	(86)	(13)	
Country	China (Shanghai)	China (Wuxi)	Spain (Granada)	Denmark	Ireland (Cork)	China (Beijing)	China (Suzhou)	China (Guangzhou)	Malay	Spain (Madrid)	Spain (Valencia and Murcia)
Units	mg/100 g	μmol/100 mL	Total (202 ± 39 nmol/mL)	mg polar lipids/total lipids	mg/100 mL	mg/100 mL	mg/100 mL	mg/100 mL	mg/L	mg/100 mL	
			Weight%								
PE	4.61 ± 2.11	7.39 ± 0.58	5.86 ± 0.63	0.41 ± 0.03	49.40 ± 13.68	7.6 ± 3.1	12.6 ± 7.4	9.9 ± 2.6	89.9 ± 25.8	12.36 ± 0.61	10.46 ± 0.75
PC	20.32 ± 6.61	7.55 ± 1.52	38.40 ± 3.09	1.26 ± 0.19	11.44 ± 2.64	10.9 ± 4.8	12.6 ± 7.7	12.5 ± 4.6	76.7 ± 55.4	4.87 ± 0.11	4.51 ± 0.28
PS		1.29 ± 0.09	7.91 ± 1.12	0.56 ± 0.03		1.8 ± 2.3	1.7 ± 0.5	1.3 ± 0.4	125.8 ± 63.0	3.49 ± 0.01	3.43 ± 0.20
PI		1.05 ± 0.06	6.03 ± 0.61	0.36 ± 0.02		1.6 ± 0.5	2.3 ± 1.0	1.8 ± 0.5	11.2 ± 3.3	3.13 ± 0.01	3.12 ± 0.15
SM	10.14 ± 3.39	8.54 ± 1.83	40.49 ± 3.57	1.82 ± 0.26	6.90 ± 1.26	10.9 ± 4.9	9.7 ± 3.1	7.7 ± 1.6	39.7 ± 25.7	13.36 ± 0.29	9.95 ± 1.75
Transition milk (Day 8–15)											
References	(58)	(58)	(58)	(81)	(23)	(82)	(84)	(7)	(85)	(86)	(13)	
Country	China (Beijing)	China (Suzhou)	China (Guangzhou)	China (Shanghai)	Denmark	China (Wuxi)	Spain (Granada)	Ireland (Cork)	United Arab Emirates	Malay	Spain (Madrid)	Spain (Valencia and Murcia)
Units	mg/100 mL	mg/100 mL	mg/100 mL	mg/100 g	mg polar lipids/total lipids	μmol/100 mL	Total (209 ± 38 nmol/mL)	mg/100 mL	mg/L	mg/L	mg/100 mL	
							Weight%					
PE	7.3 ± 2.4	10.8 ± 5.8	5.6 ± 3.7	4.79 ± 2.01	0.77 ± 0.12	7.07 ± 0.60	8.55 ± 1.16	37.86 ± 14.00	66.3 ± 27.16	100.0 ± 24.5	13.90 ± 0.98	15.90 ± 0.85
PC	8.3 ± 3.7	11.9 ± 6.1	11.3 ± 5.6	19.94 ± 5.35	1.50 ± 0.13	7.21 ± 1.20	37.69 ± 4.88	6.56 ± 3.26	66.4 ± 32.87	48.6 ± 11.6	5.97 ± 0.15	8.09 ± 0.14
PS	1.0 ± 0.4	1.3 ± 0.5	0.8 ± 0.4			1.21 ± 0.24	8.17 ± 1.04		28.5 ± 13.29	90.9 ± 18.0	4.66 ± 0.10	6.74 ± 0.08
PI	1.5 ± 0.4	2.4 ± 1.1	1.2 ± 0.7		0.40 ± 0.03	0.93 ± 0.30	5.21 ± 0.54		11.2 ± 5.5	9.6 ± 3.0	4.30 ± 0.07a	6.32 ± 0.05
SM	6.2 ± 3.8	8.5 ± 4.7	6.8 ± 2.7	10.37 ± 2.69	2.37 ± 0.40	8.37 ± 1.54	39.20 ± 3.63	4.23 ± 1.88	91.2 ± 26.38	20.9 ± 5.7	14.86 ± 1.11	16.49 ± 1.46
Mature milk												
	1 month					**(16–60 days)**				**2 months**		
References	(23)	(82)	(82)	(84)	(65)	(13)			(81)	(82)	(86)	
Country	Denmark	China (Wuxi)	China (Wuxi)	Spain (Granada)	Singapore	Spain (Madrid)	Spain (Valencia)	Spain (Murcia)	China (Shanghai)	China (Wuxi)	Malay	
Units	mg polar lipids/total lipids	μmol/100 mL	μmol/100 mL	μmol/100 mL	mg/100 mL		mg/100 mL		mg/100 g	μmol/100 mL	mg/L	
PE	0.76 ± 0.10	7.21 ± 1.20	6.92 ± 0.98	1.87 ± 0.17	6.76 ± 1.86	11.98 ± 1.09	12.68 ± 0.66	11.58 ± 0.98	3.63 ± 1.53	6.89 ± 1.22	39.3 ± 15.8	
PC	1.07 ± 0.11	1.21 ± 0.24	6.74 ± 0.72	4.59 ± 0.70	5.97 ± 1.34	5.42 ± 0.31	6.55 ± 0.13	5.67 ± 0.24	15.41 ± 5.09	6.06 ± 0.60	21.0 ± 10.8	
PS	0.84 ± 0.06	0.93 ± 0.30	1.03 ± 0.15	1.52 ± 0.19	0.75 ± 0.31	4.45 ± 0.24	5.55 ± 0.07	4.90 ± 0.18		1.15 ± 0.22	14.8 ± 7.7	
PI	0.41 ± 0.02	8.37 ± 1.54	0.64 ± 0.08	0.86 ± 0.07	1.07 ± 0.35	4.15 ± 0.24	5.20 ± 0.04	4.60 ± 0.17		1.00 ± 0.21	6.3 ± 3.6	
SM	1.97 ± 0.33	41.03 ± 3.41	8.34 ± 0.94	6.03 ± 0.50	8.47 ± 1.72	13.20 ± 1.27	12.19 ± 0.54	12.44 ± 1.11	9.07 ± 2.52	6.89 ± 1.22	57.4 ± 11.7	

(Continued)

TABLE 2 | Continued

Mature milk								
	3 months		61–135 days			4 months	6 months	
References	(65)	(82)	(13)			(7)	(87)	
Country	Singapore	China (Wuxi)	Spain (Madrid)	Spain (Valencia)	Spain (Murcia)	Ireland (Cork)	Malay	
Units	mg/100 mL	μmol/100 mL		mg/100 mL		mg/100 mL	mg/L	
PE	6.36 ± 3.11	7.13 ± 0.32	10.71 ± 0.24	9.51 ± 0.76	11.36 ± 0.73	29.15 ± 13.04	66.1 ± 0.	
PC	4.84 ± 2.06	5.89 ± 0.53	5.06 ± 0.14	4.92 ± 0.13	4.86 ± 0.16	4.50 ± 1.97	23.7 ± 0.	
PS	0.75 ± 0.33	0.85 ± 0.07	4.56 ± 0.14	4.39 ± 0.06	4.30 ± 0.13		15.5 ± 9.4	
PI	1.13 ± 0.55	0.75 ± 0.14	4.23 ± 0.11	4.13 ± 0.05	4.06 ± 0.13		5.9 ± 0.0	
SM	7.71 ± 3.01	8.15 ± 0.55	11.34 ± 0.34	9.20 ± 0.90	10.29 ± 0.83	3.29 ± 1.73	70.4 ± 36.8	

Mature milk								
Up to 8 months								
References	(65)	(85)	(58)			(13)		
Country	Singapore	United Arab Emirates (Sharjah, Dubai, and Ajman)	China (Beijing)	China (Suzhou)	China (Guangzhou)	Spain (Madrid)	Spain (Valencia)	Spain (Murcia)
Units	mg/100 mL	mg/L	mg/100 mL	mg/100 mL	mg/100 mL		mg/100 mL	
PE	8.08 ± 3.10	80.0 ± 35.35	5.3 ± 2.6	7.3 ± 3.2	7.1 ± 3.9	8.29 ± 0.40	10.24 ± 0.60	8.37 ± 0.56
PC	4.94 ± 1.88	30.2 ± 22.07	7.6 ± 4.5	8.5 ± 5.3	8.6 ± 5.1	3.79 ± 0.19	5.02 ± 0.16	4.13 ± 0.12
PS	0.91 ± 0.33-	16.1 ± 6.99	0.9 ± 1.2	1.2 ± 1.4	1.0 ± 0.6	3.39 ± 0.17	4.49 ± 0.07	3.73 ± 0.11
PI	1.67 ± 0.66	6.5 ± 3.61	1.2 ± 0.5	1.7 ± 0.8	1.5 ± 0.8	3.12 ± 0.1	4.23 ± 0.08	3.50 ± 0.09
SM	8.26 ± 2.64	82.9 ± 29.21	7.3 ± 3.9	7.4 ± 4.2	7.1 ± 4.0	7.96 ± 0.11	9.44 ± 1.09	8.39 ± 0.52

PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.
The concentration is shown as mean ± standard deviation. Units differ according to each publication.

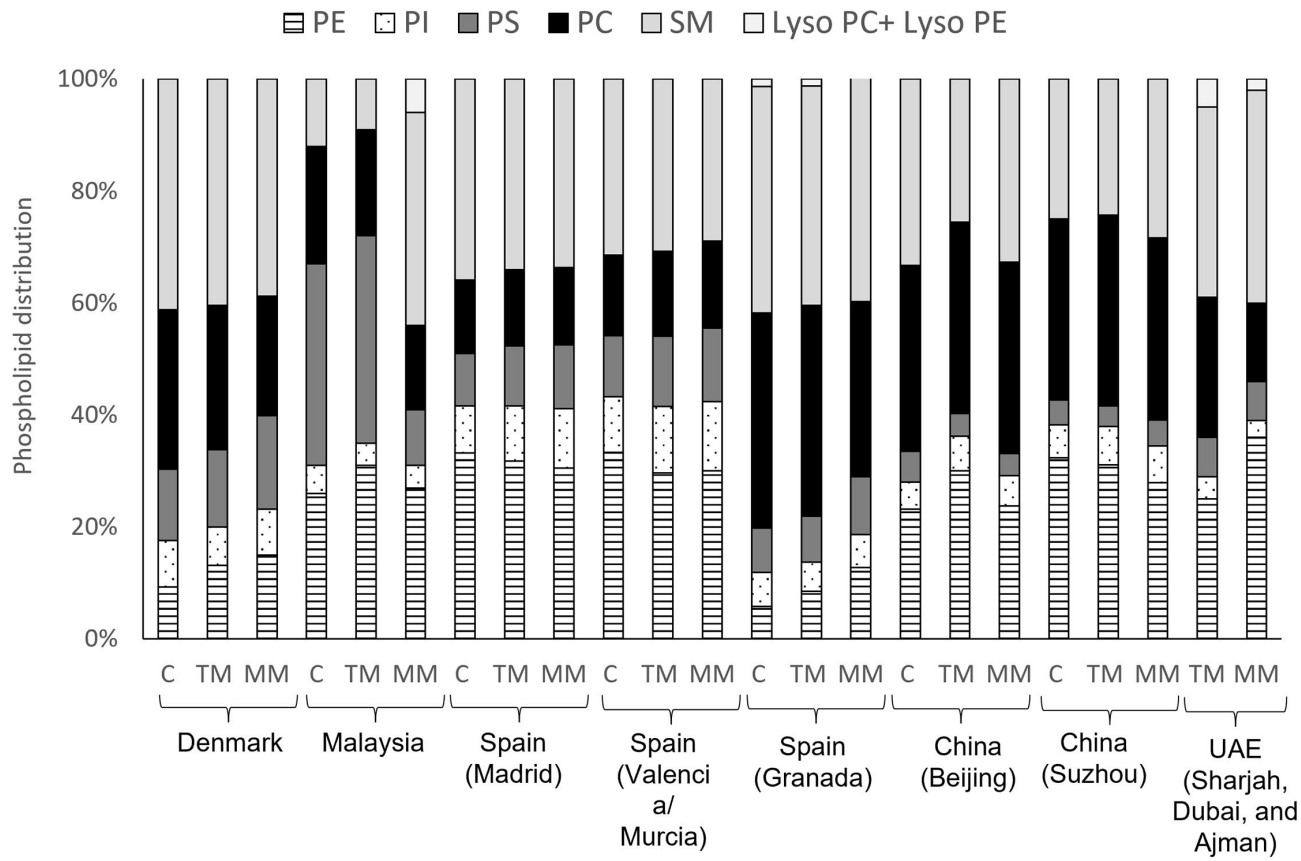

FIGURE 5 | Distribution of phospholipid classes in colostrum (C), transitional milk (TM) and mature milk (MM) in different geographical cohorts. Data from (23), Denmark; (86), Malaysia; (13), Spain (Madrid, Valencia and Murcia); (84), Spain (Granada); (58), China (Beijing and Suzhou); (85), UAE (Sharjah, Dubai, and Ajman). PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

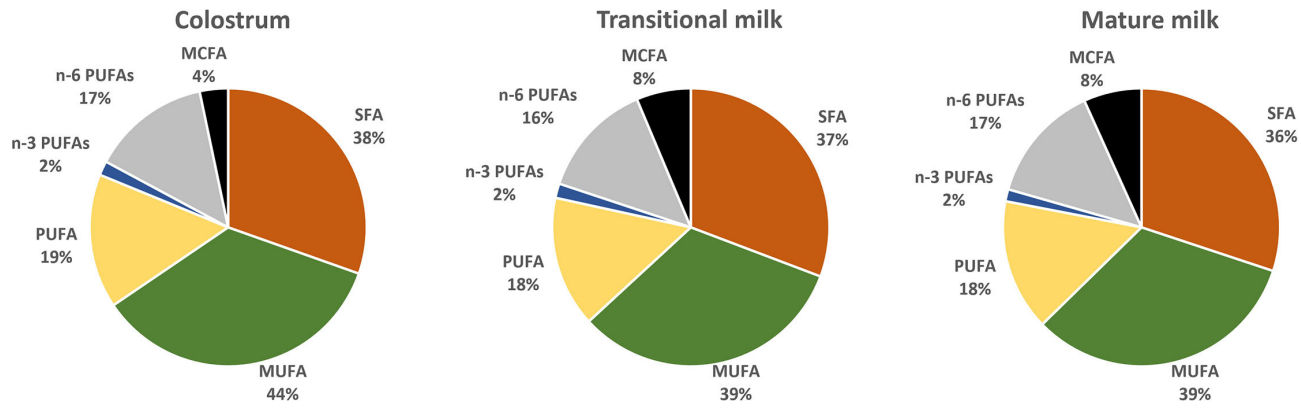

FIGURE 6 | Distribution of phospholipids fatty acids categories in colostrum (1–7 days), transitional milk (8–15 days) and mature milk (60 days) from Chinese mothers reported by Wei et al. (82). PUFAs, polyunsaturated fatty acids; n-3 PUFAs, n-3 polyunsaturated fatty acids; n-6 PUFAs, n-6 polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

discrepancies in the results could be due to differences in sampling, analytical methods (**Supplementary Table 2**) and other factors such as maternal diet and infant gender, etc. A significant positive correlation between gangliosides and total milk lipid has been described (87, 100, 104, 106). Gangliosides are found in the MFGM surrounding the fat droplet; therefore, if the gangliosides were a constant proportion of the MFGM and the MFG were a similar size,

TABLE 3 | Concentration of ganglioside molecular structures in human colostrum, transitional, and mature milks.

	Colostrum								**0–11 days**
References	(99)	(79)*	(100)	(100)	(101)*	(87)#	(102)	(58)#	(103)#
Country	Spain	Spain	Panama	Spain	Japan	Malaysia	China	China	China
Units	mg LBSA/kg	μg LBSA/g	μg LBSA/g	μg LBSA/g	μg LBSA/mL	mg/L	mg/L	mg/L	μg/mL
Total	2.3 ± 0.5	2.9	2.2 ± 0.4	3.6 ± 0.6	9.2 ± 2.0	26.8	15.9 ± 5.9	8.0 ± 5.3	8.1
GM_3	8.8 ± 2.2%	7.40%	6.2 ± 2.3%	6.2 ± 2.3%	3.7 ± 2.0%	6.5 ± 7.0	2.3 ± 0.5	3.8 ± 2.5	4.3 ± 0.9
GD_3	**63.2 ± 4.0%**	**37.40%**	**51.3 ± 7.0%**	**43.7 ± 2.5%**	**46.7 ± 9.9%**	**20.3 ± 13.0**	**13.7 ± 5.7**	**4.1 ± 4.5**	**3.8 ± 0.4**
	Transitional milk								**16 days-8 months**
References	(99)	(79)*	(100)	(100)	(101)*	(87)#	(102)	(58)#	(103)#	(99)
Country	Spain	Spain	Panama	Spain	Japan	United Arab Emirates	Malaysia	China	China	China
Units	mg LBSA/kg	μg LBSA/g	μg/g	μg/g	μg LBSA/mL	mg/L	mg/L	mg/L	mg/L	mg/L
Total	1.38 ± 0.4	1.54	3.8 ± 0.8	3.7 ± 0.6	9.2 ± 0.2	21.2 ± 11.5	18.9	12.7 ± 4.5	8.5 ± 4.5	11.0 ± 5.0
GM_3	11.6 ± 2.2%	1.6	12.0 ± 2.0%	10.2 ± 1.6%	26.7 ± 5.9%	9.5 ± 8.4	8.3 ± 4.8	2.1 ± 0.3	5.5 ± 3.2	10.1 ± 4.6
GD_3	52.4 ± 4.2%	21	34.4 ± 3.5%	38.9 ± 3.0%	31.2 ± 7.6%	11.7 ± 9.5	10.6 ± 4.3	10.6 ± 4.4	3.0 ± 3.4	1.0 ± 1.7
	Mature milk									
	1 month									
References	(101)*	(104)*	(100)	(100)	(42)	(103)#	(58)#	(65)#	(102)	(86)
Country	Japan	Spain	Panama	Spain	Spain	China	China	Singapore	China	China (Guangzhou)
Units	μg LBSA/ mL	μg LBSA/g	LBSA μg/g	LBSA μg/g	mg LBSA/kg	μg/mL	mg/L	mg/L	mg/L	mg/L
Total	9.0 ± 1.6	0.82	4.3 ± 2.1	2.1 ± 0.5	0.8 ± 0.2	9.1			9.5 ± 3.1	13.1 ± 6.7
GM_3	32.1 ± 7.6%	47.4%	27.2 ± 6.5%	22.0 ± 2.7%	50.2 ± 1.6%	7.4 ± 0.2	10.1 ± 4.6	2.3 ± 0.8	2.2 ± 1.1	8.5 ± 4.7%
GD_3	19.9 ± 2.1%	8.2%	23.2 ± 6.9%	21.5 ± 3.7%	21.3 ± 1.3%	1.7 ± 0.2	1.0 ± 1.7	2.3 ± 1.2	7.2 ± 3.2	4.6 ± 3.1%
	Mature milk									
	2 months						**3 months**			
References	(101)*	(104)*	(100)	(100)	(42)	(103)#	(58)#	(65)#	(102)	
Country	Spain	China (Guangzhou)	China	China	Singapore	Malaysia	Spain	China	China (Guangzhou)	
Units	μg LBSA/g	mg/L	mg/L	μg/mL	mg/L	mg/L	μg LBSA/g	mg/L	mg/L	
Total	0.85	18.2 ± 7.8	7.4 ± 2.2	10		14.8 ± 8.0	1.39	7.5 ± 2.3	20.9 ± 10.5	
GM_3	42.3%	11.3 ± 6.2%	2.2 ± 0.8	9.1 ± 0.3	2.9 ± 1.4	8.3 ± 5.5	56.3%	2.1 ± 0.6	17.4 ± 9.0%	
GD_3	9.3%	7.0 ± 7.7%	5.2 ± 2.1	0.9 ± 0.1	1.9 ± 2.0	6.5 ± 5.1	13.5%	5.3 ± 2.3	3.5 ± 2.5%	

(Continued)

TABLE 3 | Continued

	Mature milk							
	4 months				6 months			
References	**(65)[#]**	**(102)**	**(103)[#]**	**(87)**	**(87)**	**(87)[#]**	**(102)**	**(85)[#]**
Country	Singapore	China	China	China (Guangzhou)	China (Guangzhou)	Malaysia	China	United Arab Emirates
Units	mg/L	mg/L	μg/mL	mg/g	mg/g	mg/L	mg/L	mg/L
Total		7.0 ± 2.5	10.7	19.8 ± 9.4	22.9 ± 9.9	25.3 ± 15.7	6.5 ± 2.0	20.2 ± 9.8
GM_3	3.9 ± 1.8	2.1 ± 0.6	9.8 ± 0.3	18.3 ± 9.4%	21.4 ± 9.5%	21.4 ± 13.0	2.1 ± 0.8	18.6 ± 9.7
GD_3	1.7 ± 1.9	4.9 ± 2.4	0.9 ± 0.1	1.5 ± 1.1%	1.5 ± 1.1%	4.3 ± 5.5	4.4 ± 1.9	1.6 ± 2.2

[*]Colostrum (5 days post-partum), Transitional milk (10 days postpartum). LBSA, lipid bound sialic acid. [#]Total as sum of GM3 and GD3. The concentration is shown as mean ± standard deviation. Units differ according to each publication. LBSA, lipid bound sialic acid. [#]Total as sum of GM3 and GD3.

more gangliosides would be expected with an increase in fat content.

Due to the large range of ganglioside structures, quantification of gangliosides can be difficult. Until 2009, conventional methods to detect and quantify gangliosides were mainly based on high-performance thin-layer chromatography (HPTLC) and results were converted from lipid-bound sialic acid (LBSA). Data were often inaccurate due to limitations of these method (107). An improved HPLC-MS method was developed by Fong et al. (108), reporting the content and number and a large number of ganglioside structures in different food matrixes. In 2013–2014, this method was used to report that the total content of gangliosides in HM of Singaporean and Chinese mothers 30–120 days after delivery was 4.6–5.6 and 9.1–10.7 mg/L, respectively (65, 103). In 2015, Ma et al. reported the content of HM in mothers from South China within 8 months after delivery as 13.1–22.9 mg/L (106) and Tan et al. (102) reported the content of gangliosides in HM of Chinese mothers was 6.5–15.9 mg/L within 6 months after delivery (102). The lowest content (0.8 mg/L) was observed for Spanish samples from studies using the HPTLC methodology (99). It is interesting to note that high overall ganglioside concentrations in milk were reported in Asian mothers (7- 25.3 mg/L) (**Table 3**) (58, 101, 102, 106) where nutritional aspects (such as fat being mainly sourced from fish) may largely contribute to these findings.

Among the seven different gangliosides that have been identified in HM (99, 101, 104), GD3 and GM3, referred to as "simple" gangliosides, are the prevalent individual components of the ganglioside fraction. GD3 ganglioside is the predominant form present in human colostrum and transitional milk (30–80%) but concentrations decrease up to 4–6 months post-partum (8–25%). Conversely, GM3 is predominance in mature milk (58, 100, 106, 109). These gangliosides are likely to survive the infant's digestion, reaching the intestinal tract and having an inhibitory effect on the adhesion of pathogenic bacteria (110, 111). It has been suggested that in early milk GD3 may have a role in organ development, such as of the gut and brain (112). The increase in GM3 in mature milk has been linked to the development of the immune and central nervous systems by supporting signal transduction, cell adhesion, and growth factor receptors (79). Therefore, the variation in the ganglioside composition of HM over the course of lactation might be linked to alterations that occur in the immunological prophylactic system, and in the development of the central nervous system and the autonomic nervous system of the intestine and other organs.

Large variations in the concentration of individual gangliosides can be observed in studies using similar methodologies (**Supplementary Table 2**). Giuffrida et al. (58) reported average GM3 values, in colostrum and transitional milk, of 4.1 and 3.0 mg/L, respectively, whereas Ma et al. (87) reported 20 and 10 mg/L, respectively. Within mature milk, at 1–2 months and 3–8 months GD3 content was reported to be as low as 0.87 and 0.25–0.50 mg/L, respectively (58) and high as 4.6–7.0 and 1.5–2.7 mg/L (87, 106), respectively in Asian mothers. Interestingly, infant gender may influence gangliosides concentration with one study reporting an increase in GM3 in milk for male infants at 120 days of lactation compared to milk

for female infants (65). These authors indicated that the increase in the total amount of lipid in milk for male infants at 120 days (119%) could partly explain the observed increase in GM3 and other amphipathic molecules such as phospholipids (PC, PI, PE, and SM).

Only one study compared the concentrations of HM gangliosides in different countries across lactation stages (100). Although no statistically significant differences were observed across locations and lactation periods, the gangliosides content tended to be higher in Spanish mothers colostrum compared to Panamanian mothers colostrum. The opposite observation was found in mature milk, where fat and gangliosides content were enriched in Panamanian mothers (100). These authors indicate that although different dietary habits were observed among these countries, the use of foremilk for their investigations may have masked the effects of maternal origin on gangliosides content.

Cholesterol

The MFGM is the source of cholesterol in HM, which is essential for the synthesis of lipoproteins, bile acids, hormones and calciferols, therefore, essential to infant growth (113). Moreover, cholesterol is a crucial part of the cell membranes and myelin, and is especially required during the neuroplasticity period (from conception to up to 4 years old) (114, 115). Despite the importance of cholesterol, only a few studies have examined the concentration of this bioactive compound in HM during lactation. Most studies, not reviewed here, were published before 1990 and showed large variability, probably due to the limitations of analytical methods available at the time.

In general HM cholesterol changes dynamically throughout lactation, with the highest level in colostrum, decreasing significantly during the first month after delivery (116–118) (**Table 4**). One particular study showed that HM cholesterol decreased by 60% from colostrum to the first month postpartum (118), and another by half at 6 months postpartum (123). After the first month postpartum, the decline in cholesterol concentration was shown to be much less pronounced (117, 118, 123) and this may be associated with the MFG size and number. Changes in the MFG diameter from colostrum to mature milk (from $\sim$ 3μm in colostrum to around 5 μm in mature milk) as well as a decrease in the number of globules leads to reduced MFGM surface area and consequently, cholesterol.

Studies of HM in Iraq, Spain, Portugal, and China reported similar ranges of cholesterol in colostrum (20–29 mg/100 mL), whereas studies in Poland and Africa reported the lowest (3.4–11.9 mg/100 mL) and the highest (36.0 $\pm$ 16.2 mg/100 mL) concentrations, respectively. Similar results were observed for mature milk, with cholesterol concentrations ranging from 11 to 13 mg/100 mL for most countries but higher in the study conducted in Africa (19 mg/mL).

Proteins

Proteins represent 25–60% of the total MFGM mass and 1–4% of the total protein content of HM. The use of proteomic techniques has enabled the assessment of MFGM-derived proteins to understand their diversity and physiological roles (70–72, 125–129). Beyond a nutritional source, the main human MFGM proteins were shown to have a role on cell communication and signal transduction, immune function, metabolism, and energy production (6).

The proteomics studies generally do not provide absolute quantitative data, however by comparing relative levels they can provide information on how MFGM proteins change over the course of lactation. In one relatively early study, Cavaletto et al. (130) used proteomics to assess the MFGM butyrophilin (BTN) protein family (which comprises seven proteins); they observed only slight differences in BTN spot distribution when comparing colostrum with mature milk. More recently, relative quantification of MFGM proteins during lactation was performed by label free spectral counting and differentiation expression analysis (6). This demonstrated a change in relative levels of many minor MFGM proteins from early to late lactation; for example, alpha-1-antitrypsin, alpha-amylase, apolipoproteins D and E, alpha-enolase, insulin-like growth factor-binding protein 2 and long chain fatty acid-coA ligase 4 were expressed at higher levels during early lactation (particularly in colostrum), whereas CD9 antigen, fatty acid binding protein, folate receptor alpha, and glutathione peroxidase 3 were expressed at higher levels during late lactation (6–12 months). Interestingly, other proteins such as xanthine dehydrogenase/oxidase, complement C3, BTN A1 and Annexin 2 had a sharp increase in concentration later in lactation (3–6 months). Other studies showed that, as observed for total proteins (131), the colostral MFGM proteome contains a higher number of proteins related to the establishing immune system than mature milk (71, 129). This may indicate that proteins may be expressed to aid a particular developmental stage of the infant (73).

Individual MFGM Proteins

Within the timeframe specified for this review, there are few reports in which levels of MFGM proteins have been quantified in HM. One study of 45 mothers in León, Nicaragua measured lactadherin in HM at 3 months of age, reporting a median concentration of 5.4 μg/mL (interquartile range, 4.0–7.3) (132). It should be noted that this level was measured following a rotavirus vaccination, so it is not clear whether this represents a normal level in HM. In addition, there were no data on changes in lactadherin through lactation in this study.

In an observational study, 200 infants in Mexico were recruited at birth, and their stool monitored for rotavirus infection; at the same time, samples of mothers' milk were collected, and assayed for a range of MFGM-associated proteins (133). Similar to observations regarding levels of phospholipids, these analyses showed wide inter-individual variation, with levels of lactadherin in HM ranging from 5.6 to 180 μg/mL. This also demonstrated an association between levels of lactadherin and protection of infants from rotavirus infection, consistent with this protein playing a role in immunity and response to infection.

Glycoproteins and Phosphoproteins

Protein glycosylation, the attachment of a carbohydrate (glycan) to a protein, is one of the most common post-translational modifications of proteins. Glycosylation has been reported to be involved in several biological and cellular

TABLE 4 | Concentration of cholesterol in human colostrum, transitional, and mature milks.

Country	Sample collection	Method analysis	Colostrum	Transitional	Mature milk						Reference
					1 month	2 months	3 months	4 months	5 months	6 months	
Spain	Pooled milk	E-S			11.3 ± 0.4						(119)
Iraqi	Partial expression (5–10 mL) of two breasts combined morning	E-S	28.3 ± 4.2								(120)
USA	NR	GC						14.2 ± 3.3			(121)
Netherlands	24 h sample	GC-FID		16.6			12.8 ± 1.0				(122)
Spain	Pooled milk	GC	20.7 ± 0.6	14.8 ± 0.8	12.8 ± 0.5		10.9 ± 0.5		10.1 ± 0.4		(123)
		E-S	23.2 ± 1.1	17.1 ± 0.8	13.6 ± 0.5		12.8 ± 0.2		11.7 ± 0.1		
Poland	NR	ATR-FTIR	3.4–11.9	4.4–13.0							(124)
Portugal	NR	HPLC-DAD	29.2 ± 0.01		17.4 ± 0.5	12.0 ± 0.1		9.5 ± 0.1			(117)
African	Manual expression, mid-way through nursing	GC	36.0 ± 16.2	19.7 ± 0.7	19.0 ± 0.8						(116)
China	Full single breast expression, mechanical expression, morning	HPLC	20	17.1	12.6						(118)

NR, not reported; E-S, enzymatic–spectrophotometric; GC, gas chromatography; ATR-FTIR, attenuated total reflectance–Fourier transform infrared spectroscopy; HPLC-DAD, high-performance liquid chromatography with diode array detector.
The concentration is shown as mean ± standard deviation (mg/100 mL).

functions, including protein folding, immune response, and pathogen binding (134, 135). Many MFGM proteins, for example, the major proteins mucins, lactadherin, and butyrophilin (136). LC-MS/MS analysis of pooled samples from a total of 60 mothers (30 colostrum, 30 mature milk) identified 220 MFGM N-glycoproteins differentially expressed in mature milk compared with colostrum, demonstrating a significant shift in N-glycoprotein composition of HM across lactation (72). Among those proteins differently glycosylated, the proteins involved in immune system maturation and microbial colonization, such as lactoperoxidase, major histocompatibility complex (MHC) and cell adhesion molecules (CAMs) showed increased N-glycosylation levels in colostrum compared to mature milk (72). This may play a significant role in the formation of the immune system of infants. Also, it has been proposed that not only the overall concentration of a protein is crucial for its overall activity but understanding glycosylation pattern during lactation could also reflect the individual needs of infants during their growth (137).

Protein phosphorylation is another common posttranslational modification, regulating various cellular processes such as protein location, interaction, and overall function (138). Therefore, understanding the variation in phosphopeptides is very important to recognize the changes of many biological processes in health and disease (139). Recently, a quantitative phosphoproteomics analysis of human MFGM demonstrated that colostrum and mature milk have different phosphorylation profiles (129). Among 203 phosphoproteins identified, 48 proteins were differentially expressed between the different stages of lactation. Of those, phosphoproteins related to the cellular process and immunity (27 and 24 phosphorylation sites, respectively) were identified only in human colostrum milk.

FACTORS INFLUENCING MFGM COMPONENTS

Stage of lactation is clearly one factor that impacts on MFGM composition. There are also several maternal factors influencing MFGM content of HM, and these have recently been reviewed (17, 73). Some of them, such as stage of lactation, circadian rhythms, infant birth weight, gender (140), development at delivery (pre-term vs. term) (141), maternal diet and weight, and method of breast milk expression (17) can directly affect the total lipid content in milk, and MFG size and numbers. Because lipids and proteins are the major components of the MFGM, these have been most widely assessed in this context.

Lipids

Although FAs in the core of the globule and those in the MFGM have not been separately analyzed in many studies of milk lipids (73), some insight has been gained into key maternal factors that influence the lipid composition of the MFGM. In addition to lactation stage, the following factors also influence MFGM-derived lipid levels in HM.

Method of Sample Collection

There are two main methods of HM collection, by hand or mechanical expression by electric pump. An electric pump cycles the negative pressure with a rhythmic action simulating suckling, which provides a standardized method to collect milk samples (142). Milk hand expression generally requires breast massage, which can increase release of milk fat (143). Variation in nutrient content across expression methods needs to be considered when interpreting data.

Time of Collection and Subsampling

Lipid content varies over the course of the day (with higher concentrations found in the evening milk) and ideally milk samples representative of 24h production should be obtained. The difficulty to obtain these samples generally leads to the collection of full expression from one breast, a few mL of hindmilk, foremilk or a combination of both (142). The total amount of fat changes over time during nursing; foremilk has a lower fat concentration compared to hindmilk (16, 17) and fat also follows a circadian rhythm (morning milk has lower concentration of fat compared to evening milk) [reviewed by Italianer et al. (18)]. It has also been suggested that nursing frequency and breast (left or right) may affect milk macronutrient composition. The impact of breast (left or right) on macronutrient composition has been linked to the level of fullness of the breast before sampling, in turn linked with the last feed (144). This highlights the importance of standardization of milk collection methods or at least a thoughtful reporting of the conditions of milk collection in studies that report milk composition data.

Genetic Factors

Few studies have been published on maternal genetics regulating levels of MFGM phospholipid classes. One such study describes a polymorphism in the diacylglycerol acyltransferase 1 (DGAT1) gene which was associated with altered phospholipid composition and phospholipid/TAG ratios (145). However, this study was on bovine milk, hence more research is needed to understand the extent of the influence of genetic variation in HM.

Diet

Of particular relevance to this review, milk ganglioside, FAs and phospholipid concentrations have been reported to differ according to geographical locations, suggesting that diet may influence the amounts in HM (58). Indeed, several studies have reported associations between maternal diet and milk lipid composition [reviewed by Bravi et al. (146)]. Crossover (147–149) and observational studies (146, 150) indicate that maternal lipid intake plays an important role on the HM total fat content and FA profile. Lipids from maternal diet is one of the three known sources of milk lipids, the other being *de novo* synthesis and FAs from maternal adipose tissue. Other food components, such as choline dietary supplementation has been shown to be positively correlated with HM PC, especially in choline-deficient diets (151). The impact of maternal diet, however, may vary for particular MFGM components. The content of the long-chain fatty acid DHA within particular phospholipids in HM, for example, appears to be independent of the maternal intake of these compounds (152).

Gender of the Infant

The potential for the infant's gender to influence maternal milk composition has recently been reviewed (153). Evidence to support this idea is largely from animal studies which suggest gender is a predictive determinant of milk composition. In human studies, the lipid content in mature milk produced for males was higher than milk produced for female infants (65, 154). Authors hypothesized that higher suckling response (longer and more frequent) from male infants may feedback as a message for additional energy content that results in increased energy output from the mother. It is important to note that most studies did not assess individual milk production or infant intake, restricting the ability to account for volume and overall fat production.

Gestation Length (Term vs. Pre-term)

There is evidence that pre-term delivery may result in a different phospholipid profile in HM (7), with some evidence that sphingomyelin and PE may decrease in full-term colostrum, whereas other phospholipids such as PC, PI, and PS showed no correlation with delivery term (155). However, as this review is focused on normal, term infants we have not considered this aspect further.

Maternal Factors

Maternal factors such as body weight have been shown to correlate with milk lipid concentration in early (20, 39, 156) and late lactation (after 6 months post-partum) (157, 158). In a recent systematic review of 63 datapoints, a meta-regression analysis demonstrated a positive association between maternal BMI and human milk fat (115). It has been hypothesized that while in early lactation fat stores accumulated during pregnancy are mobilized for milk production. Later in lactation, where fat accumulated during pregnancy is depleted, the effects of maternal weight (and blood triglyceride concentration) may become more apparent (157).

Maternal age was shown to affect milk volume, with an average fall of up to 40% in the yield of breastmilk from the age of 20 to 30 years and above, as reported in several studies (159–161). Dewey et al. (160), hypothesized that milk yield is dependent on the amount of functional breast tissue, which may decrease with age due to atrophy. Interestingly, maternal age was shown to affect fat concentration in colostrum, which was increased in mothers over 35 years old compared to younger mothers, but not in transitional or mature milk. Remarkably, maternal weight was similar between the groups leading to the hypothesis that changes in maternal metabolism with age may be linked to the observed results (162, 163). Other maternal factors such as tobacco smoking (164) was associated with a lower content of milk lipids, while no link between exercise (165) and maternal genetic factors on changes in milk fat concentration have been reported (166).

Proteins

In addition to stage of lactation, environmental influence is one of other key factors affecting proteins within the MFGM. As stated above, MFGM proteins showed various functions, such as immune defense, leading to health benefits that will be described further in following section. Therefore, fluctuations in MFGM immune-related proteins were observed as part of immune response during environmental challenges, e.g., bacterial infection. MFGM proteins that are up-regulated in response to such a challenge include those involved in host defense, inflammation, and oxidative stress. It should be noted that these observations are from other mammal species such as cows and sheep, however similar phenomena likely occur in human MFGM. Whether those changes have implications for the breast-feeding infant is not yet clear (73).

HEALTH BENEFITS OF THE MFGM

The health benefits of MFGM have recently been reviewed (167, 168). There is strong evidence to demonstrate several health benefits derived from the MFGM, including cognitive and immune function, gut health and maturation, metabolism (including cholesterol and insulin metabolism) and even skin health. Some of these benefits, such as mobility, have been investigated exclusively in the context of aging humans, with a focus on the potential development of functional foods to enhance the health of aging population. There are several benefits of relevance for the growing infant, including immune and cognitive development and function, and gut maturation and health.

Most of evidence suggesting health benefits of the MFGM come from preclinical and clinical studies testing the effects of purified MFGM components or ingredients from bovine milk. Commercially available MFGM ingredients are extracted from bovine buttermilk, beta serum or whey producing products with different total composition (lactose, protein, ash, total lipids and phospholipids) and the distribution of phospholipid species (**Table 5**). Although beta serum provides the highest concentration of phospholipids (≥14%), whey offers a high concentration of phospholipids (7.5%) to be used as supplement and provides an excellent source of protein (73%), especially for infant formula supplementation. The profile of proteins in MFGM-enriched dairy products are not usually described in product label information, however, it is likely that products sourced from cream, beta serum and buttermilk contain MFGM protein as well as other components like gangliosides, cholesterol, lactoferrin, sialic acid and IgG.

Most proteins in human and bovine MFGM are from the same classes such as BTN, ADPH, FABP, MUC1, XDH and lactadherin (MFGE8). BTN was shown to be the most abundant protein, in both bovine (24.8%) and human (16.3%) MFGM (71). The main difference between human and bovine MFGM proteins is that human MFGM has a higher level of proteins involved in immune response and in lipid catabolism than bovine MFGM (169). Human MFGM, for example, is enriched in immunoglobulins whereas bovine MFGM is enriched in antimicrobial proteins (131). However, the MFGM proteome and protein functionalities between the two species are mostly similar suggesting that MFGM proteins could have a positive impact in infant health such as anti-adhesive and antimicrobial functions (169).

The lipid profile and the distribution of phospholipid species of bovine MFGM enriched ingredient and HM are described in **Table 5**. The large variation found in lipid composition in mature HM demonstrate the potential of this ingredients to be tailored to supplement IF with polar lipids, closing the gap between breast milk and infant formula composition. Although it is important to note that the phospholipid fatty acid profile differ between human and bovine milk (170), the tolerance and beneficial effects of dietary bovine milk polar lipids has been described in many clinical studies, recently reviewed by Brink and Lonnerdal (167).

In the following sections, we briefly summarize the key evidence of the health benefits of MFGM (including mechanistic studies in animals, and studies in human populations) and discuss how changes in specific components across lactation may reflect the particular role of these components in the health of the growing infant.

Cognitive Function

Cognitive function is the most widely studied potential benefit of the MFGM, because of the rapid cognitive development that occurs during early human life (in particular gestation and lactation) and the consequent influence on cognitive function throughout life. So far, five clinical trials have been published reporting the effects of dietary bovine MFGM or MFGM components (through supplemented infant formula) on neurological development (171–175). Although it is generally acknowledged that more research is needed in infants, the evidence supports the hypothesis that there are cognitive (173, 176), neurodevelopmental (171, 172, 174, 177–179) and vision functional (175) benefits from MFGM for infants. This evidence is also supported by pre-clinical studies where individual components of MFGM, or diets enriched with bovine MFGM derived product were fed to various animal models.

Gangliosides and sialic acid may be the active components within the MFGM mediating cognitive effects, possibly by ensuring sufficient amounts of these nutrients are available for the developing brain (76, 180, 181), although there is also evidence that these compounds when supplied exogenously may influence growth signaling in the brain leading to improvements in learning and (spatial) memory outcomes (76, 177, 182, 183). It is well established that there are key periods during human brain development when there is rapid accumulation of particular lipids, for example gangliosides in the forebrain at 32 weeks of gestation and plasmalogens in both cerebrum and cerebellum from 32 weeks of gestation to 6 months of age and beyond (184). Both plasmalogens and gangliosides are components of the MFGM, associated with essential processes [myelination and synaptic development, respectively (184)] for appropriate growth and development of the brain.

Phospholipids, especially choline (which is found attached to the phosphate group of PC) sourced during pregnancy were associated with improved infant cognitive scores (176) and neurodevelopmental outcomes (178, 179). Choline is a nutrient

TABLE 5 | The composition of commercially available MFGM-enriched dairy ingredients compared to mature human milk.

	Bovine MFGM enriched ingredient			Mature human milk
Concentration in product (g/100 g)	**Buttermilk[#]**	**Beta serum[#]**	**Whey[#]**	
Lactose	± 50	≤10	≤3	
Protein (N × 6.38)	≥30	>52	73	
Ash	≤9	≤6	≤3	
Total lipids	5–13	3–27	12–26	4 (range 2.0–6.1)
Total phospholipids (PL) (g/100 g of fat)*	1.6–22	≥14	5–16	0.5–1
Phospholipids (% of total PL)				
Phosphatidyl ethanolamine (PE)	35–43	22–29	19–41	12–36
Phosphatidyl choline (PC)	19–32	27–47	19–25	13–34
Phosphatidyl serine (PS)	8–18	1.2–23	8–12	4–16
Phosphatidyl inositol (PI)	4–9	1–8	3.6–7	3–12
Sphingomyelin (SM)	11–19	14–27	16–24	28–41
Gangliosides (g/100 g of fat) *	NR	NR	NR	0.02–0.05
Cholesterol (g/100 g of fat) *	NR	NR	NR	0.2–0.48

[*]Based on the variation of milk fat content average of (4 g/100 mL), phospholipid (23–43 mg/100 mL), gangliosides (0.7–2.0 mg/100 mL) and cholesterol (11–19 mg/100 mL) in mature milk described in this review. [#]Data compiled by Fontecha et al. (168).

that affects DNA methylation, long term potentiation and neural cell populations in the hippocampus as demonstrated in fetal rats (185–187). Its role in postnatal brain development is assumed to be equally important as postnatally, as its concentration in human milk was shown to be 15 times higher than in maternal blood (188).

PUFAs, in particular ARA and DHA, have been extensively studied for their impact on brain development. Pre- and postnatal development of infant brain and retina, for example, require a rapid accumulation of long-chain PUFAs (189). Healthy brain tissue consists of about 60% structural fat; of this, about 25% is DHA and 15% ARA (190, 191). Postnatally ARA and DHA are supplied mostly by human milk and, to some extent, by the infant's adipose tissue (192) influencing the PUFA profile of infant blood and tissue (193). ARA and DHA can be synthesized by chain elongation and desaturation of essential FAs, such as LA (C18: 2n-6) and ALA (C18: 3n-3), however, in infants, due to low enzymatic activity, this synthesis is very low (194) and influenced by genetic heritability (195). Thus, dietary intake of ARA and DHA are essential for infant's brain development.

It is important to understand the relevance of changes in components such as these across lactation for cognitive development, as these changes may reflect particular times at which these components may influence infant development.

Immune Function

Although not as well researched as the impact on cognitive function, there have been reports on the effects of MFGM components on immune function. Human studies have largely been in infants and relate to prevention from infection by micro-organisms (both bacteria and viruses) (196–198), with a large body of *in vitro* and animal studies investigating mechanisms of action and potential active molecules (199–202). In one clinical trial, bovine MFGM supplementation in infant formula was found to reduce febrile episodes in infants and young children and the number of days with fever among 2.5 to 6 year old children (203). Dietary supplementation of bovine milk complex milk lipids (197) or whey-derived MFGM (protein rich) (196), lowered the duration (197) and/or incidence of diarrhea (196) in infants. Reduction of the incidence of respiratory illness was also reported as a result of bovine MFGM supplementation in infants (174).

Much of this research relates to the ability of a range of MFGM components such as gangliosides (204, 205), sialic acid (206), proteins [butyrophilin, lactadherin, and fatty acid binding protein (207)], and glycoproteins to act as decoys for pathogens and therefore prevent infection. The reducing effects of MFGM on the expression of *E. coli* virulence gene (202) and the ability to bind to human epithelial cells (208) were suggested as a possible mechanism to prevent infection. However, there is also good evidence of other mechanisms, including neutralizing viral and bacterial toxins [gangliosides (209)], direct toxicity to the invading organisms, or prevention of growth and invasion [XDH (210, 211), MUC 1 and 4 (212)]. The lipid component of the MFGM and its digestion products were shown to have bactericidal activity against rotavirus (213) and food borne pathogens (214).

There is also evidence of MFGM components having immune-modulatory effects such as influencing cytokine production and macrophages (215, 216), although these effects are not as well characterized as those relating to protection from pathogens.

Gut Maturation and Gut Health

Studies have shown that MFGM components may have an impact on the development of the gut epithelium and immune system, however, these findings largely come from pre-clinical studies in animal models, in particular pigs, rats, and mice during the pre-weaning period, which is critical for gut maturation.

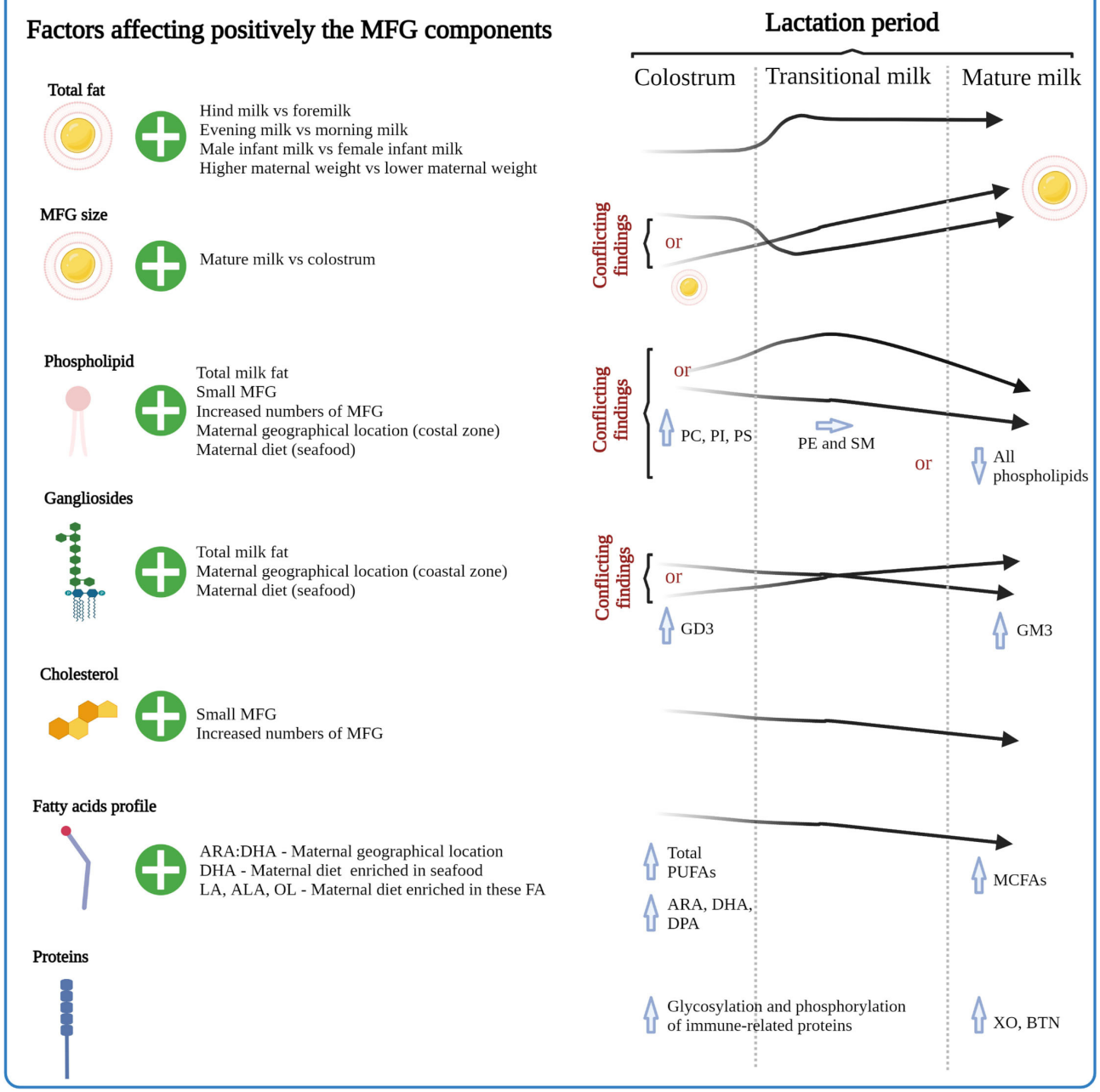

FIGURE 7 | Summary of the factors affecting MFG composition emphasizing the effects of lactation period. MFG, milk fat globule; ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ALA, α-linolenic acid; LA, linoleic acid; OL, oleic acid; XO, xanthine oxidase; BTN, butyrophilin.

Intestinal maturation being influenced by dietary bovine MFGM was shown in piglets and rats, with specific effects being improvement of intestinal morphology, increased enzymatic activity, and reduction of the proportions of pathogenic bacteria (78, 217). *In vivo* studies showed that MFGM components purified or extracted from bovine milk protect the gut from injury [for example by carcinogens (199) and bacterial lipopolysaccharide (LPS) (218)]. These results also suggest there is a role for MFGM on gut maturation. Phospholipids in particular were shown to protect the gut from injury in a dextran sodium sulfate challenge model (213) and reduced the depletion of goblet cells by decreasing the overactivated Notch signaling

pathways (219). In a lipopolysaccharide challenge model, bovine MFGM supplementation to suckling mouse pups decreased epithelium injury, inflammatory cytokines and increased the expression of gap junction proteins (220–222). The MFGM protein lactadherin was shown to support wound healing by binding to intestinal cell *in vitro* (223). These studies indicate that MFGM components are able to support the development of the infant intestine by directly strengthening, protecting and up regulating the intestinal barrier.

As summarized by Rueda (79), dietary gangliosides may play various roles relating to gut development and health, including modifying the microbiota, influencing the development of the gut immune system, and modulation of oral tolerance during early life. Overall, they appear to promote gut immunity development in the neonate, and consequently play a role in the prevention of infections during early infancy. Other MFGM components also clearly have an impact on gut maturation, affecting factors such as villus height, cell maturation, and gut enzyme activity (for example, lactase) (224).

Gut Microbiota

In addition to being important in gut maturation, barrier function and resilience and modulation of inflammation, the MFGM appears to have beneficial effects through promotion of a beneficial gut microbiota (78). To date, only one clinical study showed direct effect of bovine whey-derived MFGM on the oral microbiome of formula fed infants, with the species *Moraxella catarrhalis* being significantly reduced in the supplemented group (225). Later, the same group published data on the fecal microbiome and metabolome of infant fed formula supplemented with MFGM, standard formula or breast milk, as a reference (226). The effect of MFGM on fecal microbiota was moderate and did not override the effect of formula. Much of this research has used a fairly general MFGM preparation from bovine milk, or even a mixture of MFGM with other compounds (probiotics, prebiotics and lactoferrin) (217, 227, 228) which makes it difficult to isolate the effect of MFGM on the microbiota. The effects of polar lipids, such as phospholipids (229) and gangliosides (230) on the gut microbiota has been demonstrated.

In one *in vivo* study, artificially reared newborn rats supplemented with bovine whey-derived MFGM had similar microbiota to the dam-reared pups compared to non-supplemented pups (78). Another study found that feeding formula with bovine MFGM to piglets decreased the proportions of *Firmicutes* and increased *Proteobacteria* and *Bacteroides* in the gut compared to piglets fed formula with vegetable oils. The effects of undigested MFGM *in vitro* have been demonstrated (231) suggesting that MFGM components may play a role on the infant's gut microbiota development. There is a need for further research to more clearly identify the specific MFGM components that may confer these benefits.

Metabolic Health

Intake of milk sourced cholesterol in early life was shown to correspond to infants' serum cholesterol levels, which are high in breast-fed compared to formula-fed infants (232). Higher levels of serum cholesterol were shown to prevent cardiovascular diseases in adult life (121, 233) by downregulating hepatic hydroxymethyl glutaryl coenzyme A reductase *via* epigenetic modifications (234).

CONCLUSIONS

Although the body of literature describing compositional analysis of MFGM components within HM is not standardized in terms of methodology, the results do show certain patterns. For example, the relative concentration levels of MFGM-specific phospholipids, gangliosides, cholesterol, FAs and proteins appear to alter over the course of lactation, and such changes are likely to reflect the changing requirements of the growing infant (review summary, **Figure 7**). There is also evidence that factors such as maternal diet and geographical location can influence certain aspects of HM MFGM composition.

Although the majority of the research has been conducted using MFGM materials derived from bovine milk and employs animal models, the body of evidence for specific health benefits of MFGM components is increasing, and clearly demonstrates that in addition to its role in encapsulating and delivering lipids within milk, the MFGM also contains a range of components that have important implications for the health of the growing infant. Health outcomes include cognitive development, intestinal development and function, and immune health.

The small number of clinical trials in which infant formula products have been tested provide some evidence that the inclusion of MFGM, or components such as gangliosides, in infant formula has benefits for the health of the growing infant. Although current infant formula products are adequate to support growth and development, comparisons with breast-fed infants suggest that these formula products are not optimal, in particular regarding cognitive outcomes. The data from *in vivo* studies and clinical trials, suggest that the inclusion of MFGM or components within it in infant formula is important to ensure optimal cognitive, immune, and intestinal development and function.

Collectively, the information presented here suggests that infant formula products require further development to mimic the composition of HM more closely, including the MFGM. The production of MFGM ingredients to supplement infant formulations could be tailored to meet the different stages of lactation, or even the different maternal geographical locations. This will ensure that those mothers for whom breast feeding is not possible can have the best possible alternative to their growing infant. To enable this, further work is required to fully understand patterns of MFGM components across lactation, and their efficacy in supporting the health of the infant at different stages of growth and development.

AUTHOR CONTRIBUTIONS

CT and MB wrote the manuscript. LD, IS, FL, and YY critically revised the manuscript. All authors

have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Body Composition in Preschool Children and the Association With Prepregnancy Weight and Gestational Weight Gain: An Ambispective Cohort Study

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Objective: To describe the body composition in preschool children and to evaluate the association with prepregnancy BMI and gestational weight gain (GWG).

Methods: Children were recruited in their first year in kindergarten (3 years old) and followed up for the next 2 years. Information during pregnancy and birth was retrieved from medical records. Height, weight, fat mass, fat-free mass, and percentage of body fat (FM%) were measured through a bioelectrical impedance analysis for each child visit, and BMI, fat mass index (FMI), and fat-free mass index (FFMI) were calculated. Generalized linear mixed models (GLMMs) were used to evaluate the associations between prepregnancy weight, GWG, and adiposity indicators.

Results: A total of 3,329 single-birth 3-year-old children were recruited as the baseline population and were followed at 4 and 5 years old. During the 3 years of follow-up, the mean (±SD) values of BMI, FMI, FFMI, and FM% of the children were 15.6 (±1.6) kg/m^2, 2.8 (±1.3) kg/m^2, 12.8 (±0.7) kg/m^2, and 17.2% (±5.8%), respectively. The prevalence rates of overweight and obesity in mothers before pregnancy were 16.6 and 3.2%, respectively. Mothers were divided into three groups based on GWG: appropriate (1,233, 37.0%), excessive (767, 23.0%), and insufficient (1,329, 39.9%). GLMMs analyses showed that the preschool children's BMI, FMI, FFMI, and FM% were all significantly positively related to maternal prepregnancy BMI and GWG (all $P < 0.001$); the children of mothers who were overweight/obese before pregnancy were more likely to be overweight/obese, high FMI, high FFMI, and high FM% at preschool age (all $P < 0.001$); although maternal excessive GWG was not correlated with offspring's overweight/obese ($P = 0.156$), the children of mothers with excessive GWG are more likely to have higher FMI, but not to be with a higher FFMI status than the children of mothers with appropriate GWG. For prepregnancy overweight/obese women, compared with the GWG-appropriate group, maternal excessive GWG was related to the risk of high FMI (coefficient = 0.388, 95% CI: 0.129–0.647) and high

FM% (coefficient = 0.352, 95% CI: 0.097–0.607), but was not related to the risk of overweight/obese or high FFMI of the offspring at preschool age.

Conclusion: Fat mass index decreased with age, while FFMI increased with age among 3- to 5-year-old children. It is necessary to optimize maternal weight prior to conception and GWG management to improve the health outcomes of the offspring.

Keywords: body composition, body fat, obesity, cohort study, gestational weight gain, prepregnant BMI, preschool children child adiposity, gestational weight

INTRODUCTION

The worldwide prevalence of overweight and obesity has approximately doubled since 1980 to the extent that over one-third of the world's population is now classified as overweight or obese (1). According to a prediction, 57.8% of the world population will be overweight or obese by 2030 if current trends continue (2). The prevalence of overweight/obesity in Chinese adults has increased linearly (3). The course of obesity can begin very early, especially *in utero* and in the first 2 years of life (4, 5). The prevalence of obesity among Chinese preschool children increased continuously from 1986 to 2016, especially after 3 years of age (6).

Body mass index (BMI) is a widely used indicator to evaluate general adiposity, but its use is limited because it does not discriminate for body composition (7). Parental environmental factors affect the health and chronic disease risk of people throughout their lives (8–10). An increased prevalence of prepregnancy obesity and excessive gestational weight gain (GWG) is associated with a child's weight status or other components of metabolic disease both in childhood and adulthood, and it is time to redirect efforts and optimize maternal weight prior to conception (11–13).

Previous studies that evaluated the consequences of prepregnancy BMI or GWG for the child mostly focused on birth outcomes or the health outcomes at a single age point, and few studies followed up on the health conditions of offsprings at multiple time points, especially lack comprehensive assessment of the children's growth and development, including not only anthropometric indicators but also multi-time evaluation of body composition. Studies with larger sample sizes and an extended follow-up period are also warranted to generate stronger evidence and to evaluate the influence of prepregnancy weight status and GWG on the long-term body composition of children.

This study prospectively followed preschool children in kindergartens for 3 years between the ages of 3 and 5 and linked their mothers' medical records during pregnancy. This ambispective cohort study aimed to describe the body composition of preschool children and to evaluate whether and how prepregnancy weight status and GWG affect the body composition of 3- to 5-year-old children.

MATERIALS AND METHODS

This study was carried out by the Tianjin Women's and Children's Health Center and Capital Institute of Pediatrics in Tianjin from 2017 to 2020 and was approved by the IRB of Tianjin Women's and Children's Health Center (BGI-IRB 17116-201711). Parental written consent forms were obtained when the children were recruited in junior class.

Participants

In general, children enter kindergarten at 3 years old in China, and they usually stay there for 3 years (junior, middle, and senior class) before school. Children were recruited at kindergarten junior classes with informed consent obtained from their parents, and their mothers' information during pregnancy was matched by retrieval from medical records. The exclusion criteria for the children were as follows: (1) any condition or chronic diseases or use of any drug known to affect growth and development; (2) acute diseases that prohibit children from participating in the physical examination; (3) twins or other multiple births; (4) mothers without GWG records in the antenatal healthcare system.

By using a stratified cluster sampling method, 11 districts (including 6 central urban districts, 4 loop urban districts, and 1 suburban district) were selected from the 16 municipal districts in Tianjin, the city where the prevalence of obesity in school-aged children is the highest in China. Then, 42 state-owned kindergartens were selected from 11 districts starting in 2017. Children in the junior classes of the 42 kindergartens were recruited into the cohort study, which has good population representativeness of the preschool children in Tianjin, and they were followed up and completed physical examinations in junior, middle, and senior classes from September 2017 to September 2020. Their mothers' clinical information on antenatal and postnatal healthcare was collected from the Tianjin Hospital healthcare system, including anthropometry results collected at each follow-up phase.

Measurements of Gestational Weight

Prepregnancy weight and height were self-reported and registered at the first visit during the 1st trimester. Maternal gestational weight was measured at antenatal clinics during each visit. The median number of repeat weight measurements per woman was 7, and P_{25}–P_{75} was 6–8 times. GWG was calculated as weight at the last visit (within 1 week of delivery) minus the self-reported weight for the first visit at the antenatal clinic.

Anthropometric Measurements of Children

For each child followed up in kindergarten, height was measured without shoes at each visit. Bodyweight, fat mass, fat-free mass, and percentage of body fat mass (FM%) were measured by trained nutritionists through bioelectrical impedance analysis (see higher BAS-H, China), which measured the impedances of the body and each limb in the standing position and is suitable for children aged 3 years and older, at frequencies of 1 kHz, 5 kHz, 50 kHz, 250 kHz, 500 kHz, and 1 MHz. Children were required to be on fasting and to have an empty bladder. When in the measurement, children in light clothing stood on the platform without shoes, and held both hands at a 45-degree angle away from the body; four tactile electrodes were in contact with the palm and thumb of both hands, and the other four were in contact with the anterior and posterior aspects of the sole of both feet. BMI, fat mass index (FMI), and fat-free mass index (FFMI) were calculated for each subject as body weight, fat mass, and fat-free mass in kilograms divided by height in meters squared, respectively.

Classification of Health Outcomes and Conditions

GWG was classified as insufficient, appropriate, or excessive according to the 2009 Institute of Medicine (IOM) criteria (14). Prepregnancy weight status was defined as two groups (BMI $<$ 25 kg/m^2 group and $\geq$ 25 kg/m^2 overweight/obese group).

The Chinese gender-specific and age-specific BMI cutoffs for overweight and obesity for children from 2 to 18 years (15) were used to define preschool children as having normal weight, overweight, or obesity. The BMI cutoffs were established based on representative data from two national representative cross-sectional surveys in China: The National Growth Survey of Children under 7 years of age in the nine cities of China in 2005 and The Physical Fitness and Health Surveillance of Chinese School Students in 2005, with estimates of L, M, and S parameters, values of percentile and Z-score calculated. High FMI was defined as an age- and gender-specific Z-score of FMI at each follow-up $\geq$1 during the 3 years; low FFMI was defined as an age- and gender-specific Z-score of FFMI at each follow-up <-1 during the 3 years; and high FM% was defined as an age- and gender-specific Z-score of FM% at each follow-up $\geq$1 during the 3 years.

Statistical Analyses

Data analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, Illinois) and R software (version 4.1.2). Comparisons of characteristics were conducted using one-way ANOVA or chi-square tests. The loess smooth curve of body composition indicators (95% CIs) with age during the 3-year follow-up was developed. Generalized linear mixed models (GLMMs) (16, 17) were used to evaluate the associations between prepregnancy weight, GWG, and the adiposity indicators, with a linear regression link or a binary logistic regression. Considering potential confounding factors (12), gestational week, parity, children's age, and gender, whether exclusive breastfeeding within 6 months of birth, annual family income, maternal educational level, paternal educational level, maternal occupation, and paternal occupation were adjusted in the GLMMs.

TABLE 1 | Characteristics of children at junior class with and without GWG data that as participants and non-participants in the follow-up study.

	Participants (n = 3,329)	Non-participants (n = 419)	P-value
Age, mean (SD), y	3.79 (0.3)	3.78 (0.31)	0.450
Male, n (%)	1,732 (0.52)	282 (0.53)	0.737
Weight, mean (SD), kg	16.53 (2.31)	16.55 (2.58)	0.837
Height, mean (SD), cm	102.77 (4.51)	102.46 (4.43)	0.186
BMI, mean (SD), kg/m^2	15.6 (1.43)	15.7 (1.53)	0.185
FMI, mean (SD), kg/m^2	2.87 (1.15)	2.84 (1.2)	0.623
FFMI, mean (SD), kg/m^2	12.73 (0.73)	12.86 (0.76)	0.001
Overweight, n (%)	408 (0.13)	49 (0.13)	0.803
Obese, n (%)	177 (0.06)	27 (0.07)	0.353

BMI, body mass index; FMI, fat mass index; FFMI, fat free mass index.
Overweight and obesity was defined according to the Chinese gender-specific and age-specific BMI cutoffs for overweight and obesity for children from 2 to 18 years of age.

RESULTS

Participants and Cohort Follow-Up

A total of 3,822 children were recruited in their kindergarten junior class and completed physical examinations, including body composition analysis, from October 2017 to October 2018. Excluding 74 twins or other multiple-birth children and 419 mothers who had missing GWG data in medical records, 3,329 single-birth children had complete maternal GWG data in the antenatal healthcare system, and they provided the cohort baseline data. In total, 3,329 children remained participants and the other 419 children were non-participants. There were no significant differences in demographic and growth parameters between the participant and non-participant groups in the kindergarten junior class except that the FFMI showed a slight difference (12.73 vs. 12.86 kg/m^2, **Table 1**). When the children were followed up through kindergarten middle class, 144 of them were not approached, 26 of them did not have height and weight examinations, and 3,159 remained in the study. In the senior kindergarten class, 2,382 children were followed up and had complete adiposity indicator data (**Figure 1**). A total of 144 and 777 children were not approached during the middle and senior years because they did not continue staying in the kindergartens.

Characteristics of the Participants

During the 3 years of follow-up, the mean ($\pm$SD) values of BMI, FMI, FFMI, and FM% of the children were 15.6 ($\pm$1.6) kg/m^2, 2.8 ($\pm$1.3) kg/m^2, 12.8 ($\pm$0.7) kg/m^2, and 17.2% ($\pm$ 5.8%), respectively. The change trends of adiposity indicators (95% CIs) with age during the 3-year follow-up are shown in **Figure 2**. FMI decreased with age, while FFMI increased with age among 3- to 5-year-old children.

As shown in **Table 2**, mothers were divided into three groups based on the 2009 IOM guidelines of GWG: appropriate (37.0%), excessive (23.0%), and insufficient (39.9%). Child gender and

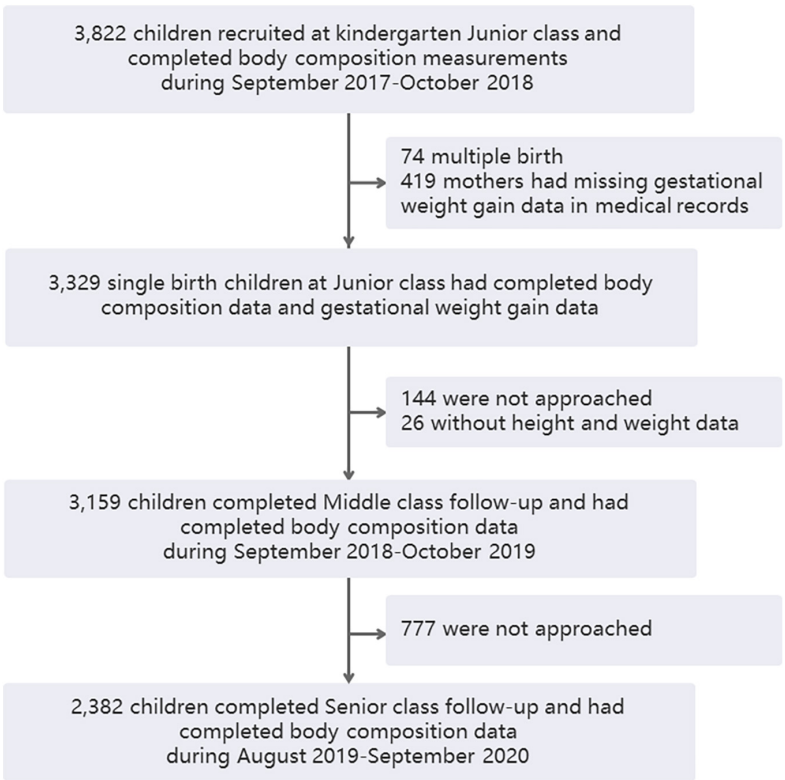

FIGURE 1 | Flowchart of the study cohort.

age during the 3-year follow-up were not significantly different between the prepregnancy BMI <25 kg/m^2 and ≥25 kg/m^2 groups. Parity, child gender, gestational week, and age at kindergarten during the 3-year follow-up were not significantly different among the three different GWG groups.

For maternal characteristics, the mean prepregnancy BMI was 22.5 (*SD* = 5.8) kg/m^2, and the prevalence rates of overweight and obesity of mothers before pregnancy were 16.6 and 3.2%, respectively.

Of the children during the 3-year follow-up in kindergarten, BMI, FMI, FFMI, and FM% were all significantly different between the prepregnancy BMI <25 kg/m^2 and ≥25 kg/m^2 groups, and these indicators were also significantly different among the three GWG groups (all $P < 0.001$). The chi-squared test showed that the prevalence of overweight and obesity during the three years were all significantly different between the prepregnancy BMI <25 kg/m^2 and ≥25 kg/m^2 groups (all $P < 0.001$), and the chi-squared test showed that these indicators increased with increasing GWG status (all $P_{fortrend} < 0.001$).

Associations Between Prepregnancy BMI, Gestational Weight Gain, and Adiposity Indicators in Kindergarten Children

Adjusted by gestational week, parity, age and gender of children, annual family income, exclusive breastfeeding within 6 months of birth, maternal educational level, paternal educational level, maternal occupation, and paternal occupation, GLMMs analyses with a linear regression link between target distribution and relationship showed that the adiposity indicators of the offspring were all significantly positively related to maternal prepregnancy BMI and GWG (all $P < 0.001$) (**Table 3**).

Associations Between Prepregnancy Weight Status, Gestational Weight Gain Groups, and Adiposity Classification Indicators

After the diagnosis of the adiposity indicators, GLMMs analyses with a binary logistic regression link between target distribution and relationship were executed. Compared to the children of mothers with appropriate GWG, insufficient GWG was a protective factor against overweight/obesity, high FMI, and high FM%, but they were more likely to have a low FFMI at preschool age (all $P < 0.01$). Although excessive maternal GWG was not correlated with offsprings' overweight/obese ($P = 0.156$), the children of mothers with excessive GWG were more likely to have a higher FMI (coefficient = 0.179, 95% CI: 0.015–0.342) but not correlated with the possibility of having a higher FFMI (P = 0.064) compared with the children of mothers with appropriate GWG. Compared with the children of prepregnancy BMI <25 kg/m^2 mothers, the children of mothers who were overweight/obese before pregnancy were more likely to be overweight/obese

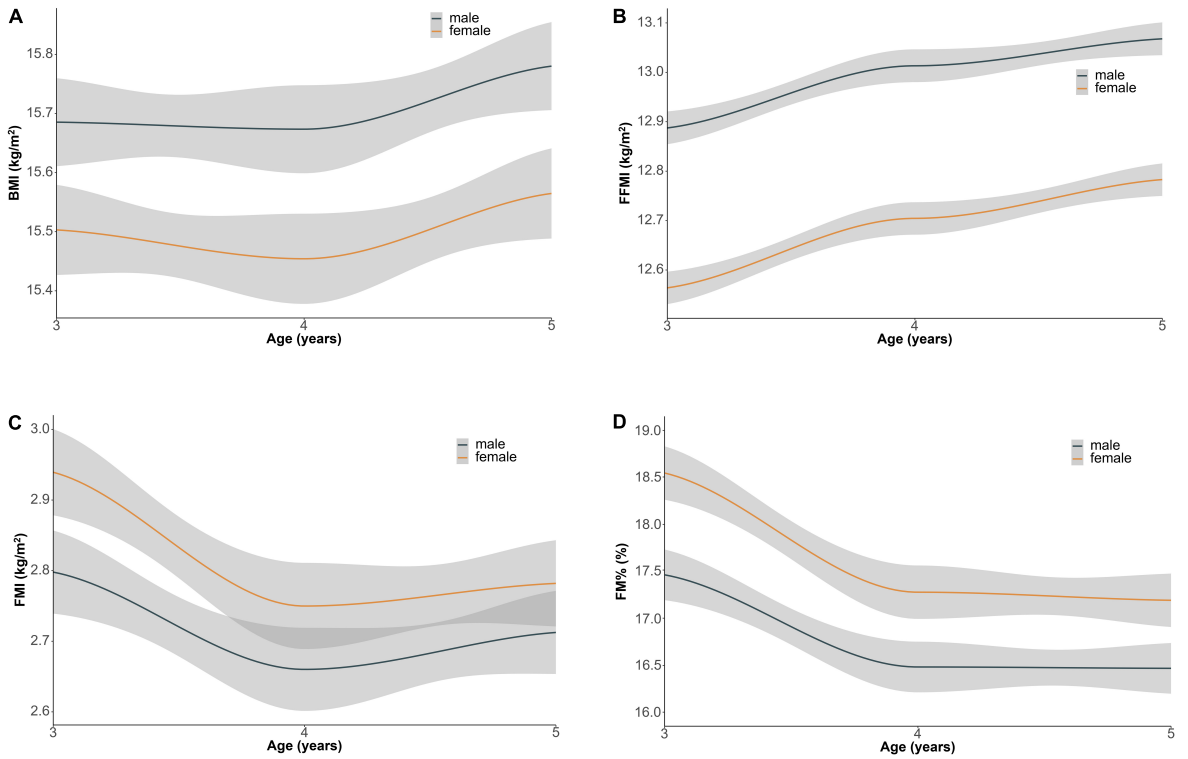

FIGURE 2 | Loess smooth curve of body composition indicators (95% CIs) with age during 3-year follow-up. **(A)** BMI, **(B)** FFMI, **(C)** FMI, and **(D)** FM%. BMI, body mass index; FFMI, fat free mass index; FMI, fat mass index; FM%, percentage of body fat mass.

(coefficient = 0.779, 95% CI: 0.647–0.912), and have a high FMI/high FFMI/high FM% at preschool age (coefficient: 0.755, 0.563, 0.658, respectively, and all $P < 0.001$) (**Table 4**).

Stratified by prepregnancy weight status (BMI $<$ 25 kg/m^2 and $\geq$ 25 kg/m^2), associations between GWG groups and the adiposity indicators are shown in **Table 5**. For prepregnancy BMI $<$25 kg/m^2 women, compared to the GWG-appropriate group, maternal excessive GWG was not related to the risk of overweight/obesity, high FMI, or high FM% of the offspring but was related to high FFMI (coefficient = 0.248, 95% CI: 0.048–0.447). GWG insufficiency was a protective factor against overweight/obesity, high FMI, and high FM%, but it was a risk factor for low FFMI (coefficient = 0.238, 95% CI: 0.095–0.382). For prepregnancy overweight/obese women, compared with the GWG-appropriate group, maternal excessive GWG was not related to overweight/obese or high FFMI of the offspring at preschool age but was related to the risk of high FMI (coefficient = 0.388, 95% CI: 0.129–0.647) and high FM% (coefficient = 0.352, 95% CI: 0.097–0.607), which provides supporting evidence for the association between maternal weight and offsprings' body composition at preschool age.

DISCUSSION

A meta-analysis (18) demonstrated that 27.8% of women had insufficient GWG and 39.4% had excessive GWG in the global population compared with the meta-analysis results in an Asian population. The total prevalence of insufficient GWG in our study was similar to that of an Asian population (39.9 vs. 35.6%), and the rate of excessive GWG in our sample was higher than that in the Asian population (23.0 vs. 16.8%).

A systematic review showed that prepregnancy maternal overweight is associated with higher offspring adiposity, and the relationship between maternal gestational weight gain and offspring lean mass or fat-free mass was not consistent (12). Based on the 3-year follow-up of the offspring, the present cohort study illustrated that both prepregnancy status and maternal GWG were related to preschool child adiposity. Overall, BMI, FMI, FFMI, and FM% in preschool children increased with increasing prepregnancy BMI and GWG. Preconception care offers a unique opportunity to address the pressing public health goal of reducing pregnancy-related morbidity and mortality (19). Women who plan to become pregnant should, before becoming pregnant, receive care that includes lifestyle changes, such as smoking cessation, guidance on exercise habits, dietary intake, and the use of vitamins and supplements. Preconception assessment of nutritional status should identify those individuals who are underweight or overweight (20). Our results also indicated that women with overweight/obesity before pregnancy should pay more attention in maintaining appropriate weight gain during pregnancy. Because excessive maternal GWG increased the risks of high FMI in offspring at preschool age, but is not related to classified high FFMI status,

TABLE 2 | Characteristics of the mothers and their preschool offspring in different groups (n = 3,329).

	Prepregnancy BMI		*P*-value*	Gestational weight gain			*P*-value*
	<25 kg/m^2 (n = 2,670)	≥25 kg/m^2 (n = 659)		Insufficient (n = 1,329)	Appropriate (n = 1,233)	Excessive (n = 767)	
	Mean (SD)/n (%)	Mean (SD)/n (%)		Mean (SD)/n (%)	Mean (SD)/n (%)	Mean (SD)/n (%)	
Mother characteristics							
Pre-pregnancy BMI (kg/m^2)	21 (2.1)	27.7 (2.4)	<0.001	21.5 (2.9)	22.4 (5.3)	24.2 (9.1)	<0.001
Gestational weight gain (kg)	12.2 (4.0)	10.7 (4.4)	<0.001	8.4 (2.3)	12.6 (2.3)	16.7 (3.4)	<0.001
Parity							
1	2,345 (81.6)	530 (18.4)	<0.001	1,130 (39.3)	1,071 (37.3)	674 (23.4)	0.130
2	317 (71.9)	124 (28.1)		196 (44.4)	157 (35.6)	88 (20.0)	
3	8 (61.5)	5 (38.5)		3 (23.1)	5 (38.5)	5 (38.5)	
Total	2,670 (80.2)	659 (19.8)		1,329 (39.9)	1,233 (37.0)	767 (23.0)	
Children characteristics							
Gender							
Male	1,412 (81.5)	320 (18.5)	0.047	688 (51.8)	660 (53.5)	384 (50.1)	0.312
Female	1,258 (78.8)	339 (21.2)		641 (48.2)	573 (46.5)	383 (50.0)	
Total	2,670 (80.2)	659 (19.8)		1,329 (39.9)	1,233 (37.0)	767 (23.0)	
Gestational weeks (week)	39 (1.3)	38.9 (1.5)	0.003	39.0 (1.5)	39.0 (1.3)	39.0 (1.4)	0.694
Age 3-at Kindergarten Junior class							
Age (years)	3.8 (0.3)	3.8 (0.3)	0.608	3.8 (0.33)	3.8 (0.3)	3.8 (0.3)	0.973
FM% (%)	17.6 (5.4)	19.6 (6.0)	<0.001	17.3 (5.4)	18.2 (5.5)	18.9 (5.9)	<0.001
FMI (kg/m^2)	2.8 (1.1)	3.2 (1.3)	<0.001	2.7 (1.05)	2.9 (1.5)	3.1 (1.3)	<0.001
FFMI (kg/m^2)	12.7 (0.7)	12.9 (0.8)	<0.001	12.7 (0.73)	12.8 (0.7)	12.8 (0.8)	<0.001
BMI (kg/m^2)	1.6 (15.5)	1.3 (16.2)	<0.001	15.4 (1.3)	15.7 (1.5)	15.9 (1.6)	<0.001
Overweight	284 (10.6)	124 (18.8)	<0.001	123 (9.3)	170 (13.8)	115 (15.0)	<0.001
Obese	103 (3.9)	74 (11.2)		45 (3.4)	71 (5.8)	61 (8.0)	<0.001
Age 4-at Kindergarten-Middle class							
Age (years)	4.6 (0.3)	4.6 (0.3)	0.821	4.6 (0.3)	4.6 (0.3)	4.6 (0.3)	0.376
FM% (%)	16.4 (5.6)	18.8 (6.8)	<0.001	16.1 (5.6)	17 (6.0)	17.9 (6.2)	<0.001
FMI (kg/m^2)	2.6 (1.2)	3.2 (1.5)	<0.001	2.5 (1.2)	2.7 (1.3)	2.9 (1.4)	<0.001
FFMI (kg/m^2)	12.8 (0.7)	13.1 (0.7)	<0.001	12.8 (0.7)	12.9 (0.7)	13.0 (0.8)	<0.001
BMI (kg/m^2)	15.4 (1.5)	16.2 (1.9)	<0.001	15.3 (1.5)	15.6 (1.7)	15.9 (1.7)	<0.001
Overweight	267 (10.6)	121 (19.2)	<0.001	124 (9.9)	146 (12.6)	118 (15.9)	<0.001
Obese	141 (5.6)	98 (15.6)		58 (4.6)	92 (7.9)	89 (12.0)	<0.001
Age 5-at Kindergarten-Senior class							
Age (years)	5.6 (0.3)	5.6 (0.3)	0.181	5.6 (0.3)	5.6 (0.3)	5.6 (0.3)	0.832
FM% (%)	15.9 (6.3)	19.6 (8.5)	<0.001	15.8 (6.5)	16.7 (6.9)	18.1 (7.7)	<0.001
FMI (kg/m^2)	2.6 (1.4)	3.5 (2.1)	<0.001	2.5 (1.4)	2.8 (1.5)	3.1 (1.9)	<0.001
FFMI (kg/m^2)	12.9 (0.8)	13.2 (0.9)	<0.001	12.9 (0.8)	13.0 (0.8)	13.1 (0.9)	<0.001
BMI (kg/m^2)	15.5 (1.8)	16.7 (2.5)	<0.001	15.4 (1.8)	15.7 (2.0)	16.2 (2.3)	<0.001
Overweight	187 (11.0)	74 (17.9)	<0.001	83 (9.8)	106 (13.6)	72 (14.7)	<0.001
Obese	117 (6.9)	84 (20.3)	<0.001	51 (6.0)	77 (9.9)	73 (14.9)	<0.001

**One-way ANOVA or chi-square test.*
BMI, body mass index; FM%, percentage of body fat mass; FMI, fat mass index; FFMI, fat free mass index.

especially among prepregnancy overweight/obese mothers. This study demonstrated that maternal weight not only has an impact on the birth outcome but also influences the body composition of the offspring at preschool age. The results provided supporting evidence of the long-term impact of maternal weight and presented core component data for guiding clinical practice, including preconception care and prenatal care.

Excessive GWG is a risk factor for cesarean delivery, large for gestational age (LGA), and macrosomia (21). GWG has significant health implications for both the mother and the child, and it is one of the non-genetic-based associations between maternal and infantile excess body mass; this demonstrates an independent role in mother-to-child obesity transmission (22). Considering that this study has good population representativeness of preschool children in Tianjin, where the prevalence of obesity in school-aged children is the highest in China (23), more attention should be given, and increasingly effective interventions should be implemented on

TABLE 3 | Generalized Linear Mixed Models (GLMMs) associations between prepregnancy BMI, GWG and the adiposity indicators in kindergarten children.

Dependent variable	Main effect	Estimate	95%*CI*		S.E.	*t*-value	*P*-value	*F* value of the GLMM models	*P*-value of the GLMM models
			Lower	Upper					
BMI	Prepregnancy BMI	0.139	0.129	0.148	0.005	28.754	<0.001	22.030	<0.001
	GWG	0.042	0.035	0.050	0.004	10.557	<0.001		
FMI	Prepregnancy BMI	0.094	0.086	0.102	0.004	24.047	<0.001	15.918	<0.001
	GWG	0.027	0.021	0.033	0.003	8.279	<0.001		
FFMI	Prepregnancy BMI	0.046	0.042	0.050	0.002	20.670	<0.001	16.273	<0.001
	GWG	0.016	0.012	0.020	0.002	8.557	<0.001		
FM%	Prepregnancy BMI	0.407	0.371	0.443	0.018	22.065	<0.001	14.814	<0.001
	GWG	0.113	0.082	0.143	0.015	7.331	<0.001		

Prepregnancy BMI, GWG, gestational week, parity, children's age and gender, annual family income, whether exclusive breastfeeding within 6 months of birth, maternal educational level, paternal educational level, maternal occupation, and paternal occupation were entered in the GLMMs, adiposity indicators during the 3 years' follow were dependent variables.
BMI, body mass index; FMI, fat mass index; FFMI, fat free mass index; FM%, percentage of fat mass.

TABLE 4 | Generalized Linear Mixed Models (GLMMs) associations between prepregnancy weight status, GWG groups and the adiposity indicators in kindergarten children.

Dependent variable	Main effect	*F* value of the GLMM models	*P*-value of the GLMM models	Estimate	95%*CI*		S.E.	*t*-value	*P*-value
					Lower	Upper			
Overweight/obese	Prepregnancy overweight/obese*	132.880	<0.001	0.779	0.647	0.912	0.068	11.527	<0.001
	GWG insufficient#	17.699	<0.001	–0.340	–0.480	–0.200	0.071	–4.764	<0.001
	GWG excessive#			0.103	–0.039	0.245	0.073	1.418	0.156
High FMI	Prepregnancy overweight/obese*	96.667	<0.001	0.755	0.604	0.905	0.077	9.832	<0.001
	GWG insufficient#	10.728	<0.001	–0.247	–0.413	–0.080	0.085	–2.906	0.004
	GWG excessive#			0.179	0.015	0.342	0.083	2.140	0.032
High FFMI	Prepregnancy overweight/obese*	57.276	<0.001	0.563	0.417	0.709	0.074	7.568	<0.001
	GWG insufficient#	9.103	<0.001	–0.217	–0.369	–0.065	0.078	–2.794	0.005
	GWG excessive#			0.148	–0.009	0.304	0.080	1.851	0.064
Low FFMI	Prepregnancy overweight/obese*	20.171	<0.001	–0.421	–0.605	–0.237	0.094	–4.491	<0.001
	GWG insufficient#	4.693	0.009	0.232	0.082	0.383	0.077	3.029	0.002
	GWG excessive#			0.081	–0.098	0.261	0.092	0.887	0.375
High FM%	Prepregnancy overweight/obese*	78.523	<0.001	0.658	0.512	0.804	0.074	8.861	<0.001
	GWG insufficient#	9.410	<0.001	–0.252	–0.408	–0.096	0.080	–3.172	0.002
	GWG excessive#			0.117	–0.040	0.274	0.080	1.463	0.144

Prepregnancy BMI, GWG, gestational week, parity, children's age and gender, annual family income, whether exclusive breastfeeding within 6 months of birth, maternal educational level, paternal educational level, maternal occupation and paternal occupation were entered in the GLMMs, adiposity indicators during the 3 years' follow were dependent variables.
**Prepregnancy normal weight group as reference.*
#Appropriate gestational weight gain group as reference.
BMI, body mass index; FMI, fat mass index; FFMI, fat free mass index; FM%, percentage of fat mass.

weight gain control during pregnancy in obstetrics clinics in Tianjin, especially among women who are overweight and obese before pregnancy, to control childhood obesity and diminish mother-to-child obesity transmission.

Children with obesity before puberty can develop obesity in early adulthood, with early-life fat deposition associated with a later risk of adult obesity (24, 25). The WHO developed guidelines on physical activity, sedentary behavior, and sleep

TABLE 5 | Generalized Linear Mixed Models (GLMMs) associations between GWG groups and the adiposity indicators in kindergarten children, stratified by prepregnancy weight status.

Dependent variable	Main effect[#]	*F*-value of the GLMM models	*P*-value of the GLMM models	Estimate	S.E.	*t*-value	*P*-value	95%*CI*	
								Lower	Upper
Prepregnancy BMI < 25 kg/m^2									
Overweight/obese	GWG insufficient	14.991	<0.001	–0.348	0.0757	–4.602	<0.001	–0.497	–0.200
	GWG excessive			0.080	0.0928	0.864	0.388	–0.102	0.262
High FMI	GWG insufficient	5.016	0.007	–0.259	0.0896	–2.888	0.004	–0.435	–0.083
	GWG excessive			0.004	0.1117	0.031	0.975	–0.216	0.223
High FFMI	GWG insufficient	6.753	0.001	–0.119	0.0829	–1.442	0.149	–0.282	0.043
	GWG excessive			0.248	0.1019	2.430	0.015	0.048	0.447
Low FFMI	GWG insufficient	6.001	0.002	0.238	0.0731	3.262	0.001	0.095	0.382
	GWG excessive			0.036	0.0994	0.361	0.718	–0.159	0.231
High FM%	GWG insufficient	5.482	0.004	–0.264	0.0828	–3.190	0.001	–0.427	–0.102
	GWG excessive			–0.047	0.1051	–0.450	0.653	–0.253	0.159
Prepregnancy BMI ≥ 25 kg/m^2									
Overweight/obese	GWG insufficient	5.341	0.005	–0.391	0.1827	–2.141	0.032	–0.750	–0.033
	GWG excessive			0.187	0.1206	1.552	0.121	–0.049	0.424
High FMI	GWG insufficient	8.391	<0.001	–0.325	0.2091	–1.554	0.120	–0.735	0.085
	GWG excessive			0.388	0.1319	2.939	0.003	0.129	0.647
High FFMI	GWG insufficient	3.012	0.049	–0.434	0.2047	–2.122	0.034	–0.836	–0.033
	GWG excessive			0.056	0.1332	0.424	0.672	–0.205	0.318
Low FFMI	GWG insufficient	Model analysis is not feasible							
	GWG excessive								
High FM%	GWG insufficient	6.746	0.001	–0.262	0.2034	–1.289	0.198	–0.661	0.137
	GWG excessive			0.352	0.1300	2.704	0.007	0.097	0.607

Prepregnancy BMI, GWG, gestational week, parity, children's age and gender, annual family income, whether exclusive breastfeeding within 6 months of birth, maternal educational level, paternal educational level, maternal occupation, and paternal occupation were entered in the GLMMs, adiposity indicators during the 3 years' follow were dependent variables.

[#]*Appropriate gestational weight gain group as reference.*

BMI, body mass index; FMI, fat mass index; FFMI, fat free mass index; FM%, percentage of fat mass.

for children under 5 years of age in 2016 (26) and 2019 (27), which called for more political commitment to face the increasing problem of childhood obesity with specific practical implementations for children. BMI has been widely used as a simple measure of defining obesity. However, it cannot differentiate between fat mass and fat-free mass (7, 28). Furthermore, simply relying on BMI to assess obesity could hinder future interventions aimed at obesity prevention and control (1). Prepregnancy BMI and GWG have attracted significant scientific attention, yet little is known about maternal factors that influence children's health in the long term (22). In this prospective cohort of preschool children from kindergarten in our study, body composition was measured in all 3 years. Combined with our previous results, the fat-free mass had a protective impact on impaired fasting glucose in children with normal weight but not in children with overweight and obesity (29). This calls for urgent attention to the importance of maintaining normal weight before pregnancy and avoiding excessive GWG because the children of mothers with excessive GWG may have higher FMI, which is harmful to their health, and not more muscle or bone mass.

The strength of this study is that both the continuous follow-up of child adiposity development in the 3 years of kindergarten and obstetric records during pregnancy were included in an ambispective cohort study. Some potential confounding variables were adjusted in the analysis which reveals the relatively causal link between prepregnancy BMI, GWG, and preschool child adiposity at the body composition level. Body composition and

anthropometric measurements were involved in the continuous 3-year measurements, which made it possible to draw continuous lines during the 3 years in preschool children. Furthermore, maternal gestational weight was measured at antenatal clinics at each visit, and the medical records of the gestational weight improved the reliability of the GWG data.

Our study has some limitations. First, prepregnancy weight was self-reported at the first visit to the antenatal clinic: there may be a recall bias in the self-reported weight data. Although a systematic review concluded that self-reported pregnancy-related weight has a high correlation with weight measurement (30), women generally underestimate their own weights (31), which may lead to a higher GWG than the actual value. Furthermore, the association between GWG and preschool child adiposity can be enhanced if this is the case. Another limitation is due to the ambispective cohort study method: we failed to retrieve some of the maternal GWG data records, and there was a different loss of follow-up rates among children in 3 years, which could have resulted in bias. Third, we measured the children's adiposity indices using bioelectrical impedance analysis rather than the gold standard, which improves the feasibility, but the accuracy should be further considered. Fourth, lifestyle behaviors, such as maternal smoking, diet, sleep, and physical activities, might be confounding factors for the relationship between pregnancy weight and offsprings' body composition, but they were not collected ideally, so they were not adjusted in the analysis. In addition, the percentage of mothers with prepregnancy BMI <18.5 kg/m^2 was 10.6%. Limited by the sample size, prepregnancy BMI of 25 kg/m^2 was used to divide the mothers into two groups, which may have a certain impact on the analysis results when combined with underweight and overweight as a reference category.

CONCLUSION

Fat mass index decreased with age, while FFMI increased with age among 3- to 5-year-old children. Maternal overweight/obesity before pregnancy increases the risk of overweight/obesity, high FMI, high FFMI, and high FM% in preschool offspring. The preschool children of prepregnancy overweight/obese mothers with excessive GWG are more likely to have a higher FMI and FM% level but not a classified high FFMI level, which is required to remain healthy. It is necessary to optimize maternal weight prior to conception and GWG management to improve the health outcomes of the offspring.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the fund supporting the project does not allow the research data to be transferred to others or other institutions without consent. Requests to access the datasets should be directed to FC, airechen@126.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB of Tianjin Women's and Children's Health Center (BGI-IRB 17116-201711). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FC conceptualized and designed the study, carried out the analyses, drafted the manuscript, and reviewed and revised the manuscript. XX and GL conceptualized and designed the study, supervised data analyses, and reviewed the manuscript. TZ conceptualized and designed the study and supervised data analyses. JW, ZL, and XZ involved in data acquisition and critically reviewed and revised the manuscript. All authors critically reviewed the manuscript for interpretation and intellectual content and approved the final manuscript as submitted.

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MINI REVIEW
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Exercise Outcomes in Childhood Obesity-Related Inflammation and Oxidative Status

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Childhood obesity is identified as one of the major public health issues to increase the risk for cardiometabolic diseases and related complications in adulthood. The literature has supported inflammation and oxidative stress as the primary underlying mechanisms involved in the pathogenesis of obesity-related diseases. Epidemiological evidence consistently shows the benefits of physical activity in the improvement of obesity-mediated inflammation and oxidative stress status. In this narrative mini-review, the available scientific evidence on the potential effects of exercise in alleviating these susceptibilities in childhood obesity will be assessed.

Keywords: adipokines, antioxidant systems, cytokines, children, exercise, inflammation, oxidative stress, physical activity

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INTRODUCTION

Childhood obesity is considered as one of the most important public health issues, with approximately 5 times more likely to develop obesity in adulthood (1). Obesity-attributable illnesses, such as cardiovascular disease have contributed to more than two-thirds of deaths globally, according to data from the Global Burden of Disease study (2). One of the underlying mechanisms contributing to obesity-related diseases is the development of systemic low-grade inflammation derived from adipose tissue (3, 4). While chronic inflammation is often associated with increased oxidative stress (5), the literature has previously reported the role of oxidative stress in the pathogenesis of obesity-related diseases (6). Other possible contributors to elevated oxidative stress in obesity include, but not limited to, hyperglycemia, vitamin and mineral deficiencies,

Abbreviations: 8-epi-PGF2α or 8-isoprostane or F2 isoprostate or F2-IsoP, 8-epi-prostaglandin F2 alpha; AGEs, advanced glycation end products; AOPPs, advanced oxidation protein products; BAP, biological antioxidant potential; BMI, body mass index; CASP-1, caspase 1; CG, carbonyl groups; CD, cluster of differentiation; $CD14^{++}CD16^{-}$, classical monocytes; $CD14^{+}CD16^{++}$, non-classical monocytes; ConA, concanavalin A; CRP, C-reactive protein; d, days; d-ROMs, diacron reactive oxygen metabolites; EGF, epidermal growth factor; F, female; GPX or GSH-Px, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HGF, Hepatocyte growth factor; HRR, heart rate recovery; hs-CRP, high sensitivity CRP; IFN-γ, interferon-gamma; IL, interleukin; IP-10, IFN-γ-induced protein 10; LC, lymphocyte count; LPO, lipoperoxides; LPS, lipopolysaccharide; M, male; MCP-1, monocyte chemoattractant protein 1; MDA, malonaldehyde; MIF, macrophage migration inhibitory factor; NC, neutrophil count; NF-kB, nuclear factor NF-kappa-B; NLR, neutrophil/lymphocyte ratio; NLRP3, nod like receptor family pyrin domain containing 3; ob, obese; OPN, osteopontin; ow, overweight; OSI, Oxidative stress index; ox-LDL, oxidized low-density lipoproteins; pb, pubertal; PBMCs, peripheral blood mononuclear cells; PCs, protein carbonyls; ppb, prepubertal; RANTES, regulated on activation, normal T-cell expressed and secreted; RBP, retinol-binding protein; sCD163, soluble hemoglobin scavenger receptor CD163; sICAM, soluble intercellular adhesion molecule; SOD, superoxide dismutase; sTNFR, soluble tumor necrosis factor receptor; TAC, total antioxidant capacity; TAG, triacylglycerols; TAS, total antioxidant status; TBARS, thiobarbituric acid reactive substances; tGSH, total glutathione equivalents; TIMP-1, metalloproteinase inhibitor 1 TLR4, toll like receptor 4; TNF-α, tumor necrosis factor-alpha; TOC, total oxidant capacity; TOS, Total oxidant status; VO_{2max}, maximal oxygen uptake; *vs.*, versus; w, week; WBC, white blood cell.

hyperleptinemia, endothelial dysfunction, and impaired mitochondrial function (7). Intricately, the damage of cellular proteins by reactive oxygen species (ROS) triggers the inflammatory responses to target the pattern recognition receptors, located either on the cell membrane [e.g., Toll-like receptors (TLRs)] (8) or in the cytoplasm [e.g., NOD-like receptors (NLR)] (9), thereby leading to the activation of various transcription factors, including NF-kB signaling (10) (**Figure 1**).

Increased physical activity in childhood obesity is an effective intervention to establish a healthy adult lifestyle in reducing the incidence of chronic diseases, such as diabetes (11, 12). In fact, the decline or lack of physical activity in different environments, especially in schools, could contribute to the development of overweight and obesity and their related health problems (13, 14), along with the associated proinflammatory state (15). In this regard, physical activity has been found to be the main intervention to lose or at least maintain body weight in overweight, obese, or severely obese adults, with the corresponding improvement in the cardiorespiratory fitness level (16). The maintenance of a physically active lifestyle in childhood is the basic to prevent or reduce the risk for cardiometabolic diseases and related complications in adulthood (17). Research in high-fat diet (HFD)-induced obese mice age ranged from 4 to 20 weeks has proven that exercise could prevent the obesity-entailed infiltration of inflammatory macrophages into adipose tissue, in support of a reduction in the pro-inflammatory cytokine production (18, 19). Furthermore, the suppression of neutrophil infiltration into fat tissue, along with the inhibition of the expression of elastase or monocyte chemoattractant protein-1 (MCP-1), was also observed with exercise training in this mouse model (20). Of important note, downregulation of inflammatory mediators/cytokines in an *in vivo* rat model of early obesity in response to exercise training has been found (21). In obese children, a meta-analysis has also revealed the beneficial effect of regular physical activity in the modulation of leptin, adiponectin, and interleukin (IL)-6 inflammatory markers (22). Furthermore, regular exercise contributes to the treatment of non-alcoholic fatty liver disease (NAFLD), with a reduction on intrahepatic fat, along with an increase in β-oxidation of fatty acids and a reduction in ROS *via* increased antioxidant enzyme activity (23). Although elevated oxidative stress has been shown to correlate with a deficiency of vitamin D (24) and index of insulin resistance in obese children (25), it is likely that lifestyle interventions including physical activity and dietary modification would improve oxidative status in this population (26) (**Figure 1**). Thus, this mini-review aims to evaluate the impact of childhood obesity on inflammation and oxidative stress status and the beneficial effects of exercise on these modifications.

EXERCISE-INDUCED INFLAMMATORY MODIFICATION IN CHILDHOOD OBESITY

A recent review has reported significant alterations in the quantity and activation of both peripheral and tissue immune cells in obese children, although the obesity-related immune function in childhood still remains to be further investigated due to the limited data from the literature (27). As presented in **Table 1.1A**, increased levels of C-reactive protein (CRP) and neutrophil count were found to positivity correlate with body fat mass in children and adolescents aged 7–18 years (28, 29). Similarly, Vehapoglu et al. (30) reported increased levels of CRP, white blood cell, and neutrophil count, as well as the neutrophil/lymphocyte ratio, which were positively correlated with BMI and index of insulin resistance, demonstrating the obesity-induced chronic low-grade inflammation in childhood (2–11 years). In agreement with these findings, an increase in the circulating levels of proinflammatory cytokines such as leptin, tumor necrosis factor (TNF)-α, IL-6, and CRP was also observed in obese compared to normal-weight children and adolescents aged 2–18 years (30–39), with a concomitant reduction of adiponectin levels in a similar age group (5–18 years) (34, 37, 40). Additionally, higher levels of plasma hs-CRP, TFN-α, and leptin were reported in both overweight and obese children, while MCP-1 was only found to be higher in the obese than overweight and control subjects with no differences in IL-6 and adiponectin among groups (41). In this regard, obese children (4–14 years) with carbohydrate metabolic impairment have been shown to exhibit an elevation in the concentrations of pro-inflammatory cytokines (e.g., CRP, IL6, TNF-α) compared to controls (36). Importantly, Carolan et al. (34) further examined the phenotype and activation of immune cells and found a shift of pro-inflammatory M1 macrophages as evidenced by elevated soluble CD163 level, along with a greater capacity of peripheral blood mononuclear cells (PBMCs) to produce IL-1β *ex vivo* following lipopolysaccharide (LPS) stimulation in obese *vs.* non-obese children and adolescents aged 6–18 years. A similar observation was also reported by Schipper et al. (42), showing that obese children (6–16 years) elicited an increase in the numbers of classical ($CD14^{++}CD16^{-}$) monocytes, as well as the levels of CD11b and IL-6 after LPS treatment compared to non-obese children. Taken together, these findings might partially support the macrophages infiltration and activation associated with obesity-induced inflammation as previously discussed. Additionally, in an interesting study evaluating the levels of several inflammatory cytokines in the saliva of children with dental caries, results showed increased levels of IL-6 and IL-15 in overweight/obese compared to normal-weight children aged 3–8 years (43). In an attempt to evaluate the gender effect of childhood obesity in the circulating levels of inflammatory cytokines, Murdolo et al. (38) showed that obese girls presented elevated IL-8, IL-18, MCP-1, and soluble intercellular adhesion molecule-1 levels (sICAM-1), whereas only obese boys exhibited increased macrophage migration inhibitory factor (MIF) levels. However, although the concentration of appetite-stimulating hormone, adiponectin, was higher in normal-weight girls compared to boys age ranged from 5 to 13 years, no sex by BMI group interaction was found in this study (38). Overall, these outcomes confirm the obesity-induced chronic low-grade inflammation during childhood and propose the elevation of immune cell activation, such as macrophage infiltration, with limited gender influence during puberty.

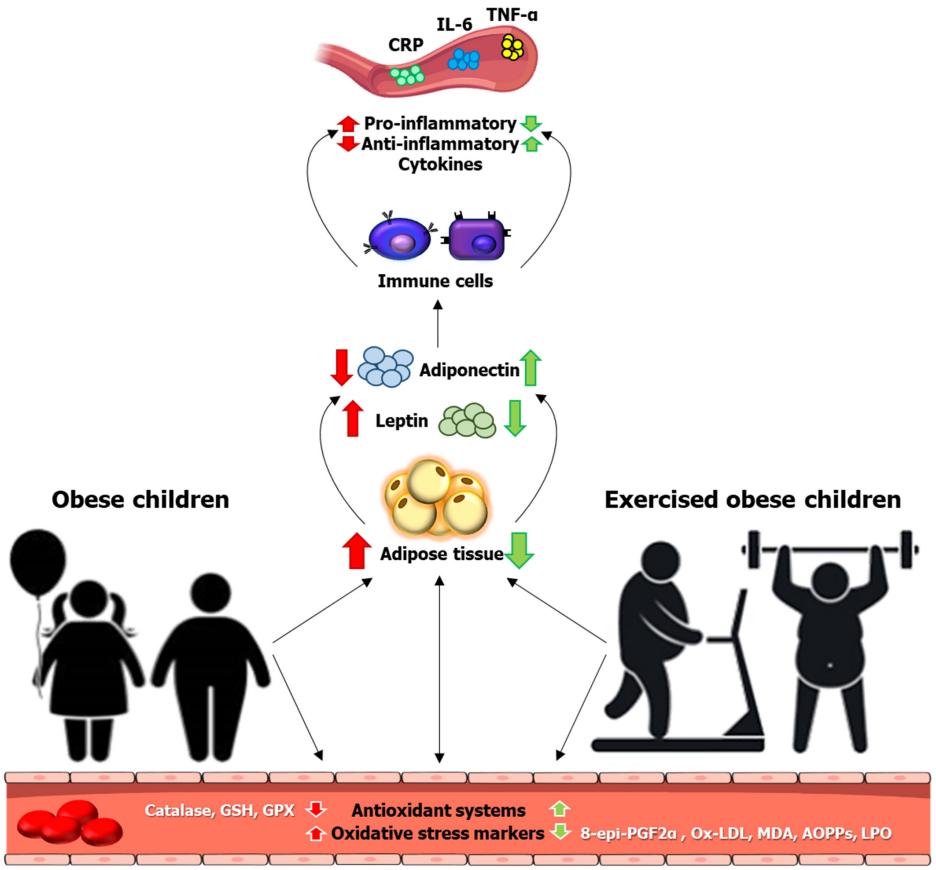

FIGURE 1 | Inflammation and oxidative stress responses to childhood obesity and exercise in obese children. Localized inflammation in adipose tissue related to obesity triggers low-grade systemic inflammation, manifesting in a general increase in circulating inflammatory markers in obese children. Childhood obesity concomitates with an oxidative status imbalance, increasing the oxidative stress markers and decreasing the antioxidant systems. Different protocols of physical activity might revert the consequences of obesity on inflammatory processes and oxidative stress during child growth. 8-epi-PGF2α, 8-epi-prostaglandin F2 alpha; AOPPs, advanced oxidation protein products; CRP, C-reactive protein; GPX, glutathione peroxidase; GSH, reduced glutathione; IL-6, interleukin 6; LPO, lipoperoxides; MDA, malonaldehyde; ox-LDL, oxidized low-density lipoproteins; TNF-α, tumor necrosis factor alpha.

Both epidemiologic and longitudinal research have supported that regular exercise or increased physical activity is an effective strategy in reducing systemic low-level inflammation in individuals with obesity and related conditions (44–46). As summarized in **Table 1.2A**, Nascimento et al. (47) conducted an 8-month longitudinal study in investigating the effects of aerobic exercise training on the inflammatory status in childhood (5–17 years) obesity and observed a reduction in the levels of TNF-α and CRP, along with an improvement in lipid profile and insulin resistance. These findings are partially supported by Nemet et al. (48), showing an increase in adiponectin and a decrease in CRP, along with no changes in leptin and IL-6 levels in obese self-referred children aged 6–13 years following a 3-month combined nutritional-behavioral-physical activity intervention. Interestingly, Woo et al. (37) found a significant increase but a reduction in the serum levels of adiponectin and leptin, respectively, following a minimum of 12 weeks of aerobic training in obese children (the average age of 11 years), despite no change in the percentage of body fat. In contrast, Kelly et al. (49, 50) did not observe any effect of 8 weeks of stationary cycling intervention in plasma CRP, IL-6, TNF-α, adiponectin, and leptin levels, as well as change in weight or body composition in overweight children in the same range of age. Similarly, no significant difference was found in serum adiponectin, IL-6, and CRP levels in overweight and obese girls aged 9–15 years in response to 12-week aerobic training (51). Additionally, although Blüher et al. (51) reported a decrease in serum leptin following the obesity therapy program, including 150 min/week of physical activity for 39 weeks, no change was observed in adiponectin and CRP levels in these overweight/obese children, independently of gender, age, or pubertal stage. Finally, in a recent study examining the effect of a 12-week combined strength and endurance training program in PBMCs of obese pediatric patients aged 7–12 years, a lower NLRP3 inflammasome (a regulator of innate immune activity/pro-inflammatory response) activation, but not TLR4 was reported (52). Similar contradictions have been described in obese adolescents conducting long-term resistance and aerobic exercise programs, showing either decreases or no changes in pro-inflammatory markers such as CRP, IL-6, leptin, or TNF-α (53–58). Additionally, while a reduction in the number of

TABLE 1 | Effects of childhood obesity (1) and exercise (2) in obese children on inflammation (A) and oxidative stress (B) markers.

1A. Inflammation

Research article	Age (years)	Subjects	Tissue	Results
Araki et al. (40)	9.9	Obese (*n* = 44; F/M: 16/28) and non-obese (*n* = 28; F/M: 13/15)	Plasma	(↓) Adiponectin
Carolan et al. (34)	12.8 ± 3.2 13.0 ± 3.0	Obese (*n* = 29; F/M: 16/13) Non-obese (*n* = 20; F/M: 7/13)	LPS-stimulated PBMCs supernatant	(↓) $CD3^+$ T lymphocytes (↑) sCD163, IL-1β, TNF-α
			Serum	(↑) Leptin (↓) Adiponectin
Chang et al. (41)	9.32 ± 0.45 10.70 ± 0.37 10.06 ± 0.60	Obese (*n* = 19; M) Overweight (*n* = 10; M) Lean (*n* = 16; M)	Whole blood	(↑, ob) hs-CRP, IL-6, MCP-1, Leptin, TNF-α (↑, ow) hs-CRP, IL-6, Leptin, TNF-α (↓, ow) MCP-1
Codoñer-Franch et al. (31)	7–14	Obese (*n* = 60; F/M: 23/37) and non-obese (*n* = 50; FM: 27/23)	Serum	(↑) CRP, IL-6, Leptin, TNF-α
Faienza et al. (35)	10.98 ± 3.2 11.98 ± 3.2 10.48 ± 3.9	Simple obese (*n* = 30; F/M: 16/14) MetS obese (*n* = 25; F/M: 13/12) Normal weight (*n* = 30; F/M: 15/15)	Serum	(↑) CRP
Jaksic et al. (29)	10.83 ± 1.67 11.05 ± 1.45 10.82 ± 1.62	Obese (*n* = 35) Pre-obese (*n* = 82) Normal weight (*n* = 85)	Serum	(↑) CRP (NS) RBP
Lechuga-Sancho et al. (36)	4–14	Obese (*n* = 18; F/M: 10/8) and normal weight (*n* = 6; F/M: 2/4)	Serum	(↑) CRP, IL-6, Leptin (NS) HGF, TNF-α
Mărginean et al. (32)	10.86 ± 3.5 12.46 ± 3.4	Obese (*n* = 91) Normal weight (*n* = 102)	Serum	(↑) IL-6, Leptin, TNF-α (↓) IL-1β
Murdolo et al. (38)	5–13	Obese (*n* = 140; F/M: 70/70), overweight (*n* = 60; F/M: 28/32) and normal weight (*n* = 105, F/M: 46/59)	Serum	(↑, ob) (M) MIF, (F) IL-8, IL-18, MCP-1, sICAM-1 (↑, ow) (M) Adiponectin (NS, ob, ow) (F) Adiponectin, (M) HMW Adiponectin, (F/M) IP-10, RANTES, Resistin (↑, ob, ow, F, M) Leptin, Leptin/HMW ratio (NS, ob, F) IL-8, IL-18, MCP-1, sICAM-1
Oliver et al. (33)	12.9 ± 0.3	Obese (*n* = 55, F/M: 25/30) and normal weight (*n* = 43, F/M: 25/18)	Plasma	(↑) CRP, IL-6
Ramírez-De Los Santos et al. (43)	5.8	Overweight/obese (*n* = 37) and normal weight (*n* = 43)	Saliva	(↑) IL-6, IL-15 (↓) IL-8, IL-18
Rowicka et al. (39)	7.5	Obese children (*n* = 62)	Serum	(↑) CRP
	6.4	Non-obese children (*n* = 21)		
Schipper et al. (42)	6–16	Obese (*n* = 60; F/M: 33/27) and lean (*n* = 30; F/M: 16/14)	Plasma	(↑) Chemerin, EGF, HGF, IL-8, IL-18, IP-10, Leptin, TIMP-1, TNF-R2
			Monocytes	(↑) $CD14^{++}CD16^{-}$ monocytes (NS) $CD14^{+}CD16^{++}$ monocytes
			LPS-stimulated whole blood	(↑) IL-6 (NS) TNF-α
Singer et al. (28)	13.1	Obese (*n* = 1,207), overweight (*n* = 1,008), underweight (*n* = 130) and normal weight (*n* = 3260)	Serum	(↑) CRP
Vehapoglu (30)	7.4 ± 2.7	Obese (*n* = 90; F/M: 49/41)	Blood	(↑) WBC, NC, NLR (NS) LC
	7.0 ± 2.6	Underweight (*n* = 80; F/M: 39/41)		
	7.2 ± 2.7	Normal weight (*n* = 80; F/M: 41/39)	Serum	(↑) CRP
Woo et al. (37)	11.30 ± 1.17 11.32 ± 1.06	Obese (*n* = 20) Normal weight (*n* = 19, M)	Serum	(↑) Leptin (↓) Adiponectin

2A. Inflammation and exercise

Research article	Age (years)	Subjects	Training protocol	Tissue	Results
Blüher et al. (79)	12.5 ± 0.2	Overweight/obese (*n* = 65)	13-month (150 min/w) endurance and resistance exercise	Serum	(↑) Resistin (↓) Leptin (NS) Adiponectin, CRP, sTNFR-II
Kelly et al. (49)	10.9 ± 0.4	Overweight training (*n* = 10; F/M: 5/5) and control (*n* = 10; FM: 6/4)	8-week (4 d/w) 30–50 min stationary cycling up to 80% VO_{2max}	Serum	(NS) CRP
Kelly et al. (50)	10.8 ± 0.67 11.0 ± 0.71	Overweight training (*n* = 9; F/M: 5/4) Overweight control (*n* = 10; FM: 6/4)	8-week (4 d/w) 30 to 50 min stationary cycling up to 80% VO_{2max}	Serum	(NS) Adiponectin, CRP, IL-6, Leptin, Resistin, TNF-α
Liu and Timmons (81)	9.5 ± 1.2	Obese training (*n* = 10)	2 × 30 min acute bouts of continuous cycling at 60% VO_{2max}	PBMCs	(↑) IL-6, TNF-α

(Continued)

TABLE 1 | (Continued)

Research article	Age (years)	Subjects	Training protocol	Tissue	Results
Merlin et al. (15)	9.00 ± 1.96 7.54 ± 1.70	Obese active (*n* = 33) Obese sedentary (*n* = 17)	1-week (7 d/w) daily step count: sedentary (9338 ± 902 steps) *vs.* active (13,614 ± 1,003 steps)	ConA-stimulated lymphocyte supernatant	(↑) IL-2, IL-17, IFN-γ, TNF-α (↓) IL-10 (NS) IL-4, IL-6
Nascimento et al. (47)	10.41 ± 1.96 10.49 ± 2.67	Obese training (*n* = 80; F) Obese control (*n* = 37; F)	8-month (5 d/w) 1 h moderate-to-vigorous aerobic and strength endurance training, flexibility, coordination and balance	Plasma	(↑) Adiponectin (↓) CRP, TNF-α (NS) IL-1β, IL-6, Leptin, Resistin
Nassis et al. (51)	13.05 ± 1.75	Obese (*n* = 19; F)	12-week (3 d/w) 40 min physical training games	Serum	(NS) Adiponectin, CRP, IL-6
Nemet et al. (48)	10.41 ± 1.96 10.49 ± 2.67	Obese training (*n* = 21) Obese control (*n* = 21)	13-week (2 d/w) 1 h team sports and running games	Serum	(↑) Adiponectin (↓) CRP (NS) IL-6, Leptin
Quiroga et al. (52)	10.8 ± 0.3	Obese training (*n* = 25; F/M: 12/13) and control (*n* = 14; F/M: 7/7)	12-week (2 d/w) 1 h combined strength and endurance training	PBMCs	(↓) CASP-1, NLRP3, OPN (NS) TLR4, LPS
Woo et al. (37)	11.32 ± 1.06	Normal weight (*n* = 19)	12-week aerobic training at HRR 45–65%	Serum	(↑, 12-w, 24-w *vs.* basal, ObT, ObD) Adiponectin (↑, 24-w *vs.* 12-w, ObT) Adiponectin (NS, 24-w *vs.* 12-w, ObD) Adiponectin (↓, 12-w, 24-w *vs.* basal, ObT) Leptin (↓, 24-w *vs.* 12-w, ObT) Leptin (NS, 24-w *vs.* 12-w, ObD) Leptin
	11.30 ± 1.17	Obese/overweight training (*n* = 10)	24-week aerobic training at HRR 45–65%		
		Obese/overweight detraining (*n* = 10)	12-week aerobic training at HRR 45–65% + 12-week detraining		

1B. Oxidative stress

Research article	Age (years)	Subjects	Tissue	Results
Albuali (59)	9.5 ± 1.5	Obese (*n* = 64; F/M: 23/41), overweight (*n* = 83; F/M: 23/60), normal weight (*n* = 66; F/M: 24/42)	Erythrocytes	(↑, ob): AOPPs, MDA, Ox-LDL (↓, ob): Catalase, GSH, GSH-Px, SOD, GSSG (↑, ow): Catalase, GSH, GSH-Px, SOD, GSSG (NS, ow): AOPPs, MDA, Ox-LDL
Araki et al. (40)	9.9	Obese (*n* = 44; F/M: 16/28) and non-obese (*n* = 28; F/M: 13/15)	Plasma	(↑) 8-epi-PGF2α (↓) TAC
Calcaterra et al. (62)	11.8 ± 2.6	Obese (*n* = 53; F/M: 25/28), overweight (*n* = 76; F/M: 41/35) and normal weight (*n* = 49; F/M: 22/27)	Serum	(↑) Ox-LDL
Carmona-Montesinos et al. (65)	4.4 ± 0.12 4.1 ± 0.109	Obese (*n* = 50) Normal weight (*n* = 50)	Plasma	(↑) GSSG, Ox-LDL (↓) GSH
			Serum	(↑) MDA, TBARS
Codoñer-Franch, Boix-García et al. (63)	10.9 ± 2.5	Obese (*n* = 48; F/M: 22/26) and normal weight (*n* = 20; F/M: 8/12)	Plasma	(↑) CG, LPO, MDA (NS) α-tocopherol, β-carotene
			Erythrocytes	(↑) GPX (↓) GSH
Codoñer-Franch, Pons-Morales et al. (64)	10.9 ± 2.5	Obese (*n* = 22; F/M: 12/10) and normal weight (*n* = 16; F/M: 8/8)	Plasma	(↑) CG, LPO, α-tocopherol, β-carotene (NS) MDA
			Erythrocytes	(↑) GPX (NS) GSH
Codoñer-Franch et al. (31)	7–14	Obese (*n* = 60; F/M: 23/37) and non-obese (*n* = 50; FM: 27/23)	Plasma	(↑) 8-isoprostane, AOPPs, MDA
Faienza et al. (35)	10.98 ± 3.2 11.98 ± 3.2 10.48 ± 3.9	Simple obese (*n* = 30; F/M: 16/14) MetS obese (*n* = 25; F/M: 13/12) Normal-weight (*n* = 30; F/M: 15/15)	Plasma	(↑) d-ROMs (↓) BAP, BAP/d-ROMs ratio
Jaksic et al. (29)	10.83 ± 1.67 11.05 ± 1.45 10.82 ± 1.62	Obese (*n* = 35) Pre-obese (*n* = 82) Normal weight (*n* = 85)	Serum	(↑) TAS
Kelly et al. (61)	12.4 ± 3.3 11.7 ± 3.5	Overweight/obese (*n* = 38; F/M: 12/26) Normal weight (*n* = 40; F/M: 15/25)	Serum	(↑) Ox-LDL
Lechuga-Sancho et al. (36)	4–14	Obese (*n* = 18; F/M: 10/8) and normal weight (*n* = 6; F/M: 2/4)	Erythrocytes	(↓) Catalase, GSH, GSSG, Ox-LDL, TAC tGSH
			Serum	(NS) TBARS
			Urine	(↑) 8-isoprostane
Lentferink et al. (67)	11.8 12.2	Obese (*n* = 143; F/M: 70/73) Normal weight (*n* = 428; F/M: 210/218)	Serum	(↑) AGEs
Oliver et al. (33)	12.9 ± 0.3	Obese (n = 55, F/M: 25/30) and normal weight (*n* = 43, F/M: 25/18)	Plasma	(↑) F2-IsoP (NS) SOD, GSH-420
Rowicka et al. (39)	7.5 6.4	Obese children (*n* = 62) Non-obese children (*n* = 21)	Serum	(↑) TOC (↓) TAC (NS) Ox-LDL

(Continued)

TABLE 1 | (Continued)

Research article	Age (years)	Subjects	Tissue	Results
Rupérez et al. (66)	3–17	Obese (n = 680), overweight (n = 358) and normal weight (n = 406)	Plasma	(NS, ppb) Ox-LDL (↑, ppb) Retinol (↓, ppb) Carotenes/TAG, Tocopherols/TAG (↓, pb, ppb) TAC
Sfar et al. (60)	6–12	Obese (n = 54; F/M: 31/23) and normal weight (n = 52; F/M: 25/27)	Erythrocytes	(↑) SOD (NS) Catalase, GPX
Vehapoglu (30)	7.4 ± 2.7	Obese (n = 90; F/M: 49/41)	Serum	(↓) TAS, Total thiol (NS) TOS, OSI
	7.0 ± 2.6	Underweight (n = 80; F/M: 39/41)		
	7.2 ± 2.7	Normal weight (n = 80; F/M: 41/39)		
Woo et al. (37)	11.30 ± 1.17 11.32 ± 1.06	Obese (n = 20) Normal weight (n = 19, M)	PBMCs	(↑) GPX (NS) SOD

2B. Oxidative stress and exercise

Research article	Age (years)	Subjects	Training protocol	Tissue	Results
Ahmadian et al. (70)	11.4 ± 0.7 11.2 ± 0.7 12.8 ± 0.8 12.5 ± 1.2	Obese asthmatic (n = 10; M) Obese non-asthmatic (n = 15; M) Lightweight asthmatic (n = 10; M) Lightweight non-asthmatic (n = 7; M)	Acute progressive aerobic cycle test until volitional exhaustion	Saliva	(↓, all groups) MDA
Dennis et al. (72)	9.3 ± 1.1	Obese high-dose training (n = 36), low-dose training (n = 34) and control (n = 42)	10–15-week (every school day) high-dose (40 min) vs low-dose (20 min) aerobic exercises	Plasma	(NS) F2 isoprostate
Kelly et al. (50)	10.8 ± 0.67 11.0 ± 0.71	Overweight training (n = 9; F/M: 5/4) Overweight control (n = 10; F/M; 6/4)	8-week (4 d/w) stationary cycling up to 80% of VO_{2max}	Serum	(=) 8-isoprostane
Paltoglou et al. (71)	10.95 ± 0.99	Male obese training (n = 23) and normal weight training (n = 53)	Acute bout of aerobic exercise until exhaustion at 70% of VO_{2max}	Erytrocytes	(↑) Catalase (↓) GSH, GSH/GSSG
				Serum	(↑) GPX, PCs, TBARS, TAC
Woo et al. (37)	11.32 ± 1.06	Normal weight (n = 19)	12-week aerobic training at HRR 45–65%	Plasma	(↑, 12-w, 24-w *vs.* basal, obT, obD) GPX (NS, 24-w *vs.* 12-w, obT) GPX (↓, 24-w *vs.* 12-w, obD) GPX (NS, 12-w *vs.* basal, obT, obD) SOD
	11.30 ± 1.17	Obese/overweight training (n = 10)	24-week aerobic training at HRR 45–65%		(↑, 24-w *vs.* basal, obT, obD) SOD (↑, 24-w *vs.* 12-w, obT) SOD (NS, 24-w *vs.* 12-w, obD) SOD
		Obese/overweight detraining (n = 10)	12-week aerobic training at HRR 45–65% + 12-week detraining		
				PBMCs	(↑, 12-w *vs.* basal, Nw, ob) GPX, SOD (↑, 12-w *vs.* basal, ObT, obD) SOD (NS, 12-w *vs.* basal, ObT, obD) GPX (↑, 24-w *vs.* basal, obT) GPX, SOD (NS, 24-w *vs.* basal, obD) GPX, SOD (↑, 24-w *vs.* 12-w, obT) SOD (NS, 24-w *vs.* 12-w, obD) SOD (NS, 24-w *vs.* 12-w, obT, obD) GPX

(↓), decreased; (↑), increased; (NS), no significant; 8-epi-PGF2α or 8-isoprostane or F2 isoprostate or F2-IsoP, 8-epi-prostaglandin F2 alpha; AGEs, advanced glycation end products; AOPPs, advanced oxidation protein products; BAP, biological antioxidant potential; CASP-1, caspase 1; CD, cluster of differentiation; $CD14^{++}CD16^{-}$, classical monocytes; $CD14^{+}CD16^{++}$, non-classical monocytes; CG, carbonyl groups; ConA, concanavalin A; CRP, C-reactive protein; d, days; d-ROMs, diacron reactive oxygen metabolites; EGF, epidermal growth factor; F, female; GPX or GSH-Px, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; h, hour; HGF, Hepatocyte growth factor; HRR, heart rate recovery; hs-CRP, high sensitivity CRP; IFN-γ, interferon-gamma; IL, interleukin; IP-10, IFN-gamma-induced protein 10; LC, lymphocyte count; LPO, lipoperoxides; LPS, lipopolysaccharide; M, male; MCP-1, monocyte chemoattractant protein 1; MDA, malonaldehyde; MIF, macrophage migration inhibitory factor; min, minutes; NC, neutrophil count; NLR, neutrophil/lymphocyte ratio; NLRP3, nod like receptor family pyrin domain containing 3; ob, obese; obD, obese detraining; OPN, osteopontin; obT, obese training; ow, overweight; OSI, oxidative stress index; ox-LDL, oxidized low-density lipoproteins; PBMCs, peripheral blood mononuclear cells; PCs, protein carbonyls; ppb, prepubertal; RANTES, regulated on activation, normal T-cell expressed and secreted; RBP, retinol-binding protein; sCD163, soluble hemoglobin scavenger receptor CD163; sICAM, soluble intercellular adhesion molecule; SOD, superoxide dismutase; sTNFR, soluble tumor necrosis factor receptor; TAC, total antioxidant capacity; TAG, triacylglycerols; TAS, total antioxidant status; TBARS, thiobarbituric acid reactive substances; tGSH, total glutathione equivalents; TIMP-1, TIMP (tissue inhibitor of metalloproteinase) metallopeptidase inhibitor 1; TLR4, toll like receptor 4; TNF-α, tumor necrosis factor-alpha; TOC, total oxidant capacity; TOS, total oxidant status; VO_{2max}, maximal oxygen uptake; vs., versus; w, week; WBC, White blood cell.

neutrophils and the expression of TNF-α was observed after a 6-month high-intensity exercise, an opposite response was found with the low-intensity protocol (53). Thus, these contradictory findings in the improvement of exercise-mediated inflammation in childhood obesity may be the result of various intensities and/or duration of the intervention, and other factors including dietary changes could also alter these inflammatory profiles.

CHILDHOOD OBESITY: OXIDATIVE STRESS STATUS AND BENEFITS OF EXERCISE

Oxidative stress is another underlying mechanism involved in the pathogenesis of obesity-related diseases. Regarding the oxidative status (**Table 1.1B**), research has demonstrated lower antioxidant activities and higher oxidation products in obese *vs.* non-obese children aged 6–12 years (59). Thus, an increase in total oxidant capacity (TOC) (39) and a decrease in total antioxidant capacity (TAC) (39, 40), as well as a depletion in total antioxidant status (TAS) and total thiols, and a rise in total oxidation status (TOS) (30) were reported in obese children aged 2–13 years compared to their normal-weight counterparts. Moreover, the erythrocyte activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione reductase (GR), and reduced glutathione concentration (GSH) were lower in obese children aged 6–12 years than their counterparts, whereas these antioxidant activities in erythrocyte (59) and serum TAS (29) were increased in overweight compared to normal-weight children aged 7–15 years, suggesting the inability to counterbalance the detrimental effects or the highly production of ROS in obesity. The results of this oxidative stress status from these obese children are in agreement with the study by Lechuga–Sancho et al. (36), demonstrating lower erythrocyte levels of catalase, GSH, and TAC in children aged 4–14 years. However, the contradictory findings have been reported including an increase in SOD and no differences in catalase and GPX activity in erythrocytes (60), elevated GPX and no changes in SOD in PBMCs (37), and no differences in plasma SOD and GSH levels (33) between obese and normal-weight children aged 6–12 years. On the other hand, obese children aged 6–12 years exhibited elevated levels of erythrocyte malondialdehyde (MDA) as well as both plasma oxidized low-density lipoproteins (ox-LDL) and advanced oxidation protein products (AOPPs), while no difference was reported in these markers between overweight and normal-weight groups (59). In support of these results, elevated levels of ox-LDL (61, 62) and 8-epi-prostaglandin F2 alpha (31, 33, 36, 40) have been shown to be higher in obese than normal-weight children aged 4–18 years, along with an elevation in circulating levels of MDA, and AOPPs (31). Importantly, Codoñer–Franch et al. (63) have previously demonstrated an increase in plasma MDA and carbonyl groups (CG), as well as erythrocyte GPX activity levels, along with decreased levels in erythrocyte GSH, in severely obese children only, whereas the levels of α-tocopherol and β-carotene, lipophilic antioxidants that act in biomembranes as scavengers of free radicals, remained similar as obese and normal-weight children aged 6–14 years. Although another investigation by Codoñer–Franch et al. (64) showed that the levels of plasma lipoperoxides (LPO), CG, and the antioxidants α-tocopherol and β-carotene, as well as the GPX erythrocyte activity were increased in obese children in the same range of age, plasma MDA levels and erythrocyte GSH concentration did not differ between groups. Similar results have been presented in obese infants aged 3–5 years, showing higher serum peroxidized lipids, specifically thiobarbituric acid-reacting substances (TBARS), plasmatic ox-LDLs and oxidized glutathione (GSSG) levels, as well as lower plasmatic GSH values, compared with their matched controls (65). Thus, the inconsistence of the above findings could potentially be due to the wide range of age groups utilized in these studies. To further clarify this potential effect of age, Rupérez et al. (66) differentiated children aged 3–17 years during pre/pubertal period and found that TAC levels were lower in prepubertal but higher in pubertal overweight/obese children than normal-weight children. Furthermore, the advanced glycation end products (AGEs) formation (a facilitator of ROS), measured as skin autofluorescence, was increased in obese children and adolescents aged 4–18 years, although no differences were shown in the group of children under 10 years old (67). These observations might be partially supported by the transient decline in insulin sensitivity during puberty attributable to lack of glycemic control (68, 69). In contrast, a high level of serum ox-LDL observed in obese children (the mean age = 11 years) was not correlated with sex, age, and pubertal status (62). It is reasonably speculated that the metabolic status and the presence of the metabolic syndrome could have influenced these reported results, as evidenced by Rupérez et al. (66) with lower carotenes and tocopherols levels in metabolically unhealthy *vs.* healthy children aged 3–17 years. In this regard, Faienza et al. (35) presented similar results, demonstrating a higher level of diacron reactive oxygen metabolites (d-ROMs) in obese children and prepubertal subjects (the average age of 11 years) with metabolic syndrome, with a negative relationship between total antioxidant capacity and standardized BMI. Thus, these findings suggest that an early onset in the pathogenesis of obesity-mediated diseases by elevated oxidative stress may occur during pediatric age.

Although there is limited research investigating the impact of physical activity interventions on childhood obesity-associated oxidative stress (**Table 1.2B**), acute aerobic cycling exercise seems to exert contrary effects on oxidative markers in obese children. Specifically, Ahmadian et al. (70) reported a decrease in MDA levels after an acute progressive test until volitional exhaustion in both obese and normal-weight children under the age of 13 years, as well as in their asthmatic counterparts (70), Paltoglou et al. (71) reported an increase in the levels of TBARS and protein carbonyls (PCs), as well as in TAC, and catalase, along with a decrease in the production of GSH and GSH/GSSG in both prepubertal and pubertal obese children following an acute bout of maximal aerobic exercise. These responses to acute aerobic exercise were also observed in their prepubertal and pubertal normal-weight counterparts. On the other hand, neither an 8-week nor 13-week aerobic training program improved the levels of plasma 8-isoprostane (50) or serum F2-isoprostane (72) in overweight/obese children aged 7–11 years. Contrary to these results, a 12-week training exercise

was effective to increase both SOD and GPX mRNA expression in PBMCs from both obese and normal-weight children (the mean age = 11 years) (37). Interestingly, this study reported that the expression of SOD and GPX mRNA remained elevated until completion of a 24-week training intervention in the obese training group, but not the obese detraining group who participated in the 12-week training plus 12-week detraining program. Similarly, an elevation in plasma levels of SOD and GPX following 24 weeks of exercise program in both obese training and detraining groups was observed, whereas only obese detraining group showed a decrease in GPX and no changes in SOD level at the mid-training (12 weeks) (37). The phenomenon of this observation might present a different behavior or time course of these antioxidant enzyme responses, depending on the oxidation-reduction transcription factors involved in the regulatory processes. Overall, these results indicate that chronic exercise could possibly elicit protective adaptations against oxidative damage in childhood obesity as the effective training interventions have been administered in adult obesity (73, 74).

Finally, oxidative stress is frequently associated with obesity-induced inflammation. Although the mechanisms for childhood obesity-induced oxidative stress remain to be investigated, leptin has been proposed as an important contributor, due to its role in mediating pro-inflammatory state in obese individuals (75, 76). Particularly, increased leptin has been shown to modulate the production of oxidative stress biomarkers, such as reduced nitric oxide (NO), increased superoxide ($O2^-$) and peroxynitrite (ONOO-) in both human endothelial cells and the endothelium of obese mice (77, 78). With a reduction in the circulating level of leptin following training in children and adolescences aged 7–18 years (79) and its potential relationship with oxidative stress (80), this could partially support the role of exercise interventions for oxidative adaptions in alleviating susceptibility to obesity-associated oxidative stress.

CONCLUSION

Results from this mini-review evidence that localized inflammation in adipose tissue triggers low-grade systemic inflammation in childhood obesity. Similarly, an increase in oxidative stress markers, as well as a decrease in antioxidant markers in obese children present an imbalance in oxidative stress status. However, the effects of physical activity on the inflammatory and oxidative stress responses in growing obese kids still remain inconclusive. Regarding oxidative status, although the studies available to date are not sufficient to conclude the beneficial effect of exercise in obese children, the influence of puberty in childhood obesity-mediated oxidative stress is warranted for further investigation. Moreover, the research addressing the role of several key hormones (e.g., testosterone, estrogen, and growth hormone) on the obesity-associated inflammatory and oxidative stress processes in the different growth stages of children is needed to further discover their exercise modulation, including a limited age range with controlling for biological and chronological age. In summary, additional insight into how physical activity interventions influence the cellular adaptations of inflammation and oxidative stress is necessary to better understand the importance of exercise as an antagonist to the current childhood obesity epidemic.

AUTHOR CONTRIBUTIONS

BE, MJC, and JG-G conceptualized the manuscript. BE and MR-V performed the literature search. BE, C-JH, and MJC performed manuscript drafting. MJC and JG-G supervised and revised the manuscript. All authors contributed to the article and approved the submitted version.

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