MODERN LIFESTYLE AND HEALTH: HOW CHANGES IN THE ENVIRONMENT IMPACTS IMMUNE FUNCTION AND PHYSIOLOGY

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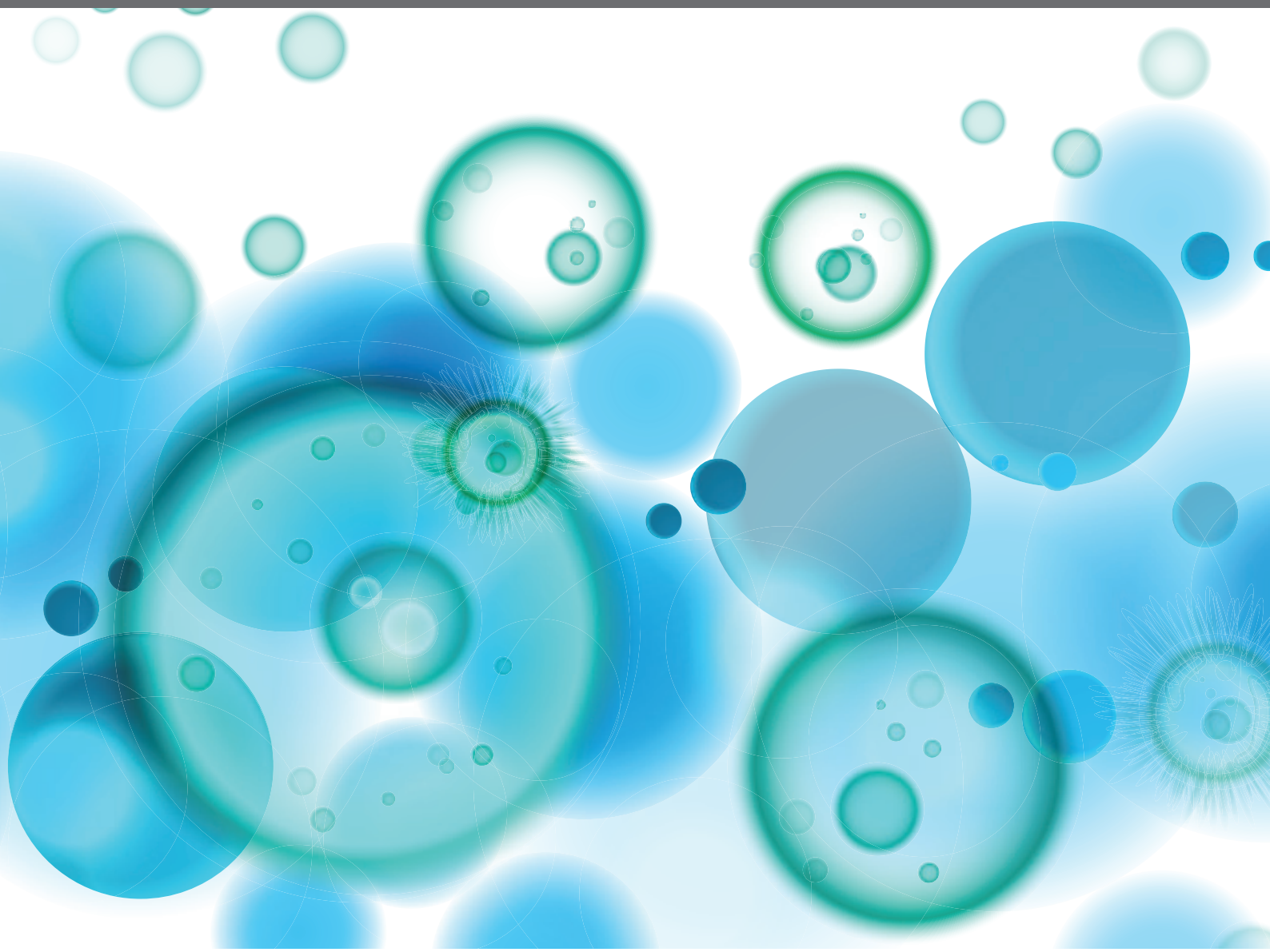

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MODERN LIFESTYLE AND HEALTH: HOW CHANGES IN THE ENVIRONMENT IMPACTS IMMUNE FUNCTION AND PHYSIOLOGY

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EDITORIAL
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Editorial: Modern Lifestyle and Health: How Changes in the Environment Impacts Immune Function and Physiology

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Editorial on the Research Topic

Modern Lifestyle and Health: How Changes in the Environment Impacts Immune Function and Physiology

Physical fitness as well as nutritional access are major evolutionary drivers across all animal species. In modern societies both of these factors have become highly modifiable principally allowing survival despite a sedentary lifestyle in an environment of virtually limitless caloric access. This has led to an evolutionary mismatch for which we are now paying the price of increasing non-communicable diseases. In addition, we are also paying the price in terms of the enormous carbon footprints transport emmissions and industrialised animal agriculture are causing. In the Research Topic *Modern Lifestyle and Health: How Changes in the Environment Impacts Immune Function and Physiology* several studies have been published providing further evidence on how this discordance between our evolutionray programming and the adoption of a modern lifestyle influence our health and wellbeing.

A series of observational investigations focused on comparing more traditional rural to urban settings in developing countries in Pacific Islands Countries and Territories. The study by Bang Nguyen Pham et al. analysed diets in under 5-year-old children in Paupua New Guinea. Interestingly dietary diversity as a proxy measure of dietary adequacy was higher in the rural settings *versus* more westernized urban settings. Dietary diversity of urban children markedly increased with maternal educational status and wealth. This was then independently reflected in childhood growth patterns, with wasting and stunting being more prevalent in urban settings and compensated again by household socioeconomic status in the urban environment (Bang Nguyen Pham et al.). Also, in terms of physical activity Wattelez et al. in a study on adolescents in New Caledonia showed that living in rural areas was associated with more physical activities and less sitting time, in contrast to urban environments associated with a more sedentary lifestyle. The common theme here is obviously that negative impacts of low physical activity and nutrition of a modern lifestyle can be partially counteracted by education and socioeconomic factors.

Physical activity partially reverse conditions associated with our modern lifestyle and can be employed as preventive strategies or medical interventions. The study by Jabbour, suggests that vigorous physical activity in type 1 diabetic adolescents did not only improve long-term blood sugar levels but also had a favourable psychological impact on fear of hypoglycaemia. In addition,

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Sooyeon Oh et al. showed that physical activity and vitamin D were associated with an increase in natural killer cell activity. These cells are targeting cells under stress such as tumor cells or virus infected cells, hence, supporting the already known benefits of outdoor physical activity often lacking in modern societies. Furthermore, Leandro dos Santos et al. demonstrated that modified forms of resistant exercise, in which blood flow to the trained muscle groups is restricted, result in an increase in lymphocyte mobilisation in the circulation. The lymphocytes are then readily available for tissue reparation, opening the door to new rehabilitation strategies by harnessing the power of immune reparation. Physical activity has indeed systemic impacts. Ma et al., reviewed the effects of the myokin irisin on cardiovascular health and its potential use as a diagnostic in cardiovascular diseases.

León, et al. provided evidence that adaptive processes towards modern diets are beginning to evolve. They investigated how wheat, a staple nutritional component of modern societies, affects cell homeostasis through the splicing of pre-mRNAs encoding key regulatory proteins.

A further series of publications were focused on the effects of diet on the immune system and more broadly on immunometabolism. In contrast to traditional plant based diets, processed foods in westernized societies are typically low in dietary fibre. However, dietary fibre influences the gut microbiome composition and is the main source of energy for intestinal bacteria. Dietary fibre is typically fermented by gut bacteria resulting in the production of short chain fatty acids (SCFA), known to have profound immunomodulatory effects beneficial in diseases such as allergies, autoimmune diseases and cancer (1). Malczewski et al. specifically focused their review on dietary fibre in the context of cancers and discuss the link between response to cancer treatments and specific gut microbiota signature. They propose that the gut microbiota metabolome signature, particularly the profile of short chain fatty acids could be a better tool to identify therapeutic responders from non-responders.

Impact of fatty acids on immunometabolism was further investigated by Xu et al, and Sawada et al. looking at the role of unsaturated long chain fatty acids on the immune system. Macrophages are highly responsive to their environment and adopt either a M1 proinflammatory profile characterised by a glycolytic activity or a M2 anti-inflammatory profile characterised by oxidative phosphorylation and fatty acid biosynthesis (2). Xu et al. identified that arachidonic acid metabolism was enriched in M2 macrophages and that manipulation of this pathway can promote or inhibit M2 differentiation. Interestingly, solid tumours can have immunomodulatory effects through these mechanisms. Furthermore, Sawada et al. reviewed the anti-inflammatory role of fatty acids, in particular Omega_3 fatty acids on different immune cell subsets. They provided an overview on how Omega_3 fatty acids and resolvins, products of Omega_3 fatty acids metabolism, affect immune cell survival, activity and migration as well as their therapeutic potentials in skin conditions including psoriasis, dermatitis, wound healing.

Finally, identifying strategies to prevent disease development associated with industrialised agricultural livestocks is critical. In this context Lagos et al. proposed feeding weaning pigs on a yeast protein diet could have numerous benefits. On top of being an environmentally friendly source of protein, it also improved gut homeostasis in weaning piglets with little to no impact on their immunity. Weaning, piglets are at high risks of developing inflammatory diseases requiring heavy use of antibiotics, which then enters the human food chain and is associated risks of antibiotic resistance. Hence there approach might alleviate the environmental impact of meat production as well as have direct health effects on humans.

To conclude, this Research Topic highlighted that further research is required to elucidate the effects of modern lifestyle on health and wellbeing of us humans but beyond on the survival of the planet. When contrasting our evolutionary past to the modern lifestyle of sedentary behaviour and nutritional abundance it becomes clear that there are more lessons to be learnt to address the health challenges of our time.

AUTHOR CONTRIBUTIONS

LM, OG, RN were editors for this topic and wrote the commentary. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Cyberlindnera jadinii Yeast as a Protein Source for Weaned Piglets—Impact on Immune Response and Gut Microbiota

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Supplying novel feed ingredients for pig production is crucial to enhance food security and decrease the environmental impact of meat production. Several studies have focused on evaluating the beneficial health effects of yeast in pigs. However, its use as a protein source has been partially addressed. Previously, we have shown that yeast at high inclusion levels maintains growth performance and digestibility, while nutrient digestibility, intestinal villi height and fecal consistency were improved. The present study combined microbiome, short-chain fatty acid, and immune parameter analysis to investigate the effect of high inclusion of yeast in diets for post-weaning piglets. Our results showed that yeast did not have a significant impact on the hematological or biochemical parameters in blood. The different immune cell subpopulations isolated from blood and distal jejunal lymph nodes (DJLN) were analyzed by flow cytometry and showed that yeast diet induced an increased number of the subtype of leukocytes CD45+/CD3–/CD8+, a special type of Natural Killer (NK) cells. Also, a very mild to moderate infiltration of neutrophilic granulocytes and lower IgA level were observed in the colon of yeast fed piglets. The microbiome profiling in different compartments of the gastrointestinal tract of piglets was performed using 16S rRNA metabarcoding. The results showed that 40% replacement of dietary protein had a statistically significant effect on the microbial communities in cecum and colon, while the microbial population in ileum and jejunum were not affected. Analysis of predicted microbial metabolic pathways analysis revealed significant upregulation of short-chain fatty acids, ether lipid metabolisms, secondary bile acids, and several other important biosynthesis pathways in cecum and colon of pigs fed yeast. In conclusion, the results showed that diet containing 40% of yeast protein positively shaped microbial community in the large intestine and increased the number of a specific subpopulation of NK cells in the DJLN. These results showed that yeast modulates the microbiome and decreases the secretion of IgA in the colon of post-weaning pigs.

Keywords: post-weaning pig, novel protein, yeast, health, microbiota

INTRODUCTION

Weaning is a critical period in a pig's life due to abrupt dietary, social, and environmental changes (1). This stressful period induces a decreased feed intake resulting in intestinal inflammation, increased susceptibility to infection, unbalanced gut microbiota, and hence post-weaning diarrhea (2). Antibiotic growth promoters (AGPs) have been extensively incorporated into pig feeds to help overcome post-weaning stress; however, cross-resistance of bacteria to antibiotics and the European ban of AGPs (EC 1831/2003) has led to a search for alternatives (3). Given the major role of antibiotics in gut microbiota dysbiosis, awareness in public health, and concerns about the spread of multiresistant bacteria, there is an urgent need to develop non-antibiotic based strategies to restore microbial balance and control diseases associated with the weaning period (4). Accordingly, probiotics and prebiotics have been used as gut microbiota modulators reducing pathogenic colonization, consequently improving animal health and welfare (5).

Supplying novel feed ingredients for pig production is crucial to enhance food security and decrease the environmental impact of meat production. Animal production can no longer continue to depend on protein ingredients that are questioned for both ethical and economic reasons (6). Thus, yeast produced from non-food resources such as second-generation sugars represents a high-quality protein and, as such, is a competitive and sustainable novel feed ingredient (7). Yeast or yeast products have been shown to promote growth performance, modulating gut microbiome and positively affect the immune system, thus reducing post-weaning diarrhea (8). Several studies have focused on evaluating the beneficial health effects of yeast in pigs (9, 10). However, its use has been limited to additives, while its health effects when used as a protein source as been partially addressed. The presence of a non-digestible cell wall in the yeast could have adverse effects on the nutritional value when used at high inclusion levels. Also, the composition and functional properties of different yeast strains can vary substantially depending on the yeast strain and processing conditions (7). Cruz et al. (11) have shown that locally produced dry yeast strain of *Candida utilis* (*C. utilis*), which is the anamorphic name of *Cyberlindnera jadinii*, can replace up to 40% of dietary crude protein while supporting high growth performance, improve digestive function, and thus reducing the incidence of diarrhea.

Gut microbiota plays a crucial role in intestinal morphology, regulation of immunity, digestion of carbohydrates, and health in livestock (12). Moreover, the composition of gut microbiota is affected by many factors, such as age, health status, and diet. For instance, Kiros et al. (13) have shown that the effect of live yeast (*Saccharomyces cerevisiae*) supplementation on the composition of the large intestinal microbiota was more pronounced when provided during the post-weaning period. The results showed that the microbiota composition was phylogenetically more homogeneous than those of control piglets, suggesting that the positive effects attributed to yeast may be partially due to its ability to modify the composition of gut microbiota (14).

The impact of yeast-derived components such as cell wall β-glucans and mannan-oligosaccharides on the gut microbiota in pigs has also been studied. Fouhse and co-workers reported a high relative abundance of *Mitsuokella* and a low relative abundance of *Coprococcus* and *Roseburia* in the cecum of piglets supplemented with yeast-derived mannan-rich fraction (14). While supplementation of yeast ingredients does seem to promote distinct intestinal bacterial groups, the modulation of short-chain fatty acid (SCFA) producing bacteria may be another intrinsic feature of such diets. Acetic, propionic, and butyric acid are among the most abundant SCFAs found in the gut. Acetate production is widely distributed among bacteria, and it is difficult to relate it to a specific bacterial group (15). However, propionic acid is mainly produced by *Prevotellaceae* and *Bacteroidiaceae* families or the *Negativicutes* class. It has been shown that SCFA have several key functions related to gut homeostasis and health (16–18).

The main objective of the present study was to evaluate the effect of inactivated *Cyberlindnera jadinii* yeast when used in higher inclusion levels as a protein source on the microbial profile and metabolites, and immune parameters in post-weaned piglets.

RESULTS

Effect of Yeast on Blood Parameter

The hematological analysis did not reveal a significant difference in the number of lymphocytes, monocytes, or neutrophils between pigs fed control and experimental diet (CJ40), both at 7 and 28 days post-weaning; however, we observed a significantly lower number of platelets at day 28 on pigs fed yeast (**Figure 1A**). We also observed that piglets fed yeast showed a substantially lower amount of hemoglobin after 28 days compared to that observed at 7 days (**Figure 1B**). All parameters are shown in **Table S1**.

Regarding the biochemical parameters in serum, there were no significant differences in the levels of aspartate aminotransferase (AST); however, the concentration of alkaline phosphatase (AP) decreased in the control pigs at 28 days compared to the control and CJ40 fed pigs at 7 days. In the yeast fed pigs the decrease in AP at 28 days compared to day 7 was no significant (**Figure 1C**). The results of all biochemical parameters are shown in **Table S2**.

Effect of Yeast on Immune Cells

Leukocytes were isolated from blood at 7 and 28 days, distal jejunal lymph node (DJLN) at 28 days and analyzed by flow cytometry. As shown in **Figure 2A**, the diet containing yeast induced an increasing number of CD3–/CD8+ cells in DJLN at 28 days post-weaning, but this increase was not observed in the blood or other subpopulations of leukocytes (**Figures 2B,C**). The gating strategy used in this study is presented in **Figure S1**. There was no statistical difference in plasma levels of IL-1A, IL1B, IL-1RA, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and TNFα between the control and experimental group on day 7 (**Table 1**) and 28 (**Table 2**) in this experiment, while IL-2 and GMCSF were undetectable at both time points.

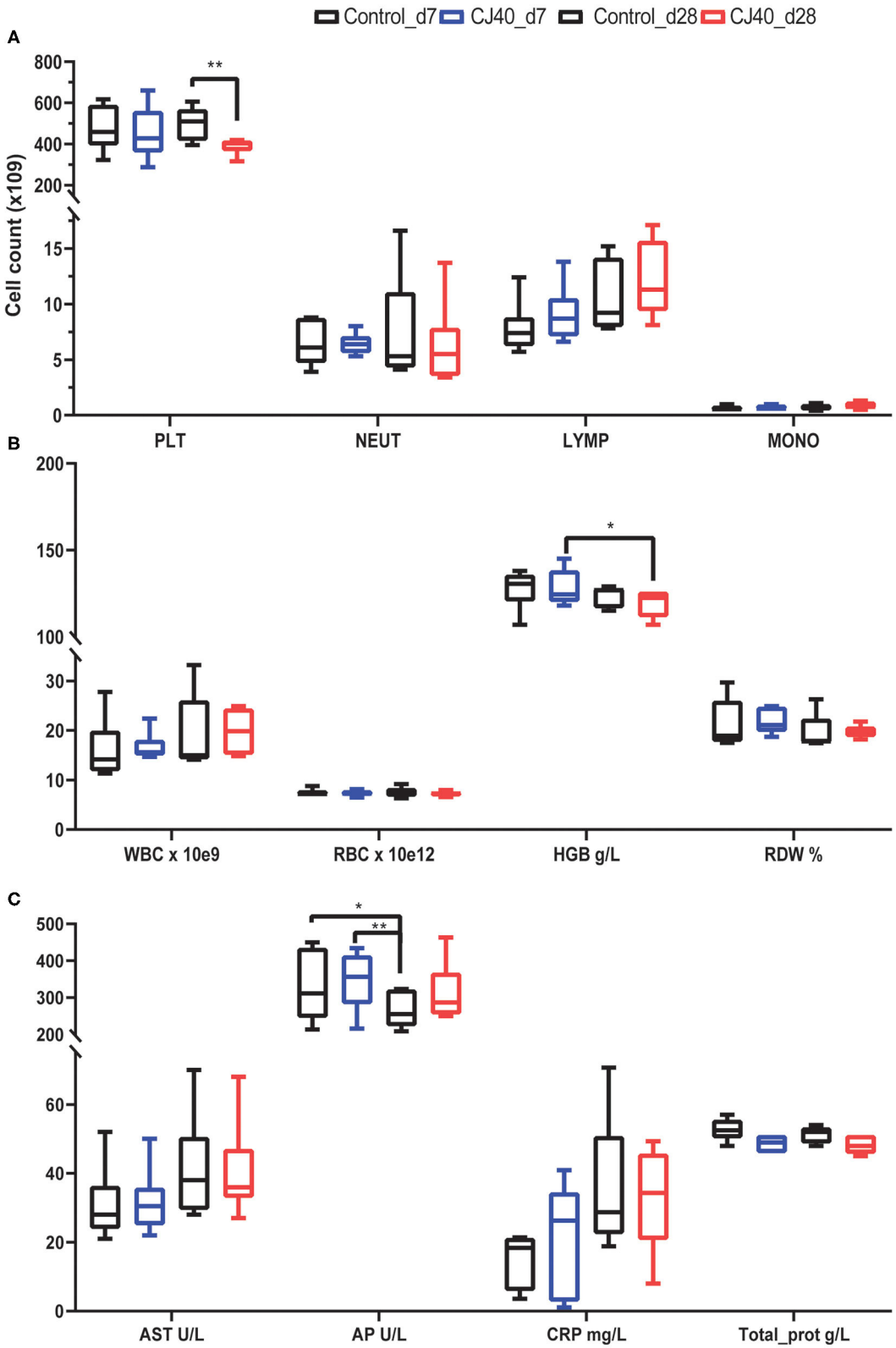

FIGURE 1 | Effect of yeast on selected hematological and biochemical parameters in the blood at 7 and 28 days post-weaning. **(A)** Bar plots showing the number of platelets (PLT), neutrophils (NEUT), lymphocytes (LYMP), and monocytes (MONO) at 7 (d7) or 28 days (28 d) after receiving a control diet or 40% yeast (CJ40). **(B)** Bar plots showing the number of white blood cells (WBC), red blood cells (RBC), the concentration of hemoglobin in grams per Liter (HGB), and percentage of red blood cell distribution width (RDW). **(C)** Biochemical metabolic profile in serum, aspartate aminotransferase (AST), alkaline phosphatase (AP), C-reactive protein (CRP), total protein (Total_prot). $n = 6$ per group. Asterisk represents statistical difference, $^{*}p = 0.03$, $^{**}p = 0.002$.

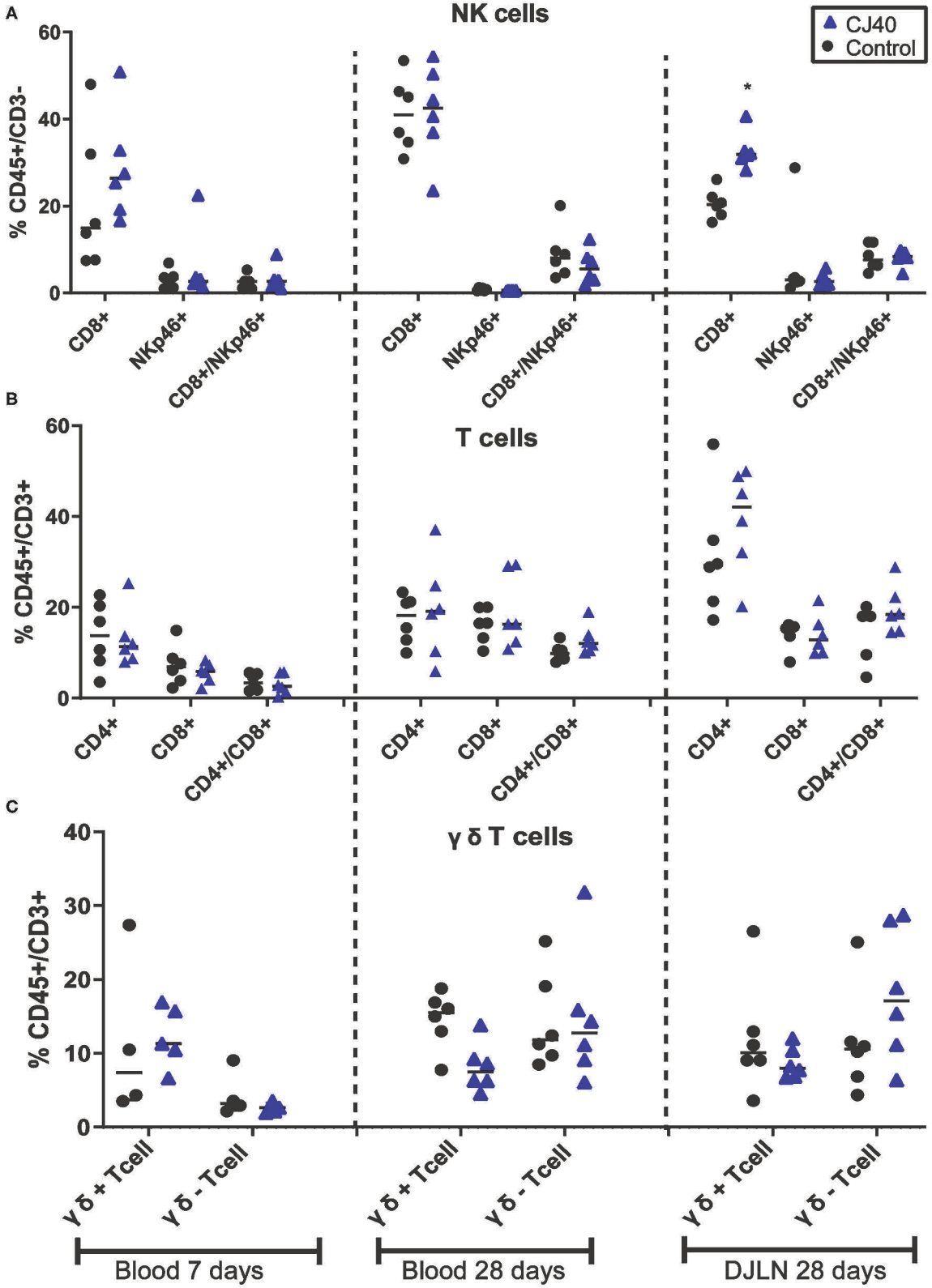

FIGURE 2 | Effect of yeast on relative immune population size in blood and distal jejunal lymph node (DJLN). Flow cytometric analysis of Natural Killers cells **(A)**, T cells **(B)**, and γδ T cells **(C)** out of the total peripheral blood mononuclear cells at 7 and 28 days or DJLN at 28 days. $n = 6$ per group. The asterisk represents the statistical difference, $^*p = 0.03$.

TABLE 1 | Inflammatory responses of weaned piglets fed a control diet or a 40% yeast protein diet at 7 days (Mean ± SD).

ng/ml	Control	CJ40	*P*
IFNγ	7.5 ± 0.5	8.3 ± 0.8	0.93
IL1a	0.01[a]	0.01[a]	>0.99
IL1b	UD	UD	
IL1ra	0.98 ± 0.3	0.14 ± 0.03	0.99
IL4	UD	UD	
IL6	UD	UD	
IL8	2.22 ± 1.2	0.50 ± 0.02	0.87
IL10	0.06 ± 0.003	0.03 ± 0.002	>0.99
IL12	0.72 ± 0.09	0.87 ± 0.07	>0.99
IL18	0.51 ± 0.19	0.22 ± 0.01	>0.99
TNFα	0.35 ± 0.2	0.35 ± 0.2	>0.99

The letters indicate that cytokine was detected in just one sample. UD, undetected.

TABLE 2 | Inflammatory responses of weaned piglets fed a control diet or a 40% yeast protein diet at 28 days (Mean ± SD).

ng/ml	Control	CJ40	*P*
IFNγ	6.83 ± 0.8	6.4 ± 0.7	0.99
IL1a	0.01[a]	0.102 ± 0.06	>0.99
IL1b	UD	1.28 ± 0.8	0.77
IL1ra	0.14 ± 0.04	0.8 ± 0.55	0.99
IL4	UD	0.35 ± 0.15	>0.99
IL6	0.07[a]	0.99 ± 0.7	0.99
IL8	1.32 ± 0.53	0.89 ± 0.21	0.99
IL10	0.04 ± 0 003	1.3 ± 0.91	0.73
IL12	0.85 ± 0.08	0.86 ± 0.11	>0.99
IL18	0.34 ± 0.09	1.17 ± 0.73	0.93
TNFα	0.24 ± 0.13	0.11 ± 0.02	>0.99

The letters indicating that cytokine was detected in just one sample. UD, undetected.

Effect of Yeast on the Expression of Genes

The gene expression of CLDN4 and TJP1 with roles in the regulation of tight junctions proteins across the intestinal epithelium was measured in the colon at 28 days post-weaning. In addition, colonic mRNA expression of toll-like receptor, TLR4, was assessed as several previous studies in pigs and poultry reported that dietary yeast supplementation could modulate the expression of pattern recognition receptors in the gut including the TLR4, thus stimulating the innate immune response (19–21). Similarly, the expression of IL1b and IL6 genes encoding the cytokines of the innate immune system was measured to study the effect of yeast on the inflammatory response. However, none of the genes included in the study showed significantly different expression levels between the diets (**Figure S2**). Because of the observed increase in the NK cell number in the DJLN of pigs fed the yeast diet, the expression of a larger set of genes with the role in immunity and NK cell function was measured. Among the selected genes were those coding for cytokines IL10, IL12a, IL18, IL6, and IL13, receptors of activated NK-cells KLRK1 (NKG2D) and NCR1 (NKp46), chemokines CXCL8 and CCL22 and FOXP3 marker of T reg. Similarly to the qPCR results in the colon, the measured mRNA gene expression profiles in DJLN showed no significant difference between the diets (**Figure S3**).

Histology of the Colon

Histopathological examination of the colon of piglets fed control or yeast diets revealed only very mild to moderate changes in the intestinal tissues of both groups (**Figure 3**). Semi-quantitative assessment of changes in the colon showed an increased presence of neutrophils in the lamina propria of piglets fed the yeast diet. A diagnosis of very mild to moderate neutrophilic colitis was made in more piglets from the yeast (8/11) than the control fed group (5/12) (**Figure 3**). No statistical differences were detected between the mean scores of the histopathological parameters.

SCFA and IgA

The short-chain fatty acids (SCFAs) and IgA levels in plasma, colon content and colon tissue were measured by LC-MS and ELISA, respectively. Regarding IgA levels, there was a tendency of lower levels of IgA in plasma ($p < 0.9$) and colon content ($p < 0.2$) in piglets fed yeast compared to the control group (**Figure 4A**), although the difference was not significant. However, in colon tissue, the levels of IgA were significantly lower ($p < 0.01$) in piglets fed yeast when compared to the piglets fed the control diet (**Figure 4A**). Moreover, pigs fed yeast showed higher levels of propionic acid in the colon content ($p < 0.05$; **Figure 4B**). We did not observe a significant difference in the levels of acetic, isobutyric, butyric, isovaleric, or valeric acid.

Effect of Yeast on Intestinal Microbiota

Analysis of microbial composition was performed on ileum, jejunum, cecum, and colon contents collected on day 28 of the experiment. The addition of 40% yeast did not significantly affect Shannon α-diversity indexes in ileum and jejunum compared to the control. The α-diversity was slightly but significantly decreased in cecum and colon samples ($\alpha = 95\%$) of the yeast fed piglets (**Figure 5A**). The Bray–Curtis dissimilarity measurements show that the inclusion of 40% of yeast protein to the diet had no impact on β-diversity in the ileum and jejunum (**Figure 5B**). Statistically significant differences were observed for the microbial populations in the cecum (PERMANOVA: pseudo—$F = 2.1$, $p = 0.03$, and $q = 0.04$) and colon (PERMANOVA: pseudo—$F = 1.9$, $p = 0.05$, and $q = 0.06$). The PCA analysis shows close clustering of the samples within two-dimensional coordinates (**Figures 6A,B**). However, no significant difference was observed in jejunum or ileum (**Figures 6C,D**). We have identified 21 differentially abundant genera in the cecum (**Table 3**), on average, representing 0.004 and 0.011% of the total OTUs (control and CJ40 diets, respectively). Out of those, 19 taxa retain the same differential abundance profiles in colon samples. *Phocea* spp. and *Roseburia* spp. were not found in colon digesta samples.

The replacement of 40% of protein diet with yeast decreased relative frequencies of *Butyricicoccus*, *Fournierella*, *Oscillibacter*, *Roseburia*, and *Streptomyces* spp., which are described as producers of natural antibiotics and butyrate in the cecum. At

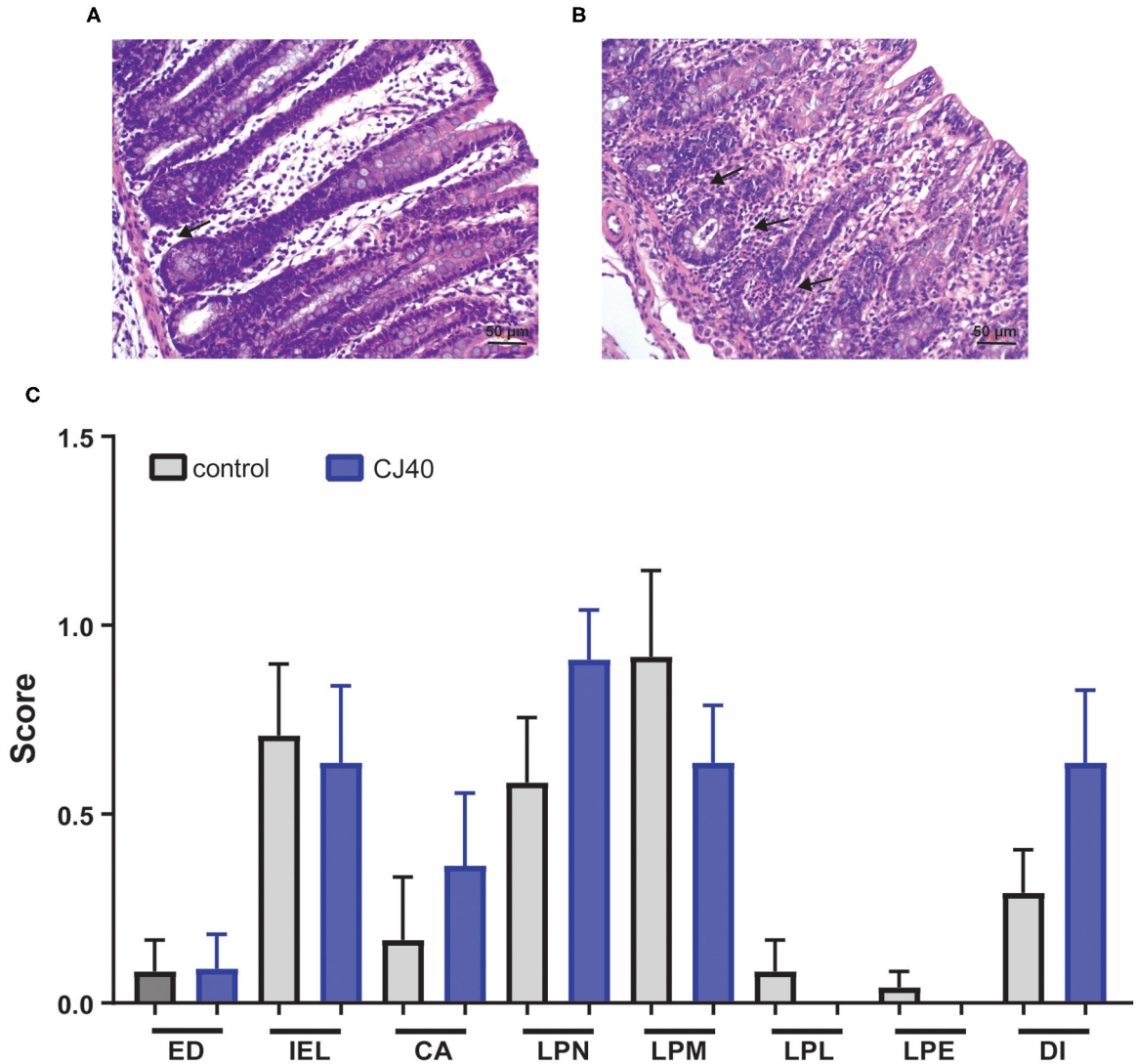

FIGURE 3 | Histology of the colon. **(A)** Colon of piglet fed the control diet, few neutrophils (black arrow) are present in the lamina propria. Bar 50 µm. **(B)** Colon of piglet fed CJ40 yeast diet. A moderate diffuse presence of neutrophils (black arrows) is evident. Bar 50 µm. **(C)** Histopathologic evaluation of colon of piglets fed the control (control) or CJ40 yeast diet. The mean and SD of semi-quantitative scoring of histopathological parameters are presented. No pathology was scored 0, very mild changes were scored 0.5; mild changes 1.0; mild-moderate changes 1.5; and moderate changes 2.0. The histopathological parameters evaluated were epithelial damage (ED); intra-epithelial lymphocytes (IEL); crypt abscess (CA); lamina propria neutrophils (LPN); lamina propria macrophages active (LPM); lamina propria lymphocytes/plasma cells (LPL); lamina propria eosinophils (LPE); and diagnosis colitis (DI). $n = 12$ for the control group and $n = 11$ for yeast fed group. Mann–Whitney testing of parameters did not reveal statistical differences, $p > 0.05$.

the same time, the propionate producing *Phascolarctobacterium* spp. was 1.4 fold more abundant in the cecum samples obtained from the animals fed with CJ40, which correlates well with the increased levels of propionic acid measured in the colon samples, as shown in **Figure 7B**. We also identify a strong correlation between *Prevotella* and propionic acid in both compartments, colon and cecum (**Figures 7A,B**). The least noticeable positive fold change in abundance (1.14) was observed for *Bacteroides* spp. The highest positive fold change (12.03) was observed for *Blautia* spp., which possess powerful quorum sensing autoinducers. Analysis of associations between the groups of differentially abundant species revealed predominantly mutualistic relations, and no statistically significant signs of biological competition were discovered in the cecum (**Figure 7A**) and colon digesta (**Figure 7B**).

The functional predictions for differentially abundant microbial communities using KEGG database integrated into PICRUSt pipeline revealed a statistically significant ($p \leq 0.05$) impact of the high yeast protein diets on 23 KEGG bacterial metabolic pathways in the cecum and 121 KEGG in bacterial metabolic pathways in colon. Among those, at least 9 pathways in the cecum (**Table 4**) and 10 pathways in the colon (**Table 5**) may

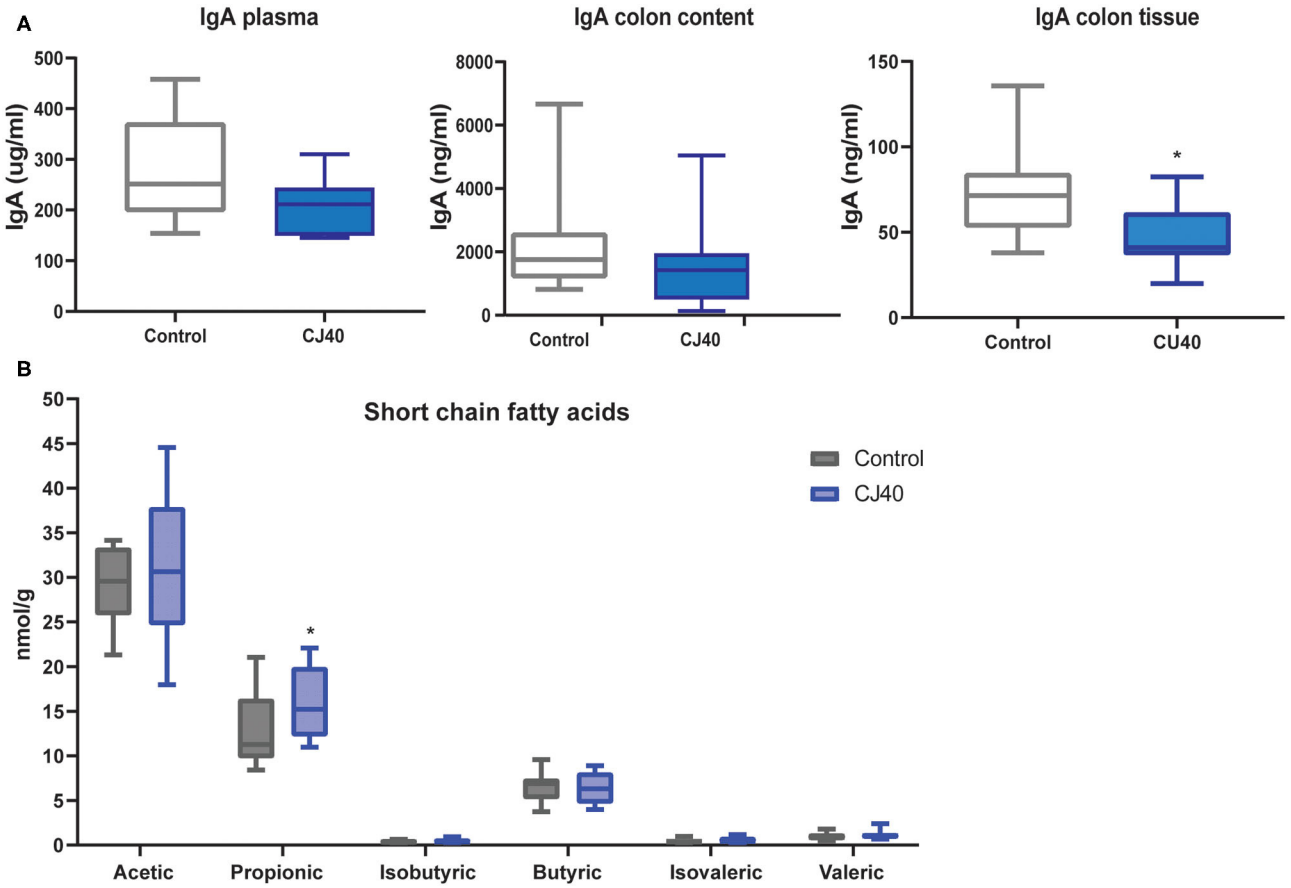

FIGURE 4 | Effect on IgA and short-chain fatty acids in piglets fed 40% yeast or control diet. **(A)** Plasma, colon content, and colon tissue immunoglobulin (IgA) concentration in pigs fed diets supplemented with 40% yeast (CJ40) or the control diet (control) at 28 days after weaning. **(B)** Short-chain fatty acid concentration in the colon digesta of piglets fed 40% yeast (CJ40) or the control diet (control) at 28 days post-weaning. $n = 12$ per group. The asterisk represents significant differences $p = 0.05$.

directly affect host performance during feeding experiments. The yeast diet had a strong positive effect on the abundance of microbial glycosphingolipid biosynthesis pathways in the cecum (708.42 fold change). In comparison, other pathways were less abundant (0.0004–0.87 fold change). The yeast diets had the greatest impact on the abundances of cyanoamino acid metabolic pathways (3325.98 fold change) and ether lipid metabolic pathways (1,115.18 fold change) in the colon. Moreover, yeast diet had a positive impact on the abundances of fatty acid biosynthesis pathways (6.08–8.69 fold change) and flavonoid biosynthesis pathways (1.89–3.18 fold change). Folate biosynthesis pathways, arachidonic acid biosynthesis pathway, as well as pathways for various glycerolipids and glycerophospholipids, were slightly less abundant in the samples collected from colon of animals fed yeast diet.

DISCUSSION

In the present study, we investigated whether yeast, when used as an alternative protein source, influence immune function and modulate microbiota in post-weaning pigs. As shown in our previous study (11), high inclusion levels of *Cyberlindnera jadinii* yeast do not affect growth performance or feed intake but increased apparent total tract digestibility of crude protein. In addition, yeast increases intestinal villi height, suggesting an increase in intestinal absorption and gut health. In this study, the effect of high inclusion of yeast on serum and DJLN, gut microbiota composition in four intestinal segments and SCFAs in the colon was investigated in post-weaning piglets. The results showed that yeast regulated the microbiota composition, selectively changing the propionic acid profile in the large intestine and increasing the number of a specific subpopulation of NK cells in the DJLN.

The proper functioning of the immune system is crucial for the transition from milk to solid feed in the weaning period. Regarding the type of immune cells assessed by flow cytometry, yeast diet induced an increased number of the subtype of leukocytes CD45+/CD3–/CD8+ in the DJLN. These cells have been described as NK cells and are numerous in blood and among splenocytes. In lambs, the recruitment of activated NK cells in the intestine is associated with the mobilization

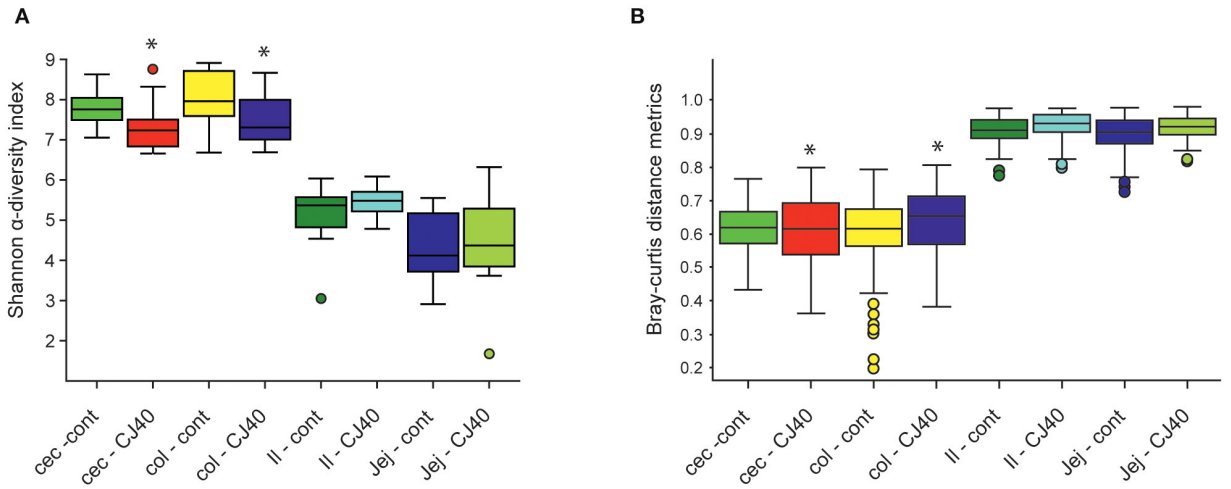

FIGURE 5 | Effects of the diet containing 40% yeast protein on **(A)** α-diversity, **(B)** β-beta diversity of microbial populations in ileum, jejunum, cecum, and colon samples. The statistically significant differences were observed for α-diversity metrics for cecum (Kruskal–Wallis: $H = 4.32$, $p = 0.04$, and $q = 0.06$) and colon (Kruskal–Wallis: $H = 3.85$, $p = 0.05$, $q = 0.07$) samples; β-beta diversity metrics for cecum (PERMANOVA: pseudo—$F = 2.1$, $p = 0.03$, and $q = 0.04$) and colon (PERMANOVA: pseudo—$F = 1.9$, $p = 0.05$, and $q = 0.06$) samples. Asterisk represent significant difference. Inclusion of 40% yeast protein had no statistically significant impact on α-diversity metrics neither for ileum (Kruskal–Wallis: $H = 0.32$, $p = 0.57$, and $q = 0.59$) and jejunum (Kruskal–Wallis: $H = 0.39$, $p = 0.53$, and $q = 0.58$) samples nor for β-beta diversity metrics for ileum (PERMANOVA: pseudo—$F = 0.52$, $p = 0.901$, and $q = 0.90$) and jejunum (PERMANOVA: pseudo—$F = 0.52$, $p = 0.834$, and $q = 0.86$) samples. $n = 12$ per group.

of innate immune responses to experimental cryptosporidiosis (22). NK cells participate in the early protective response against pathogens and secrete inflammatory cytokines to activate the adaptive immune system. Porcine NK cells have been shown to possess properties associated with antigen-presenting cells allowing them to stimulate T cell proliferation (23). Liu et al. (24) have shown the impact of early feeding on the number of this type of NK. In the current study, pigs that were fed a diet containing yeast exhibited a higher number of NK cell populations than pig fed the control diet. Because of these results, we assessed the presence of inflammatory cytokines that might be secreted by this subpopulation of cells. Although, we did not observe a significant difference in the secretion of cytokines a numerical increase was observed in the secretion of IL-10 and IL-18 at 28 days in the blood of pigs fed yeast, while a numerical lower secretion of IL-8 at 7 and 28 days was observed in the blood of pigs from the same group. Interleukin 18 is a pro-inflammatory cytokine and is key to promoting the production of interferon-gamma by NK cells against viral, fungal, bacterial and parasitic infections (25). On the other hand, IL-10, which is described as a cytokine synthesis inhibitory factor (26), is an important factor in shaping tolerant NK cells (27).

Regarding IL-8, several studies have reported that IL-8 can enhance the invasion and metastasis of solid tumors (28), and modulate the function of NK cells through the inhibition of receptors such as NKp30, NKG2D, and granzyme B (29). However, it is important to note that we did not measure cytokines in the DJLN, which might explain the low levels found in plasma and suggest a local effect rather than a systemic effect of yeast. Interestingly, a very mild to moderate infiltration of neutrophilic granulocytes was observed in the colon of more of the yeast fed piglets than the piglets fed the control diet. The presence of neutrophils in the lamina propria of the colon could suggest a local pro-inflammatory environment facilitating the mobilization of innate mucosal immune responses during weaning stress (30). Neutrophils were also present in the lamina propria of some piglets in the control group, justifying a diagnosis of very mild to mild colitis. The digestive tract of weaned piglets can be sensitive to antinutritional factors present in ingredients of the control and yeast diets such as soybean meal (31).

The present study indicates that yeast did not have a significant impact on the hematological or biochemical parameters in blood, except for the platelets count. The diet containing a high level of yeast induced significantly lower blood platelet counts at 28 days post-weaning compared with the control group; however, this value was within the value range for a healthy pig (32). Furthermore, the high inclusion level of yeast in the diet did not affect the serum concentration of AST or AP. These two enzymes are known to be increased in response to acute liver injury or liver toxicity (33). Therefore, we suggest that feed yeast for 4 weeks did not induce evident damage to the liver of piglets post-weaning.

Even though immunoglobulin G (IgG) is the main isotype in serum, a significant number of antibody-secreting cells circulating in blood secrete IgA (34). Several studies have confirmed that IgA producing cells reactive against gut-encountered antigens can be found outside the gut (35). In the present study, the yeast diet did not have a significant effect on the level of IgA in plasma or colon content at 28 days post-weaning. However, in colon tissue, piglets fed yeast for 28 days presented a significantly lower level of IgA. This result correlates with the infiltration of neutrophils observed by histology in

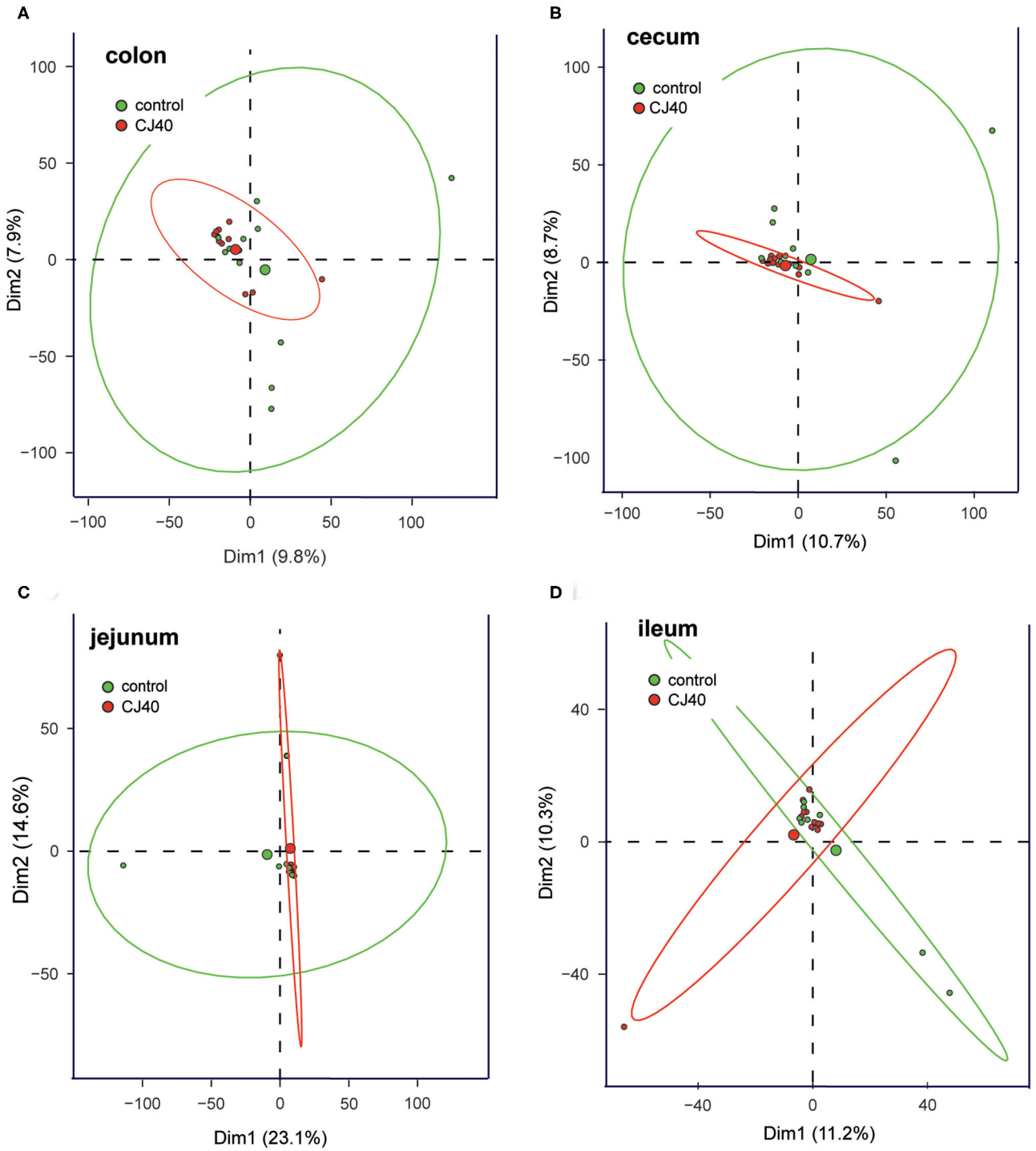

FIGURE 6 | PCA of the effects of the diet containing 40% yeast protein on the relative abundances of microbial communities in **(A)** cecum, **(B)** ileum, **(C)** jejunum, and **(D)** colon samples. The ellipsoids represent confidence intervals ($p \geq 95\%$) and the centroids represent medians for the respective groups of samples. $n = 12$ per group.

the same group. The ability of yeast to stimulate mucosal immunity needs further investigation, especially in long term feeding trials.

We also investigated the response of microbes and the secretion of SCFAs in pigs fed yeast using 16S rRNA gene sequencing and gas chromatography. Our results showed

TABLE 3 | Taxonomic characteristics and relative frequencies of differentially abundant groups of microorganisms identified in cecum digesta samples (AM, arithmetic mean).

	Control diet ($n = 12$)		CJ40 diet ($n = 12$)			
Genus	**AM × 10^{-5}**	**SD × 10^{-5}**	**AM × 10^{-5}**	**SD × 10^{-5}**	**Fold change**	**Gram's**
Anaerofustis	0.14	0.25	0.40	0.49	2.91	+
Bacteroides	1.33	4.16	1.51	3.54	1.14	−
Barnesiella	1.18	3.07	2.52	8.74	2.14	−
Blautia	0.11	0.37	1.27	2.39	12.03	+
Breznakia	4.52	8.52	11.83	18.65	2.61	+
Butyricicoccus	0.44	0.73	0.39	1.25	0.88	+
Clostridium	0.64	0.93	3.11	3.53	4.90	+
Cupriavidus	42.53	75.17	207.24	181.46	4.87	−
Dialister	225.16	432.59	799.12	758.40	3.55	−
Ethanoligenens	1.77	4.16	2.65	5.71	1.50	+
Eubacterium	0.56	1.29	0.77	1.52	1.37	+
Fournierella	0.00	0.00	0.70	1.28	0.70	+
Mitsuokella	26.22	29.59	31.36	33.06	1.20	−
Moraxella	0.54	1.32	0.72	1.90	1.34	−
Oscillibacter	2.10	2.40	1.81	0.90	0.86	−
Papillibacter	0.21	0.72	0.26	0.62	1.25	+
Phascolarctobacterium	2.44	4.43	3.43	5.31	1.41	−
Phocea	0.24	0.82	0.36	0.85	1.54	−
Prevotella	43.06	29.59	65.52	30.07	1.52	−
Roseburia	0.67	1.62	0.36	1.25	0.54	+
Streptomyces	2.11	4.42	0.56	1.95	0.27	+

agreement with previous studies (36) that the microbiota of the ileum and jejunum were structurally different from that of the large intestine (colon and cecum) and the composition and metabolism of both small and large intestinal microbiota were affected by yeast diet. The availability of yeast on the intestine and the preferential substrate utilization of microbes were the major factors that affected the composition of the microbiota. In our study, we detected a difference in the alpha bacterial diversity of the large intestine between the yeast and the control groups based on the Shannon index, where the control group appeared more diverse at the amplicon level than the group fed yeast. The yeast diet markedly increased the abundance of some bacteria from the family *Lachnospiraceae*, *Veillonellaceae*, and *Burkholderiaceae*, while decreasing the abundance of species such as *Butyricicoccus*, *Fournierella*, *Oscillibacter*, *Roseburia*, and *Streptomyces* spp. in the cecum of pigs fed the yeast diet. Even though the analysis of the microbiota composition was limited to the sequencing of the 16S rRNA bacterial gene only, the overrepresentation of the *Lachnospiraceae* family might be related to the availability of the non-digested dried yeast cells in the diet.

We found that *Clostridium* was positively correlated with the high propionic levels observed in the colon of yeats fed pigs. Besides the role of *Blautia* in acetate and propionate production, our results suggest that *Dialister* and *Cupriavidus* may have a direct contribution to the propionic pool production. Propionate is used as an energy substrate of peripheral tissues, and its health effect goes beyond the gut epithelium, as it can lower serum cholesterol levels, lipogenesis, and carcinogenesis risk. Propionate may also decrease obesity (37). Some human colonic bacteria belonging to the *Negativicute*s class of Firmicutes, such as *Dialister*, have the ability to convert succinate to propionate (38). Propionate and butyrate are formed as products from rhamnose and fucose sugars or peptides and amino acid fermentation by dominant gut commensal bacteria belonging to the Lachnospiraceae, including *Roseburia* and *Blautia* species (38, 39). It is possible to speculate that the presence of yeast cell wall glucans in the feed affects the microbial diversity by selecting microbes able to degrade these glucans.

Using a KEGG pathway analysis, we found that cyanoamino and ether lipid metabolisms were significantly upregulated in the colon of pigs fed the yeast diet. Furthermore, the biosynthesis of secondary bile acids and siderophores was significantly upregulated in the cecum of pig belonging to the same group. In this regard, several studies in mice and pigs have shown that antimicrobials (AMA), besides its effect on weight gain by altering gut microbial ecology, also can cause variations in the biosynthesis of bile acids (BA) (40, 41). Accordingly, it has been suggested that alteration on BA metabolism might be the mechanism used by AMA to promote growth (41). Ipharraguerre et al. (42) have shown that the combination of zinc oxide with different AMA promotes body weight and alters the metabolism of BA, as well as improving immune tolerance and barrier function of the intestinal mucosa. These studies provide evidence that BA has an important role mediating physiological,

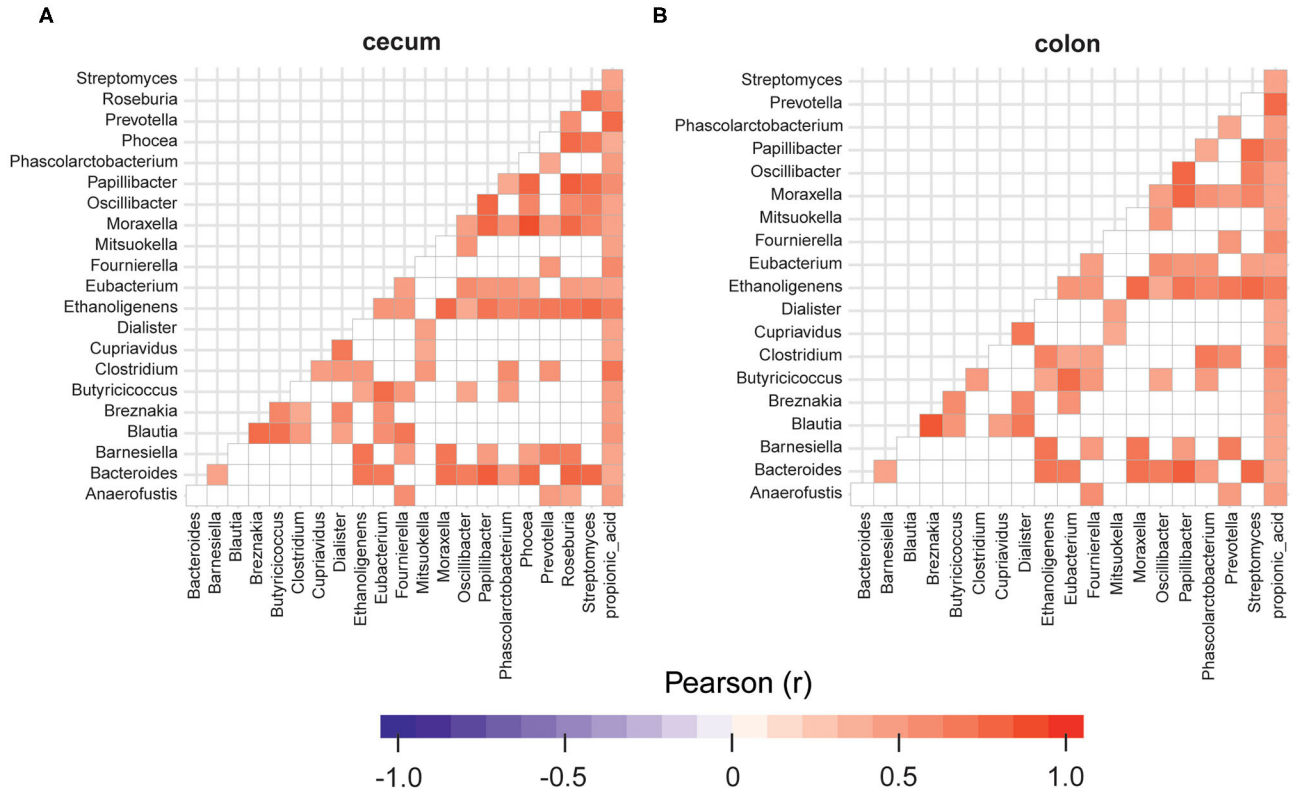

FIGURE 7 | Relationships between the groups of differentially abundant microorganisms identified in cecum **(A)** and colon digesta **(B)** of the animals fed with the diet containing 40% yeast protein. The red color corresponds to a statistically significant positive correlation ($p \geq 95\%$) and the blue color corresponds to a negative correlation. Insignificant values are colored in white. $n = 12$ per group.

TABLE 4 | Effect of the 40% yeast protein diet on the PICRUSt predicted frequencies of the selected bacterial metabolic pathways in Cecum (AM, arithmetic mean).

	Control diet		CJ40			
Function	**AM**	**SD**	**AM**	**SD**	**Fold-change**	***p*-value**
Bios. of secondary bile acids	271	140	174,034	45,487	708.42	0.037
Bios. of siderophores	50,780	19,928	165,660	48,498	1.05	0.051
Bios. of unsaturated fatty acids	241,302	51,298	107,624	32,681	0.87	0.003
Bios. of vancomycin group antibiotics	180,378	43,546	78,936	18,449	0.32	0.053
Biotin metabolism	378,470	108,202	73,471	20,620	0.08	0.081
Butanoate metabolism	1,181,758	282,339	67,934	17,397	0.06	0.062
Butirosin and neomycin biosynthesis	185,896	57,132	3,882	2,135	0.01	0.003
C5-Branched dibasic acid metabolism	613,123	165,638	372	444	0.0004	0.009
Carbohydrate digestion and absorption	72,409	24,135	1,126	869	0.0004	0.031

metabolic, and immune response, hence directly affecting growth performance. Nonetheless, the effect of yeast on the biosynthesis of BA, its profile in different tissues and its impact on feed utilization or growth in piglets needs further studies, including long term feeding.

CONCLUSIONS

The present study combining microbiome, short-chain fatty acid, and immune parameter analysis demonstrated that the diet containing high levels of yeast altered the gut microbial composition and increased the number of NK cells in the DJLN as well as the production of propionate in colon.

MATERIALS AND METHODS

Ethical Statement

All animals were handled following the applicable laws and regulations controlling experiments with live animals in Norway (Animal Welfare Act 2009 and the local legislation derived from the directive 2010/63 EU of the European Parliament and

TABLE 5 | Effect of 40% yeast protein diet on the PICRUSt predicted frequencies of the selected bacterial metabolic pathways in Colon (AM, arithmetic mean).

	Control diet		CJ40			
Function	**AM**	**SD**	**AM**	**SD**	**Fold-change**	***p*-value**
Cyanoamino acid metabolism	794,450.3	198,432.3	318,739.7	57,151	3,325.98	0.037
Ether lipid metabolism	2,085.25	1,109.904	2,325,427	401,621.4	1,115.18	0.04
Fatty acid biosynthesis	864,818.6	175,356.1	1,449,150	230,273.7	8.69	0.025
Fatty acid metabolism	354,265.4	68,911.81	572,066.5	108,274.5	6.08	0.009
Flavone and flavonol biosynthesis	23,155	11,894.66	2,748,178	488,339.2	3.18	0.019
Flavonoid biosynthesis	3,020.25	2,088.753	79,611.42	21,956.94	1.89	0.005
Folate biosynthesis	945,813.3	204,335.8	334,858.1	52,752.45	0.47	0.049
Glycan biosynthesis and metabolism	73,331.67	18,027.98	194,750.3	61,105.2	0.17	0.008
Glycerolipid metabolism	714,030	149,426.9	97,386.25	20,604.07	0.10	0.003
Glycerophospholipid metabolism	1,160,996	246,872	276.4167	285.2614	0.0038	0.034

Council of September 2010 on the protection of animals used for scientific purposes). The experiment was approved by the Norwegian Food Safety Authority (identification number 11314) and was performed at the Center for Livestock Production, Norwegian University of Life Sciences, Aas, Norway.

Experimental Design

Forty-eight crossbred piglets (Norwegian Landrace × Yorkshire × Duroc) at ∼30 days of age and an average initial body weight of 11.06 kg ± 0.84 SD, were equally distributed by litter, gender, and weight and randomly allotted to two dietary treatments, with 12 replicates per treatment. Each dietary group consisted of three replicate pens, with four piglets per pen. At the stipulated feeding times, each pig was separated from the others in an individual feeding stall for 30 min to measure individual feed intake. The experimental diets consisted of replacement of the primary sources of crude protein (CP), soybean meal, potato protein concentrate, fishmeal, and rapeseed meal with drum dried and inactivated *Cyberlindnera jadinii* corresponding to 40% of the total CP content. The yeast used in this study was produced in Lallemand, Estonia, using the lignocellulosic biomass from the Norwegian spruce tree (*Picea abies*) as a growth media as described in Cruz et al. (11). The diets were coded as control and CJ40 (40% CP from yeast). The diet composition is shown in **Table S3**. Piglets were fed three times per day during the first 14 days and two times per day during the remaining period. Feed was provided *ad libitum* during restrictive periods and the amounts of feed were adjusted weekly, based on the estimated feed intake of 3–5% of the live body weight. Water was accessible *ad libitum* via automatic drinkers. On the last day of the experiment, the piglets were euthanized with a captive bolt pistol. Intestinal content and tissue samples were collected for further analysis.

Blood Sampling and Analysis

Blood samples were collected from six piglets per diet at 7 and 28 feeding days. Samples were taken in the morning 1–2 h post-prandial by venipuncture of the jugular vein while keeping the animal on its back. Both plasma and serum were collected. For serum, blood was collected using the vacutainer with gel separator (VACUETTE® TUBE CAT Serum Separator Clot Activator) and kept for 30 min in an upright position followed by centrifugation at 2,000 × g for 10 min at room temperature and immediately stored at −20°C and then later on the same day transferred to −80°C. Serum was used for the analyses of enzymes (e.g., ALT, AST) metabolites (e.g., albumin, glucose, creatinine, and urea), acute-phase protein CRP, total protein, and globulins (alpha, beta; **Table S2**). For plasma, blood was collected in EDTA coated vacutainer (Beckman Dickson Vacutainer System), centrifugated at 2,000 × g for 10 min at 4°C and immediately stored at −20°C and then at −80°C. Plasma samples were used for cytokine and chemokine analyses (Immunology Multiplex Assay) and IgA ELISA assay. Samples of non-centrifuged whole blood collected in EDTA vacutainer were used for hematological indices; erythrocyte count (RBC), leukocyte count (total leukocyte count and differential leukocyte count), platelets (PLT), red blood cell distribution width (RDW), hemoglobin (HGB), mean cell volume (MCV), mean corpuscular hemoglobin (MCH; **Table S1**).

The analysis was performed with an Advia® 2120 Hematology System using Advia 2120 MultiSpecies System Software, while serum was tested using Advia 1800 Chemistry System (Siemens healthcare diagnostics Inc., Tarrytown, NY 10591, United States) at the Central Laboratory, Norwegian University of Life Sciences.

For flow cytometry analysis, whole blood was diluted 1:1 in RPMI 1640 and kept on ice until single-cell isolation. Peripheral blood mononuclear cells (PBMCs) were recovered using a density gradient Percoll ($d = 1.77$; Sigma–Aldrich) after centrifugation at 1,200 × g, 30 min without brake at 20°C and washed twice in PBS with 2 mM EDTA. After counting, isolated PBMCs were incubated with Fixable Yellow Dead Cell Stain Kit (Life Technologies, Thermo Fisher Scientific Inc.) followed by primary monoclonal antibodies (mAbs), brief incubation with 30% normal pig serum to block Fc-receptors, and finally fluorescence-labeled secondary antibodies. To detect the intracellular CD3 epitope, surface-labeled cells were permeabilized with Intracellular Fixation and Permeabilization Buffer Set (eBioscience, Affymetrix Inc.) according to the manufacturer's instructions. Labeled cells were analyzed in a

Gallios flow cytometer and data were processed using Kaluza 1.5 software (both Beckman Coulter, Inc.). Cell gates were designed to select for single and viable mononuclear cells. Defined markers were used to identify the different immune subpopulations. To detect T cells, the following antibodies were used: CD45, CD3, TCR γ/δ, CD4, CD8, FOXp3, and CD25. To identify T and NK cells, we used CD45, CD8, NKp46, CD4, Ki67, and CD27. The list of antibodies is shown in **Table S4** and the gating strategy is shown in **Figure S1**.

Quantification of GMCSF, IFNγ, IL-1A, IL1B, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and TNFα was measured in serum samples using MILLIPLEX MAP Porcine Cytokine and Chemokine Magnetic Bead Panel—Immunology Multiplex Assay (Merck Millipore) following the manufacturer's instructions.

Gene Expression Analysis

Total RNA was extracted from colon and DJLN of twelve control diet-fed piglets and twelve yeast fed piglets following the RNeasy Plus Universal Kits protocol (Qiagen). The RNA concentration and quality were determined using NanoDrop TM 8000 spectrophotometer (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies). All samples had a RIN-value ≥ 7. The cDNA synthesis was performed according to the AffinityScript qPCR cDNA synthesis protocol (Agilent Technologies). The qPCR reactions were performed in a total volume of 20 μL using 10 μL of LightCycler 480 SYBR Green I Master, 2 μL primers (5 μM), 3 μL H2O, and 5 μL template. The PCR conditions were as follow: 95°C 10 min, 40 cycles of 95°C 10 s, 60–64°C 10, and 72°C 10 s. As a final step, a melting curve was included. Samples were analyzed using LightCycler® 480 System (Roche Diagnostics) and calculated as $2^{-\Delta\Delta Cp}$. The sequences of primers used for qPCR are listed in **Table S5**.

Histology

As described in detail in previously (11), at 28 days post-weaning, tissue segments were collected from the colon of 12 and 11 piglets fed either control or CJ40 diets, respectively. Formalin-fixed, paraffin-embedded tissues were cut in 4 μm thick sections before routine staining with hematoxylin and eosin. The tissue sections from the colon were evaluated histopathologically by a pathologist (CPÅ) and scored semi-quantitatively where no pathology was scored 0, very mild changes were scored 0.5; mild changes 1.0; mild-moderate changes 1.5; and moderate changes 2.0. The histopathological parameters evaluated were epithelial damage (ED); intra-epithelial lymphocytes (IEL); crypt abscess (CA); lamina propria neutrophils (LPN); lamina propria macrophages active (LPM); lamina propria lymphocytes/plasma cells (LPL); lamina propria eosinophils (LPE); and diagnosis colitis (DI).

Short-Chain Fatty Acids (SCFAs) Analysis

The SCFAs from colon microbiota were determined by gas chromatography (Trace 1300, Thermo Fischer), equipped with a flame ionization detector. Standards of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and 2-methyl valeric acid in 5% formic acid (as internal standard) were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, United States) and were of HPLC grade with >99% purity. Before the SCFAs analysis, 500 mg of colon content were mixed with 500 μl of cold internal standard solution and sonicated for 5 min in cold water. Then, centrifugate at 4°C with 15,000 g 15 min, the supernatant was transferred to a spin column (45 kDa) centrifugate and the resulting supernatant was injected into a capillary column (30 m × 250 μm × 0.25 μm, Restek Corporation, Bellefonte, PA, USA). The column starting temperature was 90°C (2 min) followed by 10°C/min until 150°C, then 50°C/min until 250°C (1 min).

ELISA

The concentrations of immunoglobulin A (IgA) in the plasma, colon tissue and colon content were measured with commercial kits [Pig IgA ELISA Kit (ab190536), Abcam, Cambridge, UK]. The assays were performed in duplicate and according to the manufacturer's instructions. To extract proteins from colon tissue and colon content, ∼60 mg of each samples was added to a 2 mL tube containing 1 mL of lysate buffer (Tris 20 mM, NaCl 100 mM, Triton X-100 0.05%, EDTA 5 mM, and protease inhibitor cocktail) and one 5 mm Stainless Steel Beads (Qiagen, Hilden, Germany). Samples were homogenized twice for 1.5 min at 20 Hz using a TissueLyser (Qiagen, Hilden, Germany). After homogenizing, the samples were centrifuged at 4°C, 15,000 × g for 25 min. The supernatant was aliquoted into four tubes and stored at −80°C until further analyzes. Total protein concentration was measured in the supernatant from each sample using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. Samples were normalized to the same protein concentration.

DNA Extraction, Sequencing, and Data Processing for Microbiota Profiling

At the end of the experiment, digesta of ileum, jejunum, colon, and cecum, were collected and immediately frozen in liquid nitrogen and then stored at −80°C until processing. Total genomic DNA was extracted using QIAamp Fast DNA Stool kit (QIAGEN) according to the manufacturer's instructions. The extracted DNA was quantified, accessed for purity and used for 16S metabarcoding with the primers specific to V1-V3 hypervariable region of 16S rRNA gene (27F−5′-AGAGTTTGATCCTGGCTCAG−3′ and 534R−5′-ATTACCGCGGCTGCTGG3′). The amplicons were generated using Illumina 2× 300 bp chemistry at GATC-Biotech, Germany, but only forward reads were used for subsequent data analysis.

The analysis of microbial communities was done using 2018.8 version of the Quantitative Insights into Microbial Ecology (QIIME2) pipeline (43). The taxonomic analysis was performed using 99% full-length sequences of the GreenGenes 13.8 bacterial 16S subset database (44). The samples from the ileum, jejunum, colon, and cecum were analyzed separately. Raw sequence reads were quality score filtered to retain sequences with quality score > Q20. The sequence quality control and OTU table construction were performed using DADA2 (45) algorithm within QIIME2 pipeline. The resulting OTU tables were used for subsequent

α and β diversity analysis. For differential abundance analysis, the OTU tables were filtered to retain sequences occurring in at least three samples and having at least 11 copies per dataset. The filtered OTU tables were further used to estimate α and β diversity in the filtered datasets to ensure that the filtering of the datasets did not affect initial biological conclusions. The filtered datasets were then used for differential abundance analysis with Gneiss algorithm (46). The relative frequencies of differentially abundant taxa and concentrations of propionic acid measured in colon samples were used for correlation analysis through the calculation of the Pearson correlation coefficient (47) and respective *p*-values (48). The calculation of correlation metrics and visualization were performed in R using ggplot2 package (49). Only correlation coefficients with $p \leq 0.05$ were used to draw conclusions.

The differentially abundant taxa were manually curated and analyzed for their potential functions using KEGG database (50) integrated into PICRUSt 1.1.3 pipeline (51). Predicted functions were hierarchically collapsed into KEGG level 3 pathways using standard PICRUSt tools and further analyzed using the two-tailed homoscedastic *t*-test in R. The raw sequencing data were deposited at NCBI nucleic acid collection under the bioproject PRJNA531397.

Statistical Analysis

Non-parametric data from flow cytometry were analyzed by Kruskal–Wallis followed by *post-hoc* Dunn's test with a comparison of mean rank. D'Agostino and Pearson normality test was used to test the normal distribution of data from the histopathological scoring. A non-parametric Mann–Whitney test was used to compare the mean scores of the histopathological parameters. The qPCR results were analyzed using Student *t*-test, were $p \leq 0.05$ was considered a significant difference.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/, PRJNA531397.

ETHICS STATEMENT

The animal study was reviewed and approved by the Norwegian Food Safety Authority (identification number 11314).

AUTHOR CONTRIBUTIONS

LL, AS, AB, CP, and MØ designed the experiment. LL, AS, and AB conducted animal study, lab, and statistical analysis. LL, AS, AB, CÅ, CP, and RÅ participate in the sampling and analysis. AB performed sequencing and bioinformatic analysis. LL and AS data visualization. LL and AB wrote the manuscript with the input of other co-authors. All the authors 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/fimmu.2020.01924/full#supplementary-material

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Effects of Blood Flow Restriction on Leukocyte Profile and Muscle Damage

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Muscle damage affects the blood leukocyte profile. Resistance exercise (RE) with blood flow restriction (BFR) attenuates exercise-induced muscle damage (EIMD).

Purpose: To evaluate muscle damage and the leukocyte profile in response to RE+BFR and to compare with high intensity RE.

Methods: Twenty volunteers performed the RE in the leg press apparatus in the following groups: RE80, 80% of 1RM (3 × until concentric muscle failure); RE40+BFR, 40% of 1RM with BFR (same total work of RE80 group). The BFR applied was 80% of the total occlusion pressure.

Results: There were no differences in the blood leukocyte profile among groups despite the lower exercise-induced muscle damage (EIMD) in the RE40+BFR group (RE80: 10.07 ± 2.67 vs. RE40+BFR: 8.25 ± 0.96; cell × 10^3/mm^3). Both groups showed leukocytosis (RE80: 7.59 ± 1.48 vs. 10.07 ± 2.67 and RE40+BFR: 6.57 ± 1.50 vs. 8.25 ± 0.96; cell × 10^3/mm^3) and lymphocytosis (RE80: 2.48 ± 0.83 vs. 3.65 ± 1.31 and RE40+BFR: 2.22 ± 0.23 vs. 3.03 ± 0.65; cell × 10^3/mm^3) immediately after exercise. Leukocytosis (ES 1.12 vs. ES 1.33) and lymphocytosis (ES 1.11 vs. ES 1.76) was greater in the RE40+BFR group.

Conclusion: RE associated with BFR was accompanied by a greater leukocytosis and lymphocytosis immediately after exercise, with no difference in neutrophils. This leukocyte blood profile may be related to less muscle damage, as well as faster muscle recovery after 24 and 48 h post-exercise.

Keywords: Kaatsu training, exercise-induced muscle damage, lymphocytosis, neutrophils, monocytes

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INTRODUCTION

The understanding of the changes in the leukocyte profile in face of different types of acute and chronic exercises has been frequently investigated in healthy and in clinical populations. While evidence suggests that chronic exercise has an anti-inflammatory effect, the impact of acute exercise needs to be better understood (Schlagheck et al., 2020). Leukocytosis is often associated with infection and/or inflammation. However, it is also present in the exercise as a transitory phenomenon, returning to pre-exercise levels between 6 to 24 h after its end (Simpson et al., 2015).

Muscle damage affects the blood leukocyte profile (Peake et al., 2017). Studies demonstrated the mobilization of neutrophils and monocytes after exercise (Peake et al., 2005; Neubauer et al., 2008). Lymphocytes are also mobilized with exercise, however, during the initial stages of recovery, lymphocytopenia occurs (Simpson et al., 2015). This would be linked to greater vulnerability to disease during the recovery phase of exercise, which is known as "open window" (Simpson et al., 2015). These changes have different magnitudes depending on the type, intensity and duration of the exercise, as well as physical fitness, age and other variables (Hulmi et al., 2010). In contrast, a more recent interpretation of the lymphocytopenia has been raised. It has been showed that lymphocytes are redistributed to potential sites of infection rather than a real decrease and thus would actually be related to a better immune response (Campbell and Turner, 2018). Recent studies have demonstrated that accumulation of these cells in the injured muscle has a fundamental role in its repair and regeneration post-exercise (Peake et al., 2017; Deyhle and Hyldahl, 2018).

Exercise-induced muscle damage (EIMD) is molecularly characterized by transient ultrastructural myofibrillar disruption and efflux of myocellular enzymes and proteins such as creatine kinase (CK) (Peake et al., 2017). This myofibrillar disruption leads to functional loss of muscle strength and power and increase in delayed-onset muscle soreness (DOMS). The EIMD seems to be related to the intensity of exercise and muscle contractions at 80 and 100% of maximal voluntary contraction (MVC) as well as exercise with pronounced eccentric muscle actions produce large muscle damage than exercise protocols using lighter loads, such as 40 and 60% of MVC (Chen et al., 2007).

Resistance exercise (RE) combined with blood flow restriction (BFR) is a training method that consists in applying pressure cuffs placed in the proximal region of the limbs which are inflated to a set pressure throughout exercise (Loenneke et al., 2013). BFR training has been studied as an exercise strategy for patients who are contra-indicated from performing traditional heavy-load resistance exercise. BFR training can induce skeletal muscle strength and hypertrophy even in the presence of lower muscle damage (Franz et al., 2017, 2018).

Few studies, however, have analyzed changes in the acute leukocyte profile associated with muscle damage in resistance exercise with BFR. Thus, the aim of this study was to analyze the acute effects of high intensity resistance exercise and low intensity resistance exercise combined with BFR on leukocyte profile and its association with EIMD. Our study hypothesis is that RE+BFR would induce less leukocytosis due to the lower EIMD compared to the RE group alone.

MATERIALS AND METHODS

Experimental Approach to the Problem

The participants visited the laboratory six times for data collection. At the first visit, the participants were familiarized with 1RM testing procedure, BFR and the vertical jump performance test. At the second visit (after 48 h) anthropometric measurements and one-repetition maximum test were obtained. In the third visit (after 48 h), it was determined the blood flow restriction pressure and retest the maximum repetition. The fourth visit was 7 days after the third one. In the fourth visit the participants performed the resistance exercise protocol. The fifth and sixth (each one after 24 h) visit consisted of performing only blood collections and measures of muscle damage. The present study used a randomized design to perform the exercise protocol.

Participants

Twenty healthy men volunteers (**Table 1**), with aged from 18 to 36 years, who had been involved in regular RE for at least 1 year and at least 3 days per week, were enrolled in this study. Sample size was determined using GPower 3.1 software with a statistical power of 80% and medium effect size of 0.15. The following exclusion criteria were adopted: (i) use of drugs that could affect cardiorespiratory responses; (ii) bone-, joint- or muscle-diagnosed problems that could limit the execution of elbow flexor; (iii) systemic hypertension (≥140/90 mmHg or use of antihypertensive medication); (iv) metabolic disease; and (v) use of exogenous anabolic–androgenic steroids, toxic drugs or medication with potential effects on physical performance.

All procedures and risks were explained to participants before they provided written consent to participate. This study was approved by the Ethics Committee Federal University of Espirito Santo. Participants were instructed to refrain from strenuous activities at least 72 h before the RE sessions and to avoid the use of any pain-relieving and anti-inflammatory drugs and to maintain their normal food intake and lifestyle habits throughout the study.

Exercise Protocols

The volunteers arrived at the lab between 8 and 8:30 am. Participants performed one of two different interventions on bilateral leg press exercise equated by total work (sets × repetitions × load): high intensity RE (RE80, $n = 10$): three sets of with 80% of 1RM until concentric muscle failure; and low intensity with blood flow restriction RE (RE40+BFR, $n = 10$): three sets of 25 repetitions with 40% of one repetition maximum (1RM) combined with 80% BFR. The number of repetitions in the RE40+BFR was calculated based on the total work achieved by the RE80 group (**Table 2**). Each group performed the leg press exercise with cadence fixed at 2 s at each concentric/eccentric muscle actions with 1 min of rest between sets.

Determination of the Blood Flow Restriction Pressure

Subjects were asked to lie on a supine position while resting comfortably. A vascular Doppler probe (DV-600, Martec, Ribeirão Preto, SP, Brazil) was placed over the tibial artery to

TABLE 1 | Subjects' main characteristics at baseline.

Groups	N	Age (years)	Height (cm)	BM (kg)	BMI (kg/m^2)
RE80	10	23.9 ± 5.2	167 ± 10	66.5 ± 11.5	23.6 ± 1.9
RE40 + BFR	10	26.0 ± 6.8	168 ± 8	69.8 ± 12.4	24.5 ± 3.2

TABLE 2 | Blood flow restriction and total work.

Groups	Blood flow restriction pressure (%)	Total blood flow restriction pressure (mmHg)	Total work (kg)
RE80	–	–	10,284 ± 3,695
RE40 +BFR	80	220 ± 39	11,576 ± 2,908

determine the BFR pressure (mmHg). A standard blood pressure cuff (width 18 cm; length 35 cm) attached to the proximal portion of thigh was inflated up to the point in which the auscultatory pulse of the tibial artery was interrupted. The BFR pressure was maintained constant throughout the exercise session. The cuff pressure used during the training protocol was determined as 80% of the necessary pressure for complete blood flow occlusion in a resting condition (Laurentino et al., 2012).

One-Repetition Maximum Test

The procedures adopted for 1RM test for the bilateral leg press exercise (Sickert, Brazil) were followed the recommendations described by Brown (Brown and Weir, 2001). In the first set, participants performed eight repetitions with a load correspondent to 50% of their estimated 1RM obtained during the familiarization session. In the second set, they performed three repetitions with 70% of their estimated 1RM. A 2-min interval was allowed between warm-up sets. After the completion of second set, participants rested for 3 min and then had up to five attempts to achieve their 1RM with 3-min interval enforced between attempts. The 1RM strength on the leg press exercise was recorded and reproduced throughout the study. Tests were conducted by an experienced researcher, and strong verbal encouragement was provided during the attempts.

Blood Lactate Concentration

After local cleansing of middle finger, participant's finger was lanced, and the capillary blood sample was collected using heparinized capillary tubes. The blood lactate concentration was determined with an electrochemical device (YSI 1500 Select; Yellow Springs, OH, United States).

Rating of Perceived Exertion and Pain

Immediately after each set, subjects were asked to report their rating of perceived exertion (RPE) and pain (RPP) using Borg's 6–20 scale (Vieira et al., 2015; Neto et al., 2016).

Blood Collection

Approximately 5 mL of blood samples were collected from the antecubital vein in vacutainer tubes containing EDTA in the moments before, immediately, 24 and 48 h after each exercise bout. Blood samples were centrifuged at 1,500 *g* for 10 min at 4°C and the serum was stored at −80°C for subsequent analysis for leukocytes, neutrophils, lymphocytes, monocytes, using a blood analyzer (Beckman Coulter T660; Beckman Coulter, Inc., Miami, Florida).

Indirect Markers of Muscle Damage

CK Levels Assay

The CK levels were carried in an automated biochemical analyzer Bioclin2200 using commercially available kits (Bioclin, Belo Horizonte, Brazil) following the manufacturer's specifications.

cfDNA Concentration Assay

A standard curve with seven concentrations was generated by serial dilution of commercial salmon sperm DNA (Sigma-Aldrich). The curve was evaluated in triplicate resulting a standard curve used to calculate the DNA concentrations. cfDNA concentrations were directly analyzed with a fluorescent nuclear stain (SYBR Gold) in serum samples. Briefly, SYBR Gold (1: 10,000 dilutions in PBS) was added to serum in 96-well black plates and fluorescence was recorded using a fluorometer Varioskan Flash (Thermo Fisher Scientific, Inc., Rockford, IL, United States) with an excitation wavelength of 485 nm, and emission wavelength of 535 nm (Goldshtein et al., 2009).

Vertical Jump Performance Test

Squat jump (SJ) and countermovement jump (CMJ) tests. For each protocol three jumps were performed with 30 s of interval between jumps. The SJ was carried out from a squatting position, with an approximately 90° of knee flexion and the hands fixed on the hip. This position was maintained for 3 s and then, upon verbal command, they jumped vertically to maximum height. No countermovement was allowed. In the CMJ the participants started from an erect standing position with knees fully extended (knee = 180°). Upon the verbal command, made a downward countermovement approximately to the same starting position as the SJ (knee = 90°) and then jumped vertically for maximum height in one continuous movement. A contact mat was used to perform (Jump Test-Hidrofit, Brazil). All the subjects had been previously familiarized with SJ and CMJ tests.

Statistical Analysis

Values were expressed as the mean ± standard deviation (SD) for all variables. Data were analyzed for normality (Gaussian distribution) using the Shapiro-Wilk test. As data were normally distributed, parametric analyzes were used. Statistical analyses were performed by two-way ANOVA to evaluate differences between trials and time-points. When the ANOVA showed a significant interaction effect, a Tukey's *post hoc* test was used to locate differences between variables. In addition, unpaired *t*-tests were used to analyze the differences between groups on total work. The statistical analyses were performed using Prism software (Prism 6, GraphPad Software, Inc., San Diego, CA, United States). A value of $p < 0.05$ was regarded as statistically significant.

In addition, the effect size (ES) and confidence interval 95% (CI) was used to verify the magnitudes of changes between assessments of the protocols as trivial (0–0.19), small (0.20–0.49), medium (0.50–0.79), large (0.80–1.29) and very large (1.30 or greater) (Rosenthal, 1996).

RESULTS

The **Figure 1A** shows blood lactate concentration after RE40+BFR and RE80. Blood lactate increased ∼3-fold compared to resting values in both groups. In spite of the statistically significant increase in blood lactate concentration after exercise ($p < 0.0001$) no differences were observed between the groups (RE40+BFR, 4.4 ± 1.0 vs. RE80, 4.8 ± 1.3, mmol/L) groups.

For RPP the two-way ANOVA indicated significant interaction [$F(2,36)$ 5.894; $p = 0.0061$] and main factor for time [$F(2,36)$ 56.07; $p < 0.0001$] without significant main effect for exercise condition [$F(1,18)$ 2.011; $p = 0.1733$]. RPP increased in set 2 and 3 in the RE40+BFR group, while in RE80 only in set 3 (**Figure 1B**). For RPE the two-way ANOVA indicated no significant interaction [$F(2,36)$ 3.145; $p = 0.0551$] or main factor for exercise condition [$F(1,18)$ 0.9464; $p = 0.3435$]. There was, however, a significant main effect for time [$F(2,36)$ 25.94; $p < 0.0001$]. RPE increased only at set 3 in both groups compared to set 1 (**Figure 1C**).

For SJ the two-way ANOVA indicated significant interaction [$F(3,54)$ 2.874; $p = 0.0445$] and main factor for both time [$F(3,54)$ 18.85; $p < 0.0001$] without significant main effect for exercise condition [$F(1,18)$ 0.1301; $p = 0.7226$]. SJ performance (**Figure 2A**) decreased significant immediately after exercise in both groups compared to pre-exercise values. However, only in RE80 the performance reduction persisted until 48 h after exercise.

For CMJ the two-way ANOVA indicated significant interaction [$F(3,54)$ 5,571; $p = 0.0021$] and main factor for time [$F(3,54)$ 17.44; $p < 0.0001$] without significant main effect for exercise condition [$F(1,18)$ 0.02198; $p = 0.8838$]. The CMJ performance (**Figure 2B**) had the same response of SJ, with a reduction in jump height immediately after exercise in both groups but still impaired after 48 h only in RE80.

For CK the two-way ANOVA indicated significant interaction [$F(3,54)$ 3.469; $p = 0.0227$] and main factor for time [$F(3,54)$ 7.119; $p < 0.0001$] without significant main effect for exercise condition [$F(1,18)$ 1.518; $p = 0.2347$]. Serum CK activity was statistically different only in RE80 group post-24 h compared to pre-values, without changes in the RE40+BFR group (**Figure 2C**). cfDNA (**Figure 2D**) increased significantly only in RE80 group immediately after exercise (406.3 ± 67.2, ES 0.80, CI −0.14–1.68) compared with pre (249.6 ± 82.2, ES 2.08, CI 0.92–3.07), without meaningful changes in the RE40+BFR group ($p = 0.11$).

The **Figure 3** shows the leukocytes profile response induced by the exercise protocols. For leukocytes the two-way ANOVA indicated no significant interaction [$F(3,54)$ 1.317; $p = 0.2785$] or main factor for exercise condition [$F(1,18)$ 3,906; $p = 0.0637$]. Only main effect for time [$F(3,54)$ 26.78; $p < 0.0001$] was observed. It can be observed that the total leukocytes increased immediately after exercise (10.07 ± 2.67 × 10^3/mm^3) compared with pre-exercise (7.59 ± 1.48, ES 1.12, CI 0.14–2.01) in RE80. The same was observed in the group RE40+BFR: immediately after exercise (8.25 ± 0.96) vs. pre-exercise (6.57 ± 1.50, ES 1.33, CI 0.31–2.24) (**Figure 3A**). Regarding lymphocytes, the same pattern was observed (**Figure 3B**). The two-way ANOVA indicated no significant interaction [$F(3,54)$ 1.79; $p = 0.1601$] or main factor for exercise condition [$F(1,18)$ 0.6513; $p = 0.4302$]. Only main effect for time [$F(3,54)$ 14.92; $p < 0.0001$] was observed. RE80 immediately after exercise (3.65 ± 1.31) vs. RE80 pre-exercise (2.48 ± 0.83, ES 1.11, CI 0.13–2.00) and RE40+BFR immediately after exercise (3.03 ± 0.65) vs. RE40+BFR pre-exercise (2.22 ± 0.23, ES 1.76, CI 0.67–2.71). However, no changes in neutrophils (**Figure 3C**): RE80 pre vs. immediately after exercise (4.30 ± 1.47 vs. 5.34 ± 2.07, ES 0.56, CI –0.36–1.42) and RE40+BFR pre vs. immediately after exercise (3.72 ± 1.51 vs. 4.43 ± 1.23, ES 0.51, CI −0.40–1.38), and monocytes (**Figure 3D**): RE80 pre vs. immediately after exercise (0.48 ± 0.18 vs. 0.71 ± 0.30, ES 0.93, CI −0.03–1.81) and RE40+BFR pre vs. immediately after exercise (0.37 ± 0.13 vs. 0.44 ± 0.15, ES 0.50, CI −0.41–1.37) were observed. There were no differences in the leukocyte profile in the post-exercise times 24 and 48 h in relation to the pre-exercise, as well as in the profiles of lymphocytes, neutrophils, and monocytes.

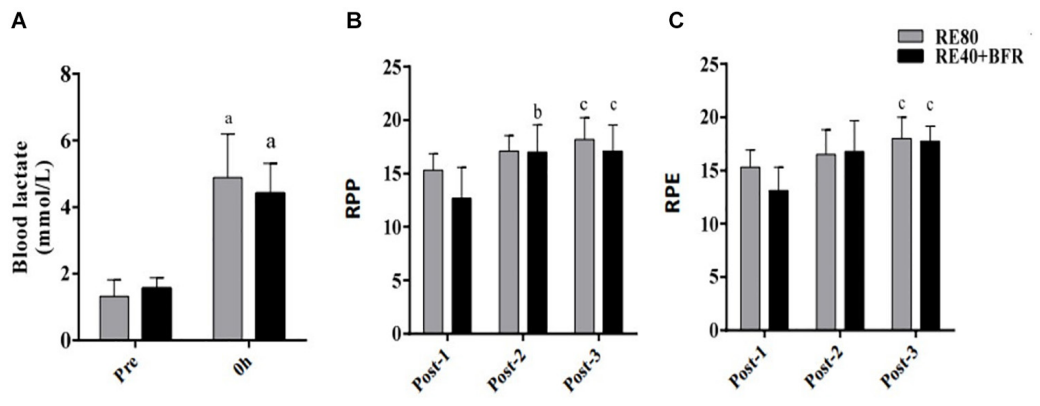

FIGURE 1 | Blood lactate concentration **(A)**, rating of perception pain **(B)**, and rating of perception exertion **(C)**. Data are shown by mean ± SD. $a = p < 0.05$ to both RE80 and RE4O+BFR vs. Pre, $b = p < 0.05$ Post-2 vs. Post-1 and $c = p < 0.05$ Post-3 vs. Post-1.

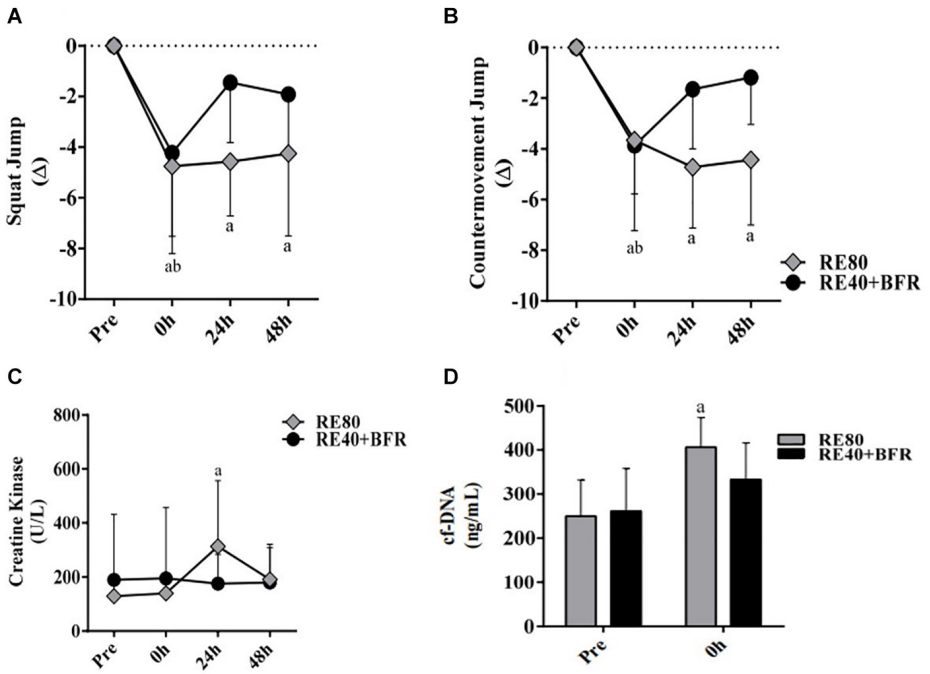

FIGURE 2 | EIMD markers. Squat jump performance **(A)**, countermovement jump performance **(B)**, Creative kinase **(C)** and Cell-free DNA **(D)**. The data are expressed as the mean ± SD. $a = p < 0.05$ RE80 compared with pre, and $b = p < 0.05$ to RE4O+BFR vs. Pre.

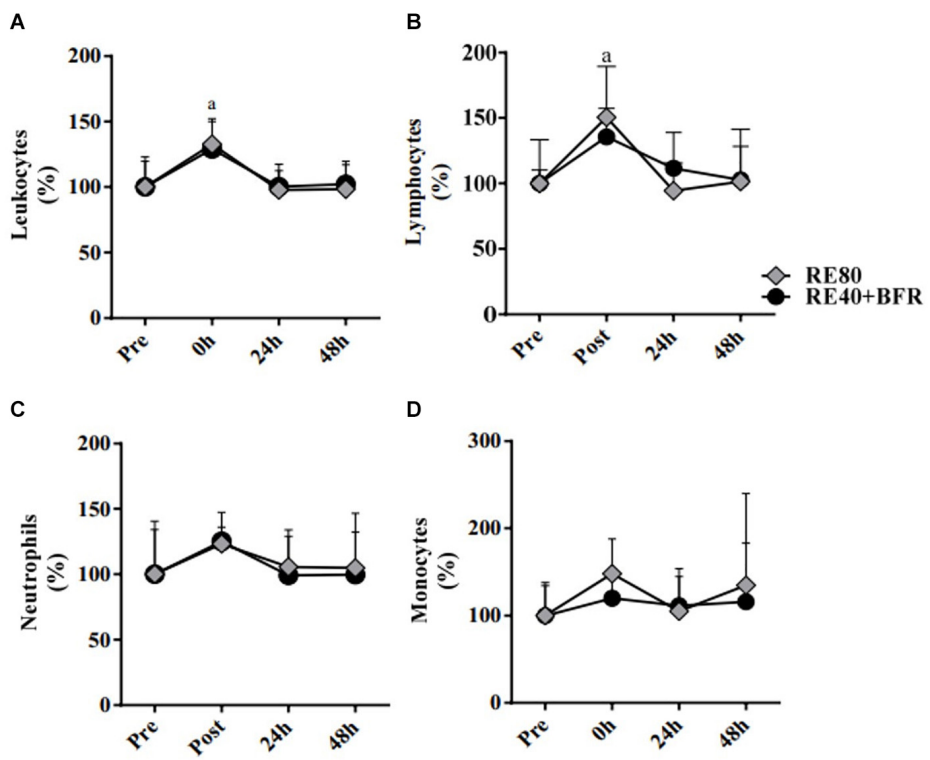

FIGURE 3 | White blood cells. Leukocytes **(A)**, Lymphocytes **(B)**, Neutrophils **(C)**, and Monocytes **(D)**. The data are expressed as the mean ± SD. $a = p < 0.05$ both RE4O+BER and RE80 compared with pre.

DISCUSSION

The aim of this study was to analyze the acute effects of high intensity resistance exercise and low intensity resistance exercise combined with BFR on leukocyte profile associated with EIMD. The main findings of our study were as follows: (1) there were no differences in the blood leukocyte profile of the RE40 + BFR group compared to the RE group, despite the lower EIMD in the RE40 + BFR, (2) both groups showed leukocytosis and lymphocytosis immediately after exercise, (3) leukocytosis and lymphocytosis was greater in the RE40 + BFR group which may help to explain the fast recovery after EIMD.

Historically, inflammation was recognized as a phenomenon that compromised post-exercise recovery. It is now accepted that the inflammatory responses occurs related to muscle damage and plays an important role in its regeneration and recovery (Peake et al., 2005). Mechanical and metabolic stress associated with EIMD, activates several types of cells in order to recover and remodel the injured muscle (Peake et al., 2017). Among these various cell types are the inflammatory cells such as lymphocytes, neutrophils and monocytes (Hyldahl and Hubal, 2014). Interestingly and unexpectedly, this smaller increase was accompanied by a larger effect size on lymphocytosis and leukocytosis, however, with no difference in effect size in relation to neutrophils.

The accumulation of leukocytes in the inflamed muscle is a gradual process that depends on the extent of the damage (Hyldahl et al., 2014). Studies report the presence of leukocytes in the muscle in response to moderate to severe muscle damage, usually induced by maximum eccentric exercise (Peake et al., 2017). There are several mechanisms by which these cells participate in the repair and regeneration of the damaged muscle. Neutrophils and macrophages act in the removal of cellular debris through phagocytosis and production of reactive species (Nguyen and Tidball, 2003; Arnold et al., 2007). Understanding the phenotypic transfer of pro-inflammatory macrophages (M1) to anti-inflammatory macrophages (M2) is essential, since M1 macrophages interact with the proliferation of satellite cells, while M2 macrophages participate in the differentiation of these satellite cells in addition to the synthesis of connective tissue (Tidball and Villalta, 2010; Schlagheck et al., 2020).

Although research has focused more on neutrophils and macrophages, recent studies have demonstrated a predominant role of lymphocytes in the regeneration process, cells that until then had been linked only to pathological muscle processes. Lymphocytes participate in the muscle repair process in two basic ways: regulate myogenic cell activity and regulate muscle immune cell infiltrate. CD8+ T-cells facilitate the expression of C-C motif chemokine ligand 2 (CCL2) by macrophages residing in the muscle, essential for the recruitment of pro-inflammatory monocytes in the injured muscle (Zhang et al., 2014). Regulatory T cells (Treg) support muscle regeneration through the expression of amphiregulin growth factor (AREG) (Burzyn et al., 2013). Burzyn et al. (2013) demonstrated that treatment with AREG normalizes the evolution of the muscle transcriptome throughout the muscle repair process and promotes myogenic differentiation *in vitro* (Burzyn et al., 2013). The absence or deficiency of Tregs in the muscles after injuries is related to decreased fiber growth and failure in phenotypic change from M1 to M2 and, therefore, exaggeration in the inflammatory process (Burzyn et al., 2013; Kuswanto et al., 2016). The main subpopulation responsible for the largest effect size also present in leukocytosis in the RE40 + BFR group was lymphocytes. therefore, we believe that lymphocytes are related to faster muscle recovery in the RE40 + BFR group compared to the RE80 group.

There are two mechanisms related to acute post-exercise Leukocytosis: (1) increased cardiac output and, consequently, blood flow in the pulmonary, hepatic and splenic vascular bed, which induces, through shear stress, leukocyte demargination; and (2) increase in the expression of β-2 adrenergic and glucocorticoids receptors in leukocytes, thus increasing their activation in response to adrenaline and glucocorticoids during exercise [13]. The greater activation of the sympathetic-adrenal-medullary axis accompanied by greater release of catecholamines that occurs in exercises with blood flow restriction (Spranger et al., 2015), may be the mechanism that explains the greater size of lymphocytosis effect observed in the RE40 + BFR group, facilitating thus the sequestration of these cells by the injured muscle.

Muscle function and performance are markers of EIMD (Peake et al., 2017). Although CK was different between conditions, both groups showed reduction in the vertical jump performance immediately after RE. Thus, we believe that the mechanism of reduced performance is different. Classically, RE cause EIMD by mechanical stress which is thought to represent the primary factor in muscle adaptive response (Tidball and Villalta, 2010) and explain the CK increase after 24 h. However, studies have shown that the reduction in exercise performed after BFR has been associated with decreased oxygen supply and increased metabolic stress (Downs et al., 2014). Since our results showed an increase in blood lactate immediately after exercise in the RE+BFR group we believe that the metabolic stress could explain the reduction in performance immediately after exercise.

Another interesting data observed in our study was the response of cfDNA, we and other have already demonstrated that it may be new marker of EIMD or a predictor of exercise performance 24 h after the exercise session (Atamaniuk et al., 2010; Andreatta et al., 2018). The second mechanism is reinforced by our data since cfDNA did not increased significantly immediately after exercise in the RE40+BFR group while exercise performance was already recovered 24 h after exercise. In addition, Tug et al. (2015) showed in 2015 that the majority of cfDNA released during aerobic exercise was derived from the hematopoietic system. One possible hypothesis could be the release of cfDNA from neutrophil, in a mechanism called neutrophil extracellular traps (NETs) (Brinkmann, 2004). Immediately after one aerobic exercise session shows increased NET-like structures in the blood (Beiter et al., 2014). However, both raised points require further investigation in future studies to deeply elucidate the contribution of different cell type in release cfDNA during both aerobic as well as strength exercise.

A limitation of this study is the fact that the leukocyte profile was analyzed only immediately after exercise and at times 24 and 48 h after. However, for a deeper understanding of the leukocyte profile dynamics it would be interestingly to evaluate

every 2–4 h. Also, it should be emphasized that only indirect markers of muscle EIMD were used in this study.

CONCLUSION

In summary, RE associated with BFR was accompanied by a greater leukocytosis and lymphocytosis immediately after exercise, with no difference in neutrophils. This leukocyte blood profile may be related to less muscle damage, as well as faster muscle recovery 24 and 48 h post-exercise. The results of the present study may have useful practical application, both in sports and clinical settings. The use of RE + BFR can be a valid alternative to promote gains in muscle mass and strength, imposing less overload on the joints and promoting faster recovery in muscle function between training sessions. It can, therefore, be an interesting strategy in the clinical environment for patients with functional limitations, and even for athletes during periods of high training volume, promoting improved recovery between training sessions and competitions.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

This study was approved by the Ethics Committee Federal University of Espírito Santo. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LS: discussion and manuscript writing. MA, VC, WM, and LF: protocols and data collection. VB: research orientation, discussion, and manuscript writing. All authors contributed to the article and approved the submitted version.

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frontiers in Physiology

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Vigorous Physical Activity Is Associated With Better Glycated Hemoglobin and Lower Fear of Hypoglycemia Scores in Youth With Type 1 Diabetes: A 2-Year Follow-Up Study

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To correlate glycated hemoglobin (HbA1c) and fear of hypoglycemia scores with physical activity (PA) levels in children and adolescents with type 1 diabetes (T1D) over a period of 2 years. Twenty-eight children and 33 adolescents with T1D have been assessed for their PA profile. Personal and medical data for the patients were collected at baseline (visit 0: V0), 1 year later (V1), and 2 years later (V2). At baseline, children with T1D engaged in less moderate to vigorous PA (MVPA) ($p < 0.01$) per day than adolescents. These results did not differ across visits. On the contrary, adolescents spent fewer time in vigorous physical activity (VPA) ($p < 0.01$) than children did ($p < 0.01$). Fear of hypoglycemia scores correlated significantly with VPA levels ($\beta = -0.41$, $p = 0.03$; $\beta = -0.44$, $p = 0.06$; $\beta = -0.61$, $p = 0.001$). For HbA1c (%), significant correlations were reported with VPA levels ($\beta = -0.54$, $p = 0.02$; $\beta = -0.47$, $p = 0.03$; $\beta = -0.62$, $p = 0.01$) across visits. Body mass index percentile correlated with total screen time ($\beta = 0.28$, $p = 0.02$; $\beta = 0.29$, $p = 0.01$; $\beta = 0.31$, $p = 0.04$) and overall PA levels ($\beta = -0.52$, $p = 0.02$; $\beta = -0.42$, $p = 0.03$; $\beta = -0.42$, $p = 0.01$). Performing more vigorous PA a day is associated with better HbA1c with lower perceived fear of hypoglycemia among youth with T1D. Therefore, dedicating more time in VPA may be an appropriate advice for patients with T1D.

Keywords: children, adolescent, type 1 diabetes, vigorous physical activity, glycated hemoglobin, fear of hypoglycemia, follow-up

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INTRODUCTION

In the context of type 1 diabetes (T1D) management, physical activity (PA) practices are widely recommended as a major preventive factor of the deleterious effects of T1D (Colberg et al., 2016). Indeed, to obtain optimum benefits from PA engagement, the Centers for Disease Control and Prevention, the American Heart Association, and the American Diabetes Association (Colberg et al., 2016) recommended that children and adolescents with T1D engage in at least 60 min/day of moderate- to vigorous-intensity aerobic activity, including vigorous muscle-strengthening and bone-strengthening activities at least 3 days/week.

Regular exercise can improve health and well-being and can help individuals achieve their target fitness and glycemic goals, and higher levels of PA are associated with improved glycemic control and a decrease in cardiovascular risk factors in individuals with T1D (Carral et al., 2013; Bohn et al., 2015). Moreover, the addition of structured exercise may potentiate these benefits (Valerio et al., 2007; Trigona et al., 2010). For Trigona et al. (2010), youth with T1D must go beyond simply being active to obtain the most benefit and improve their physical fitness profile, in addition to increasing their engagement in PA.

Despite the known benefits of PA, the numerous physiological (e.g., hypoglycemia episodes) and psychological (e.g., fear of hypoglycemia) risks associated with T1D make it challenging to incorporate PA practices (Riddell et al., 2017). Consequently, people with T1D tend to be at least as inactive as the general population, with a large percentage of individuals not achieving the minimum amount of moderate to vigorous aerobic activity per week (Øverby et al., 2009; Trigona et al., 2010). Several barriers to PA practices can exist for a person with T1D, leading to effects on the level and the mode of PA participation. These barriers have been evaluated using the Barriers to Physical Activity in Type 1 Diabetes (BAPAD-1) scale (Dubé et al., 2006; Brazeau et al., 2008, 2012b), and fear of hypoglycemia was identified as the main barrier to PA practices in adults (Brazeau et al., 2008) as well as in youth (children and adolescents) (Jabbour et al., 2016). Recently, a cross-sectional study conducted in diabetes adults supports the assumption that PA may be lower in this population due to unique barriers (Keshawarz et al., 2018). Unfortunately, reporting potential barriers, particularly fear of hypoglycemia in relation to PA levels is limited to cross-sectional studies, and no data are available whether any changes in scores for fear of hypoglycemia and/or overall perceived barriers scores over time will affect PA levels. Such data are crucial to add more emphasis on the relationship between PA levels and perceived barriers, open new insight toward engagement in PA safely, and increase overall PA.

Within this perspective, the present 2-year follow-up study aimed to explore the association between perceived barriers, mainly the fear of hypoglycemia, and PA using a detailed and validated questionnaire among youth with T1D. Since glycemic control and body weight control are important in improving overall health in individuals with T1D (Carral et al., 2013; Bohn et al., 2015) and known to be reduced by PA practices (Carral et al., 2013; Bohn et al., 2015), they will be explored throughout.

MATERIALS AND METHODS

Study Population

The participants of this project are young people (boys and girls) with T1D (diagnosed for at least 1 year) and aged between 5 and 17 years old. The study was conducted at the Pediatric Diabetic Clinic of the Dr. Georges-L.-Dumont (Vitalité Health Network), located in Dieppe, in which 61 children and adolescents with T1D were followed (from a total of ∼630 patients registered in all the affiliated pediatric clinics of the Vitalité Health Network). The ethics committee of the Vitalité Health Network approved the project, and all participants signed an informed consent form. This study complies with the principles laid down in the Declaration of Helsinki Recommendations. Of the 61 children and adolescents with T1D, all agreed to take part in the study, representing 100% of the Pediatric Diabetic Clinic of the Dr. Georges-L.-Dumont's clientele. All completed a self-administered questionnaire directly at the clinic administered by kinesiology students at the University of Moncton.

For the purpose of the present work, these same children and adolescents were asked to answer the same questionnaire 1 year (visit 1; V1) and 2 years later (visit 2; V2). Data were collected consecutively during the same period of collection (between October and November). All participants met the inclusion criteria: age between 6 and 17, duration of diabetes longer than 1 year, and no other chronic diseases.

In the present study (**Figure 1**), 61 participants completed all the relevant questionnaires at baseline [visit 0 (V0)] prior to their regular visit with the physician. They were asked to complete one questionnaire related to exercise barriers (barriers to PA in T1D score–BAPAD-1; for all, see **Table 1**) and the activity profile using the questionnaire from cycle 2 of the Canadian Health Measures Survey, which is adapted according to age (children and adolescents; < 12 years and ≥ 12 years old). More details are provided in the questionnaire section. All participants agreed to be contacted 1 and 2 years later for the same purpose [visits 1 and 2 (V1; V2)]. At both visits, all completed the same questionnaire that they completed at V0. However, three participants were excluded from the final analysis because they did not meet the age criteria (**Figure 1**). Ultimately, 58 participants separated into two groups ($n = 25$ children and $n = 35$ adolescents) were analyzed for the purposes of the present work.

Age and sex were obtained using a self-reported questionnaire completed by the child/adolescent. Height and weight were evaluated on-site by a nurse. Age- and sex-specific body mass index (BMI) percentiles were calculated according to the US Centers for Disease Control and Prevention growth charts (Lau, 2007; Centers for Diseases Control and Prevention, 2014). The number of years since the patient's diabetes diagnosis was also calculated, and mean glycated hemoglobin (HbA1c) values over the preceding 3 months were preliminarily recorded.

Barriers to Physical Activity

We administered the validated BAPAD-1 questionnaire to all study participants. The questionnaire consists of eight universal barriers to PA relevant to all study participants and four diabetes-specific barriers. For this study on children and adolescents, nine items were kept, since two items were not applicable to this age group ("the fear of suffering a heart attack" and "the fear of being tired") and "the school schedule" was added (Jabbour et al., 2016; Michaud et al., 2017). The BAPAD-1 score was obtained by calculating the average of the individual scores obtained for each type of barrier, for which the answers to exercise barriers were rated from 1 (extremely improbable) to 7 (extremely probable). For the purposes of the present analysis, scores from 1 to 4 were categorized as "barrier not present," while scores from 5 to 7 were categorized as "barrier present".

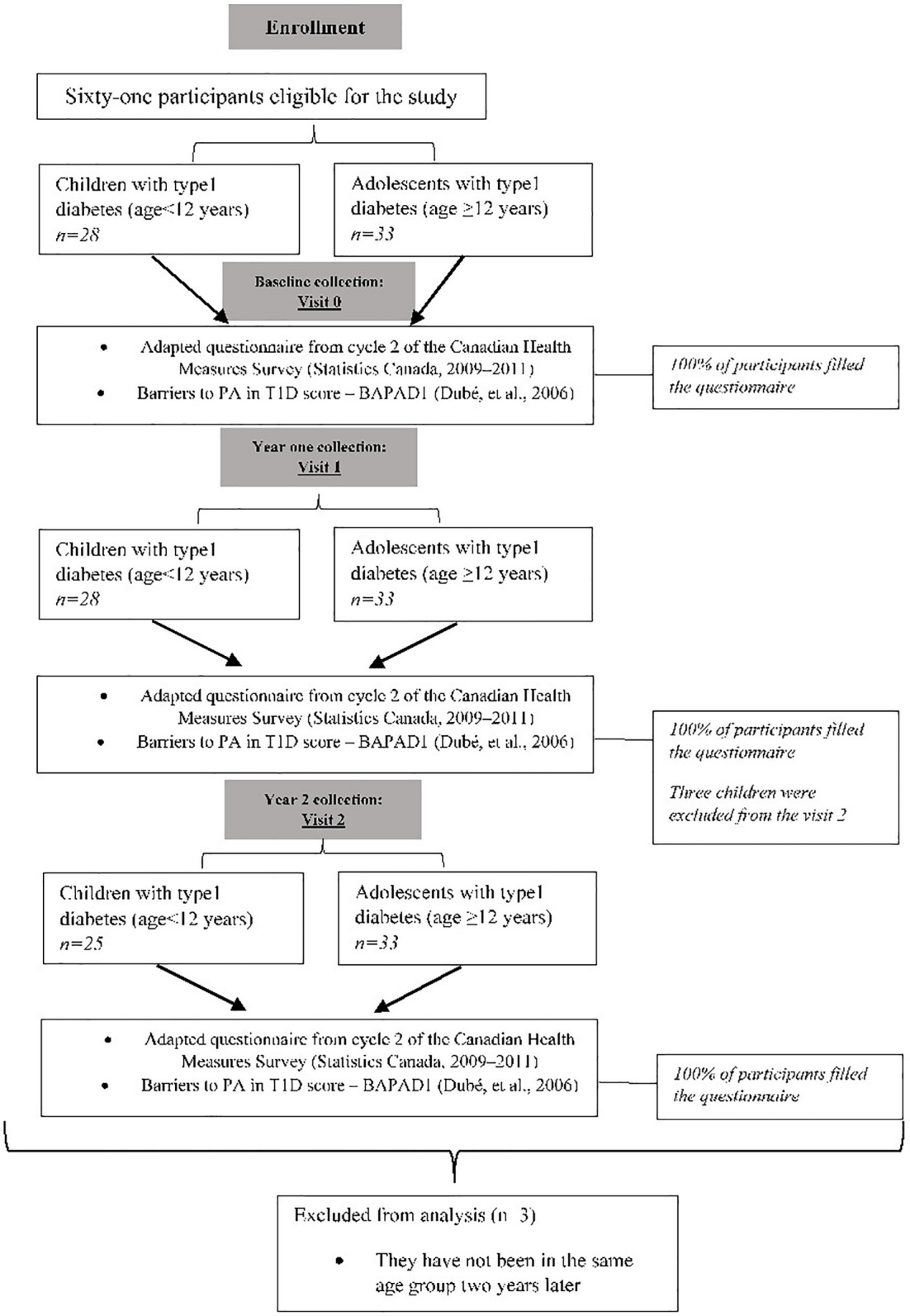

FIGURE 1 | Flow chart of study design and participants' enrollment.

TABLE 1 | Descriptive characteristics and activity profile of children and adolescent with type 1 diabetes across visits.

	Visit 0		Visit 1		Visit 2		Paired *t*-test	
	Children (*n* = 28)	**Adolescents (*n* = 33)**	**Children (*n* = 28)**	**Adolescents (*n* = 33)**	**Children (*n* = 25)**	**Adolescents (*n* = 33)**	**FT**	**p**
Study population characteristics								
Sex (% boys)	55	57	55	57	60	57	–	–
Age (years)	7.1 ± 0.6	15.2 ± 0.3[a]	8.1 ± 0.7[b]	16.1 ± 0.6[ab]	9.2 ± 0.4[b]	17.3 ± 0.3[ab]	21	<0.01
Weight (kg)	30 ± 3	65 ± 3[a]	50 ± 2[b]	70 ± 10[ab]	59 ± 1[b]	74 ± 5[ab]	29	<0.01
Height (cm)	130 ± 3	165 ± 2[a]	136 ± 6[b]	164 ± 4[ab]	148 ± 4[a]	164 ± 8[ab]	32	<0.01
BMI percentile	61 ± 11	80 ± 13[a]	69 ± 17[b]	86 ± 14[ab]	72 ± 1 3[b]	88 ± 16[ab]	29	<0.01
HbA1c (%)	7.1 ± 1.9	7.2 ± 2.19	7.2 ± 0.6	7.3 ± 0.7	7.2 ± 1.1	7.1 ± 1.2	9.3	0.43
Sometimes experience PA hypoglycemia *(%)*	45 ± 3	76 ± 2[a]	40 ± 1	86 ± 2[ab]	46 ± 1	90 ± 2[ab]	19	<0.01
Sometimes experience PA hyperglycemia *(%)*	20 ± 3	21 ± 1	18 ± 2	18 ± 1	22 ± 1	17 ± 3	8.6	0.54
Barriers to Active Lifestyles measured by the BAPAD-1								
Diabetes-specific barriers to PA with score ≥ 4 n (%)	21	44	24	39	23	47		
Loss of control over diabetes	2.2 ± 1.6	2.8 ± 1.8	2.7 ± 1.6	3.6 ± 1.9	2.6 ± 1.1	3.2 ± 1.9	10.6	0.64
Fear of hypoglycemia	2.7 ± 1.4	3.1 ± 1.9	2.7 ± 1.6	2.8 ± 1.9	2.6 ± 1.9	3.2 ± 1.8[ab]	16	<0.05
Fact that you have diabetes	2.1 ± 1.5	2.4 ± 1.6	2.2 ± 1.5	2.9 ± 1.1	1.6 ± 1.7	3.1 ± 1.9[a]	14	<0.05
Risk of hyperglycemia	1.9 ± 1.1	2.1 ± 1.7	2.1 ± 1.1	1.9 ± 1.6	2.3 ± 1.4	1.9 ± 1.1	9.6	0.44
Diabetes-universal barriers to PA with score ≥ 4 n (%)	18	20	18	19	21	24		
Fear of hurting self	2.1 ± 1.1	1.9 ± 0.1	2.2 ± 1.2	1.8 ± 0.3	1.9 ± 0.8	1.8 ± 0.6	8.7	3.2
Low fitness level	2.1 ± 1.6	2.2 ± 0.9	2.1 ± 1.3	1.9 ± 0.7	2.1 ± 0.7	1.9 ± 0.9	7.7	2.4
Weather conditions	2.6 ± 1.6	2.6 ± 1.6[a]	2.7 ± 1.1	2.9 ± 1.4[a]	2.1 ± 1.7	3.2 ± 0.9[a]	14	<0.05
Sport Center proximity	1.8 ± 1.3	1.7 ± 1.1	1.6 ± 1.1	1.6 ± 1.1	1.7 ± 1.5	1.5 ± 0.7	9.7	4.4
School/Work schedule	1.6 ± 0.9	2.6 ± 1.2[a]	1.4 ± 0.7	2.9 ± 0.9[a]	1.1 ± 0.9	2.7 ± 1.2[a]	16	<0.05
Actual physical health status (excluding diabetes)	1.9 ± 1.5	1.8 ± 1.4	1.9 ± 0.9	1.9 ± 0.7	1.7 ± 0.5	1.7 ± 0.7	4.9	2.1
Total BAPAD-1 score	2.1 ± 1.4	2.3 ± 1.3	2.2 ± 0.9	2.4 ± 0.8	1.9 ± 0.8	2.4 ± 0.9	6.3	5.1
Self-reported PA and screen time information								
Total screen time·day^{-1} (h) (TV, video games, computer)	1.8 ± 1.2	2.8 ± 1.8[a]	1.6 ± 1.3	2.9 ± 1.1[a]	2.2 ± 1.2	2.9 ± 0.8[a]	26	<0.01
Total time in MVPA.day^{-1} (min)	41 ± 11	52 ± 14[a]	44 ± 09	56 ± 12[a]	41 ± 06	56 ± 13[a]	26	<0.01
Total time in VPA.day^{-1} (min)	27 ± 09	8 ± 02[a]	23 ± 07	5 ± 04[a]	26 ± 11	4.3 ± 03[a]	19	<0.01
Number of day spent on VPA per week	3.1 ± 0.2	1.8 ± 0.2[a]	2.9 ± 0.8	1.1 ± 0.3[a]	3.2 ± 0.8	1.4 ± 0.8[a]	15	<0.01

Values are mean (standard deviation). BMI, body mass index; HbA1c, glycated hemoglobin; PA, physical activity; n (%), number in percentage; MVPA, moderate to vigorous physical activity; VPA, vigorous physical activity; BAPAD-1, Barriers to Physical Activity in Type 1 Diabetes; h, hour; min, minute. Significant difference between groups ([a]$p < 0.01$). Significant difference from previous visit values ([b]$p < 0.01$).

Activity and Sedentary Profile

To obtain the activity profile, we used the questionnaire from cycle 2 of the Canadian Health Measures Survey (Statistics Canada, 2009–2011). This questionnaire was developed and administered in two versions: one version for children under 12 and another version for adolescents 12 years and older. In this questionnaire, children and adolescents reported how many hours per day they usually spend engaged in sedentary activities, such as using a computer, playing video games, or watching TV/videos. For the data analysis, the three categories "none," "<1 h/day," and "1–2 h/day" were recoded as "≤2 h/day," and the other categories ("3–4 h/day," "5–6 h/day," and "≥7 h/day") were recoded as ">2 h/day," which is the closest possible threshold within these categories to the cutoff, according to the Canadian guidelines for screen time (<2 h/day) (Canadian Society of Exercise Physiology, 2012).

Next, the average minutes per day spent in various forms of PA were derived. Using the World Health Organization norms on metabolic equivalents of tasks (METs) (World Health Organization, 2015) and Ainsworth's Compendium of Physical Activities for children (Ridley et al., 2008), activity was categorized as low (≤3 METs), moderate (<3 METs ≤ 6), or vigorous (>6 METs) in intensity. This will allow us to calculate the amount of time spent daily in each PA intensity and to identify adolescents who follow the recommendations of ≥60 min of daily of moderate to vigorous PA (MVPA) (Ridley et al., 2008; McArdle et al., 2010).

Statistical Analysis

The analyses were performed using the IBM SPSS v. 21 software (IBM, Armonk, New York, United States). The data are presented as means (standard deviations). Normality was tested using the Kolmogorov–Smirnov test. Paired *t*-tests were used to determine whether significant differences occurred at each visit within and between groups. We used multiple linear regression to model the mean outcomes for each exposure of interest. For both linear and logistic regressions, the independent variables considered in the regression models were BAPAD-1 score, perceived barrier items, and previous experiences of PA hypo/hyperglycemia. A value of $p < 0.05$ was set as the level of statistical significance.

RESULTS

The characteristics of the study participants were compared (**Table 1**). Among the children, 55% of the subjects were boys, except for visit 2, in which 60% of the subjects were boys (three children were excluded from the final analysis, see **Figure 1**). For the adolescent group, 60% of the subjects were boys (**Table 1**). Anthropometric variables (height, weight, BMI percentile) increased for both groups across visits (**Table 1**) and were significantly higher for adolescents than for children ($p < 0.01$, respectively). There were no significant differences in HbA1c (%) between groups across visits (**Table 1**). At visit 0, 45% of children reported sometimes experiencing hypoglycemia during PA, while this figure was 40% at visit 1 and 46% at visit 2. Among adolescents, 76% reported sometimes experiencing hypoglycemia during PA at visit 0, and this percentage increased significantly at visit 1 (86%; $p < 0.01$) and at visit 2 (90%; $p < 0.01$). The total scores for PA barriers monitored by the BAPAD-1 questionnaire were similar for both groups (**Table 1**) and across visits. However, the frequency with which adolescents assigned a score of 4 or greater to diabetes-specific barriers was two times greater than that reported by children (**Table 1**). The most common diabetes-specific barrier identified was the fear of hypoglycemia. This barrier increased among adolescents at V2 compared to those at V1 ($p < 0.01$).

At baseline, children engaged in less MVPA ($p < 0.01$) per day and had less screen activity ($p < 0.01$) than adolescents did. The frequency with which participants engaged in ≥ 60 min a day of moderate- to vigorous-intensity PA and ≥ 2 h of screen time across visits is presented in **Figure 2**. Approximately 30% of children vs. 80% of adolescents have at least 2 h of screen time per day. In contrast, adolescents spent less time in VPA ($p < 0.01$) and less time in VPA bouts per day ($p < 0.01$) than children did (**Table 1**).

To examine whether participants with T1D who reported diabetes-specific barriers had low levels of PA, we present results for the linear regression analysis of PA outcomes in **Table 2**. Participants with higher scores for barriers spent significantly less time in MVPA per day than participants with lower reported barriers; this pattern was seen across all visits (β = -0.12, $p = 0.05$; β = -0.14, $p = 0.04$; and β = -0.12, $p = 0.01$ for V0, V1, and V2, respectively). Moreover, VPA is significantly correlated with total barrier scores (**Table 2**). A higher "fear of hypoglycemia" score across visits was observed for those with less VPA (β = −0.41, $p = 0.03$; β = −0.44, $p = 0.06$; and β = −0.61, $p = 0.001$ for V0, V1, and V2, respectively).

Our results reported a significant correlation between HbA1c (%) and VPA levels (β = −0.54, $p = 0.02$; β = −0.47, $p = 0.03$; β = −0.62, $p = 0.01$) for V0, V1, and V2, respectively. BMI percentile correlated with total screen time (β = 0.28, $p = 0.02$; β = 0.29, $p = 0.01$; and β = 0.31, $p = 0.04$ for V0, V1, and V2, respectively) and overall PA levels (β = −0.52, $p = 0.02$; β = −0.42, $p = 0.03$; β = −0.42, $p = 0.01$).

Finally, regression models for "sometimes experience PA hypoglycemia" and for "sometimes experience PA hyperglycemia" were examined (**Table 2**). There was a significant positive correlation between these responses of "sometimes experience PA hypoglycemia" and average time spent in MVPA per day across visits (β = 0.61, $p = 0.001$; β = 0.39, $p = 0.001$; and β = 0.21, $p = 0.03$ for V0, V1, and V2, respectively). The response "sometimes experience PA hyperglycemia" was significantly correlated with daily total screen time (β = 0.21, $p = 0.04$; β = 0.39, $p = 0.03$; and β = 0.51, $p = 0.01$ for V0, V1, and V2, respectively) as well as with the number of minutes of MVPA per day (β = 0.41, $p = 0.003$; β = 0.33, $p = 0.06$; and β = 0.31, $p = 0.04$ for V0, V1, and V2, respectively).

DISCUSSION

To the best of our knowledge, this 2-year follow-up study is the first to examine PA levels and their association with

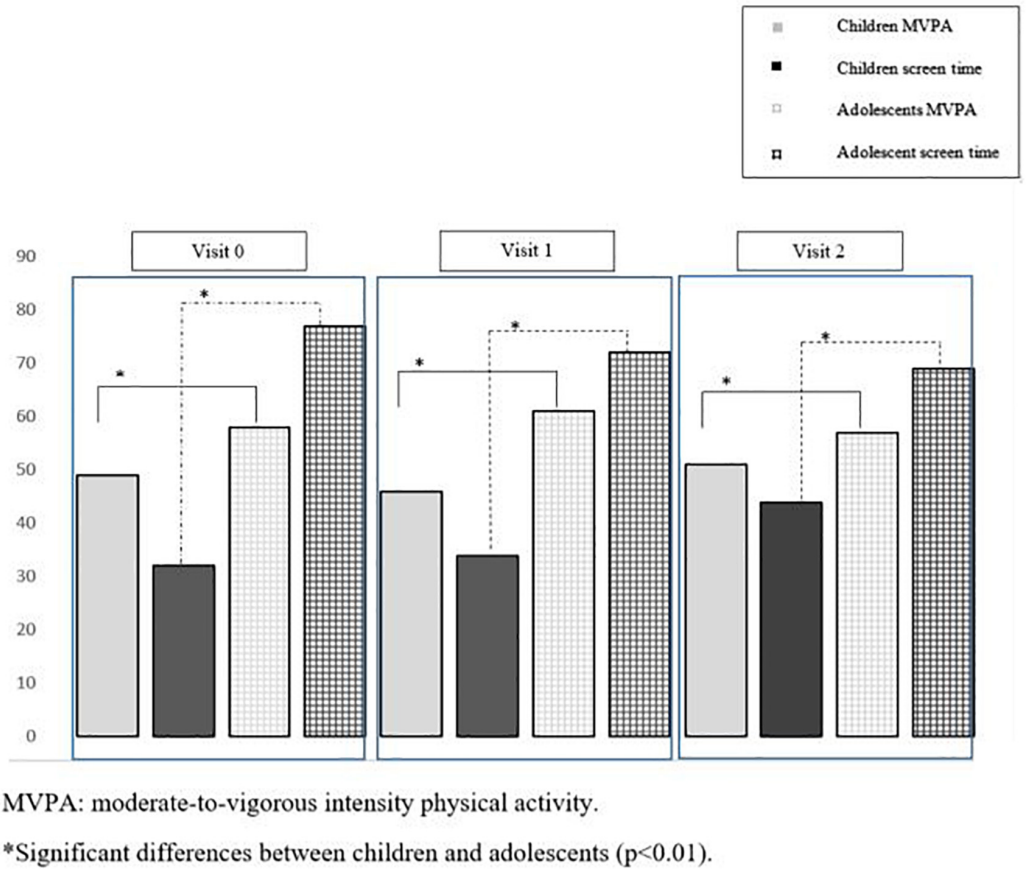

FIGURE 2 | Percentage of children and adolescents above 60 min a day of moderate-to-vigorous intensity physical activity and above 2 h of screen time across visits.

HbA1c and fear of hypoglycemia scores among youth with T1D. Our results indicated that engaging in more VPA is associated with lowered diabetes-specific barriers, primarily the fear of hypoglycemia and HbA1c. Considering that improving HbA1c with PA and increasing adherence to PA remains a major concern for pediatric diabetes, this study may highlight the importance of VPA in amplifying the benefits of PA and increasing PA engagement and overall PA.

Regardless of the diabetes group (children vs. adolescent), self-reported PA levels indicate that among our participants, 45% of children vs. 50% of adolescents cumulated at least 60 min per day of MVPA, as recommended by the Centers for Disease Control and Prevention, the American Heart Association, and the American Diabetes Association (Eckel et al., 2014; Colberg et al., 2016). Despite the greater importance of PA among individuals with T1D, our results confirm those of the literature that reported that most of the pediatric population (Troiano et al., 2008; Colley et al., 2011), and even more youths with pediatric diabetes (Michaud et al., 2017), do not meet PA guidelines.

Barriers to PA practices have been addressed as a main factor in understanding low PA levels among individuals with T1D. Indeed, those with higher scores for diabetes barriers have lower participation in PA, as reported by Michaud et al. (2017) regarding the pediatric population as well by Brazeau et al. (2012a) with respect to adults. Accordingly, for both children and adolescents, a higher barriers score has been reported for those who spent significantly less time in MVPA per day, and this pattern was seen across all visits. In fact, the accumulation of high MVPA scores may reduce overall barriers to PA practices; however, the "fear of hypoglycemia" was the only barrier's item that seemed to be unchanged by high levels of MVPA, as per our results. Given that the "fear of hypoglycemia" is the main barrier to PA (Jabbour et al., 2016; Keshawarz et al., 2018), it is still currently unclear which mode of PA may favor an appropriate glycemic balance among individuals with T1D. Consistent with our results, a meta-analysis by Hasan et al. (2018) in laboratory-based settings reported that among adults with T1D, engaging in moderate exercise appears to be associated with a high risk of hypoglycemia, and high-intensity exercise may be safer because of a lower decline in blood glucose.

Consistent with the abovementioned findings, recent strategies have indicated that certain levels of exercise intensity could prevent hypoglycemia in T1D (Guelfi et al., 2005; Iscoe and Riddell, 2011). These experimental studies suggested that high-intensity exercise might attenuate hypoglycemia due to a greater increase in catecholamines and growth hormone and

TABLE 2 | Standardized regression summary for total screen time, moderate to vigorous physical activity, and vigorous physical activity across the three visits.

	Total screen time.day^{-1} (h)			**Total MVPA.day^{-1} (min)**			**Total VPA.day^{-1} (min)**		
	Visit 0	**Visit 1**	**Visit 2**	**Visit 0**	**Visit 1**	**Visit 2**	**Visit 0**	**Visit 1**	**Visit 2**
BMI percentile	R^2 adj. = 0.07; β = 0.28; p = 0.02*	R^2 adj. = 0.08; β = 0.29; p = 0.01*	R^2 adj. = 0.065; β = 0.31; p = 0.04*	R^2 adj. = 0.039; β = −0.52; p = 0.02*	R^2 adj. = 0.066; β = −0.42; p = 0.03*	R^2 adj. = 0.054; β = −0.42; p = 0.01*	R^2 adj. = 0.81; β = 0.03; p = 0.98	R^2 adj. = 0.66; β = 0.06; p = 0.72	R^2 adj. = 0.718; β = 0.05; p = 0.92
HbA1c (%)	R^2adj. = 0.51; β = 0.04; p = 0.42	R^2 adj. = 0.66; β = 0.04; p = 0.52	R^2 adj. = 0.51; β = 0.03; p = 0.42	R^2adj. = 0.67; β = 0.08; p = 0.72	R^2 adj. = 0.46; β = 0.02; p = 0.32	R^2 adj. = 0.71; β = 0.06; p = 0.62	R^2 adj. = 0.044; β = −0.54; p = 0.02*	R^2 adj. = 0.035; β = −0.47; p = 0.03*	R^2 adj. = 0.063; β = −0.62; p = 0.01*
Sometimes experience PA hypoglycemia *(%)*	R^2 adj. = 0.71; β = 0.10; p = 0.92	R^2 adj. = 0.63; β = 0.04; p = 0.42	R^2 adj. = 0.81; β = 0.09; p = 0.45	R^2 adj. = 0.06; β = 0.61; p = 0.001*	R^2 adj. = 0.05; β = 0.39; p = 0.001*	R^2 adj. = 0.04; β = 0.21; p = 0.03*	R^2 adj. = 0.71; β = 0.05; p = 0.58	R^2 adj. = 0.62; β = 0.07; p = 0.64	R^2 adj. = 0.73; β = 0.06; p = 0.63
Sometimes experience PA hyperglycemia *(%)*	R^2 adj. = 0.05; β = 0.21; p = 0.04*	R^2 = 0.04; β = 0.39; p = 0.03*	R^2 adj. = 0.09; β = 0.51; p = 0.01*	R^2 adj. = 0.04; β = 0.41; p = 0.003*	R^2 adj. = 0.03; β = 0.33; p = 0.06*	R^2 adj. = 0.06; β = 0.31; p = 0.04*	R^2 adj. = 0.66; β = 0.07; p = 0.68	R^2 adj. = 0.41; β = 0.04; p = 0.54	R^2 adj. = 0.67; β = 0.04; p = 0.62
Loss of control over diabetes	R^2 adj. = 0.61; β = 0.05; p = 0.62	R^2 = 0.66; β = 0.07; p = 0.68	R^2 adj. = 0.72; β = 0.05; p = 0.62	R^2 adj. = 0.41; β = 0.04; p = 0.56	R^2 adj. = 0.43; β = 0.06; p = 0.64	R^2 adj. = 0.58; β = 0.03; p = 0.52	R^2 adj. = 0.36; β = 0.03; p = 0.38	R^2 adj. = 0.51; β = 0.06; p = 0.64	R^2 adj. = 0.47; β = 0.06; p = 0.42
Fear of hypoglycemia	R^2 adj. = 0.77; β = 0.04; p = 0.72	R^2 = 0.76; β = 0.05; p = 0.65	R^2 adj. = 0.62; β = 0.06; p = 0.42	R^2 adj. = 0.61; β = 0.06; p = 0.68	R^2 adj. = 0.67; β = 0.05; p = 0.61	R^2 adj. = 0.63; β = 0.04; p = 0.53	R^2 adj. = 0.05; β = −0.41; p = 0.03*	R^2 adj. = 0.07; β = −0.44; p = 0.06*	R^2 adj. = 0.08; β = −0.61; p = 0.001*
Fact that you have diabetes	R^2 adj. = 0.46; β = 0.05; p = 0.42	R^2 = 0.39; β = 0.06; p = 0.58	R^2 adj. = 0.52; β = 0.06; p = 0.52	R^2 adj. = 0.45; β = 0.03; p = 0.38	R^2 adj. = 0.43; β = 0.03; p = 0.44	R^2 adj. = 0.47; β = 0.03; p = 0.52	R^2 adj. = 0.26; β = 0.02; p = 0.32	R^2 adj. = 0.31; β = 0.04; p = 0.44	R^2 adj. = 0.37; β = 0.03; p = 0.39
Risk of hyperglycemia	R^2 adj. = 0.44; β = 0.06; p = 0.72	R^2 = 0.66; β = 0.07; p = 0.68	R^2 adj. = 0.52; β = 0.04; p = 0.62	R^2 adj. = 0.62; β = 0.04; p = 0.68	R^2 adj. = 0.56; β = 0.04; p = 0.42	R^2 adj. = 0.58; β = 0.05; p = 0.52	R^2 adj. = 0.03; β = −0.34; p = 0.05*	R^2 adj. = 0.07; β = −0.37; p = 0.04*	R^2 adj. = 0.04; β = −0.24; p = 0.02*
Total BAPAD-1 score	R^2adj. = 0.68; β = 0.08; p = 0.54	R^2 = 0.64; β = 0.05; p = 0.65	R^2 adj. = 0.67; β = 0.07; p = 0.52	R^2 adj. = 0.01; β = −0.12; p = 0.05*	R^2 adj. = 0.02; β = −0.14; p = 0.04*	R^2 adj. = 0.04; β = −0.12; p = 0.01*	R^2 adj. = 0.07; β = −0.53; p = 0.002*	R^2 adj. = 0.06; β = −0.55; p = 0.003*	R^2 adj. = 0.03; β = −0.19; p = 0.01*

**Denotes a significant relationship. BMI, body mass index; HbA1c, glycated hemoglobin; PA, physical activity; MVPA, moderate to vigorous physical activity; VPA, vigorous physical activity; BAPAD-1, Barriers to Physical Activity in Type 1 Diabetes; h, hour; min, minute.*

hence in glucose hepatic production (Bussau et al., 2007). Despite this promising alternative of reducing hypoglycemia episodes, we cannot assume that individuals with diabetes will become more active. Interestingly, a lower "fear of hypoglycemia" score with fewer "PA-hypoglycemia experiences" across visits was observed in the current study for those who had a high level of VPA, even when participants cumulated or not $\geq$ 60 min per day and had less screen time per day ($<$ 2 h). Engaging in high VPA levels has been observed in children and was maintained across visits. In contrast, adolescents with diabetes accumulated more MVPA than their counterparts did. However, due to the design of the study, causality between VPA levels and scores for "fear of hypoglycemia" and "PA-hypoglycemia experience" cannot be inferred. Moreover, an increase in VPA levels does not guarantee any increases in overall PA levels in individuals with diabetes. Therefore, determining the required level of VPA for youth with diabetes to improve and explore other associated health outcomes may constitute promising targets to align with current guidelines.

Another important result derived from our study is that performing more VPA a day is associated with better HbA1c percentage in youth with T1D. In addition, the analysis revealed that a higher HbA1c percentage in youth with T1D was associated with increased screen time. No positive impact in HbA1c was observed in relation to the time spent in MVPA. According to our results, a study by Carral et al. (2013) reported that practicing intense PA lowered HbA1c in adults with T1D. Moreover, other intervention studies (Campaigne et al., 1984; Harmer et al., 2007) have shown similar results, but it is still difficult to draw clear conclusions that can be extrapolated to clinical practice since these intervention studies have been conducted in small samples of patients for a very limited time and with highly variable protocols. Regarding the screen time exposure, it is well-established that this outcome is linked to poorer glucose regulation in youth with T1D (Galler et al., 2011). According to general pediatric recommendations, our adolescents with T1D are more exposed to screens per day compared to their counterparts, e.g., children, which is in line with the general pediatric population (Matthews et al., 2008; Colley et al., 2011). Such result stressed the need for providing specific screen time guidelines for children and adolescents with T1D.

The current study has inherent limitations. The first challenge is that the present work relies on self-reported and not objectively measured indicators, despite the use of a validated questionnaire retrieved from a recent national survey. Therefore, supplementing the self-reported data with an objective PA measure (accelerometer) will be ideal to ensure accurate PA profiling. Second, many factors (e.g., insulin dose and carbohydrate consumption), which are largely involved in glycemic control and in preventing exercise-induced hypoglycemia, have not been addressed in the current work. It is possible that those who are engaged in more VPA were simply well aware and more informed about strategies to prevent hypoglycemia. Therefore, considering these elements will be an asset in supporting such studies. Finally, in the present study, it was really difficult to evaluate the progression of outcomes (e.g., PA, HbA1c) within our participants at different visits. Some of the main barriers were the study design itself and the lack of control. Besides the low number of participants and the tool used (e.g., PA questionnaire). This important topic, e.g., outcomes variation and their influences on glycemic parameters, might be possible under a controlled study in response to an education campaign or to a program promoting PA practices.

In conclusion, although meeting the current guidelines in terms of MVPA levels might potentiate the overall health parameter in T1D youth, these recommendations are still not clear with respect to what is the best advice for PA, which may favor metabolic control and reduce the occurrence of hypoglycemia episodes. Our results indicated that youth with T1D engaged in more VPA per day had fewer diabetes-specific barriers, primarily the fear of hypoglycemia with better HbA1c. Such results may highlight the importance of integrating VPA to amplify the benefits of PA and probably increase PA engagement and overall PA among pediatric diabetic patients.

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 ethics committee of the Vitalité Health Network approved the project. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GJ contributed to the conception and design of the study and to the data collection, performed data analysis and interpretation, drafted the manuscript and revised, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Irisin: A New Code Uncover the Relationship of Skeletal Muscle and Cardiovascular Health During Exercise

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Exercise not only produces beneficial effects on muscle itself *via* various molecular pathways, but also mediates the interaction between muscles and other organs in an autocrine/paracrine manner through myokines, which plays a positive role in maintaining overall health. Irisin, an exercise-derived myokine, has been found involved in the regulation of some cardiovascular diseases. However, the relationship between irisin and cardiovascular health is not fully elucidated and there are some divergences on the regulation of irisin by exercise. In this review, we present the current knowledge on the origin and physiology of irisin, describe the regulation of irisin by acute and chronic exercises, and discuss the divergences of the related research results. Importantly, we discuss the role of irisin as a biomarker in the diagnosis of cardiovascular diseases and describe its treatment and molecular mechanism in some cardiovascular diseases. It is expected that irisin will be used as a therapeutic agent to combat cardiovascular diseases or other disorders caused by inactivity in the near future.

Keywords: irisin, myokine, exercise, skeletal muscle, cardiovascular health

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INTRODUCTION

Cardiovascular disease is the most common underlying cause of death, accounting for 31.5% of all deaths worldwide. It is estimated that 43.9% of the adult population in the United States will suffer from some form of cardiovascular disease by 2030, and the total global medical costs of this disease will reach $918 billion (Benjamin et al., 2017). Although progress in clinical treatment and care has reduced the mortality rate of patients with cardiovascular disease, the incidence of the disease continues to increase, and good prevention and treatment strategies are still needed. It is well-established that physical exercise reduces all-cause mortality and increases longevity (Moore et al., 2012). Particularly, exercise reduces the risk of cardiovascular diseases, regulates the abnormal metabolism of blood lipid, and improves vascular function (Diabetes, 2012). Exercise plays an important role in the prevention and treatment of hypertension and coronary heart disease, which has become an effective management in maintaining overall health (Fiuza-Luces et al., 2018).

As the largest endocrine organ, the skeletal muscle secretes a hormone known as myokine. Irisin is a myokine secreted by the skeletal muscle both in rodents and in humans (Bostrom et al., 2012; Lourenco et al., 2019), which enters into circulation during or immediately

after physical activity (Cooper et al., 2018). Owing to properties of driving white adipose tissue browning (Wu and Spiegelman, 2014), alleviating insulin resistance (Bostrom et al., 2012; Yuksel Ozgor et al., 2020), improving glucose homeostasis, and liver lipid accumulation (Zhang et al., 2013; Kurdiova et al., 2014), irisin is emerging as a key molecular for metabolic diseases and other disorders known to improve with exercise (Seo et al., 2020). An increasing number of studies show that the concentrations of circulating irisin in patients with some kinds of cardiovascular disease has changed compared with normal people (Shen et al., 2017; Wang et al., 2017; Hisamatsu et al., 2018). Especially, it has been demonstrated that the application of irisin can affect the pathological processes and improve the disease state of certain cardiovascular diseases (Li et al., 2018; Fan et al., 2020; Yin et al., 2020).

In this review, we summarize the origin of irisin and discuss the regulation of irisin by exercise. Equally, we focus on the key role of irisin in the diagnosis of cardiovascular diseases, as well as the therapeutic effects and molecular mechanisms of certain cardiovascular diseases. We hope to at least partially bridge the knowledge gap between skeletal muscle and cardiovascular health during exercise and provide new ideas for the prevention and treatment of cardiovascular diseases.

SECRETION OF IRISIN FROM SKELETAL MUSCLE

Irisin is a hormone composed of 112 amino acids, which was first discovered in 2012 by Bostrom et al., and named after the Greek messenger goddess Iris (Bostrom et al., 2012). In their original work, the muscle of transgenic mice overexpressing peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) stimulates an increase in the synthesis of transmembrane fibronectin type III domain-containing protein 5 (FNDC5), which is cleaved and secretes irisin (Bostrom et al., 2012). Following the initial study, it is soon reported that the start codon of human FNDC5 gene is atypical ATA rather than ATG of rodents, and the translation efficiency of human FNDC5 gene constructed with ATA start codon is found to be very low in HEK293 cells (Raschke et al., 2013). Erickson et al. considered that Bostrom's experiment had serious flaws, and FNDC5 may be just a transmembrane receptor (Erickson, 2013). The claim that human FNDC5 gene codes for irisin has been questioned (Albrecht et al., 2015), and some scholars believe that the beneficial effects of irisin observed in mice are unlikely to appear in humans (Raschke et al., 2013; Elsen et al., 2014). However, there are indeed cases of proteins being expressed from unusual start codons of genes in humans, such as VANGL2, FGFR1, KCNN4, and TRPV6 (Ivanov et al., 2011). Is irisin also expressed from the unusual start codon in human? To address the issue, Jedrychowski et al. used improved mass spectrometry technique with synthetic peptides rich in heavy stable isotopes (six 13c atoms) as the internal standards and found that irisin is mainly expressed from the non-canonical start codon of FNDC5 (Jedrychowski et al., 2015; Polyzos et al., 2015). The *in vitro* experiments conducted by Raschke et al. (2013) may not reflect the real situation *in vivo*. The level of circulating irisin is higher than insulin and lower than leptin, with the molecular weight of 12 kDa (Jedrychowski et al., 2015). While other studies have reported that the molecular weight of irisin is 15–32 kDa due to glycosylation or incomplete deglycosylation (Bostrom et al., 2012; Lee et al., 2014; Zhang et al., 2014). Recently, it was illustrated that irisin reversed intestinal epithelial barrier dysfunction after intestinal ischemia reperfusion injury *via* binding to integrin αVβ5 (Bi et al., 2020). Similarly, Kim and colleagues have shown that irisin binds to proteins of αV class of integrins, and further biophysical studies identify interacting surfaces between irisin and αVβ5 integrin. Additionally, chemical inhibition of the αV integrins blocks the signaling pathway activated by irisin both in osteocytes and fat cells (Kim et al., 2019). These studies suggest that the membrane receptors of irisin exist as some scholars have predicted, and the αVβ5 integrin is the receptor of irisin in osteocytes, adipocytes, and enterocytes (Greenhill, 2019; Kim et al., 2019; Bi et al., 2020). Even so, whether there are irisin receptors in other cells, such as cardiomyocytes or vascular endothelial cells, needs further study. Anyhow, the discovery of irisin receptors above mentioned has opened a window for scholars to conduct in-depth studies.

In humans, FNDC5 mRNA is mainly expressed in muscles and other muscle containing organs, such as pericardium, rectum, and, artery (Huh et al., 2012), and can be detected in the blood, saliva, cerebrospinal fluid, and bronchoalveolar lavage fluid (Aydin et al., 2013; Sanchis-Gomar et al., 2014; Shen et al., 2017; Ruan et al., 2018). Muscle mass is the main predictor of higher circulating irisin levels in humans (Huh et al., 2012), and age-related muscle mass loss may lead to lower circulating irisin levels in elderly people (Tu et al., 2018). Nevertheless, it was also demonstrated that circulating irisin levels increased with the increase of fat mass, particularly in obesity (Perakakis et al., 2017) and correspondingly decreased with the decrease of fat mass after bariatric surgery (Huh et al., 2012). Given the irisin expression in the muscle is 200-fold of that in adipocytes (Moreno-Navarrete et al., 2013), and its key roles in lipid metabolism, it is possible that there is an irisin compensation mechanism, particular in the obese. A large number of studies have shown that irisin has a potential role in some metabolic diseases such as diabetes, obesity, and participates in the regulation of energy metabolism. For example, it promotes browning of white adipose tissue (Wu and Spiegelman, 2014), increases thermogenesis (Bostrom et al., 2012), reduces lipid accumulation, and maintains glucose homeostasis in skeletal muscle, liver, and other organs (Kurdiova et al., 2014; Perakakis et al., 2017; **Figure 1**). Abnormal glucose and lipid metabolism, diabetes, and obesity are risk factors of cardiovascular diseases. As an important regulator of energy metabolism, irisin has a potential role in maintaining cardiovascular health.

REGULATION OF IRISIN BY EXERCISE

Acute Exercise Increases Circulating Irisin Concentration

As a myokine, irisin is produced by the contraction of skeletal muscle. According to the reports, circulating irisin concentrations increase significantly when the muscle ATP

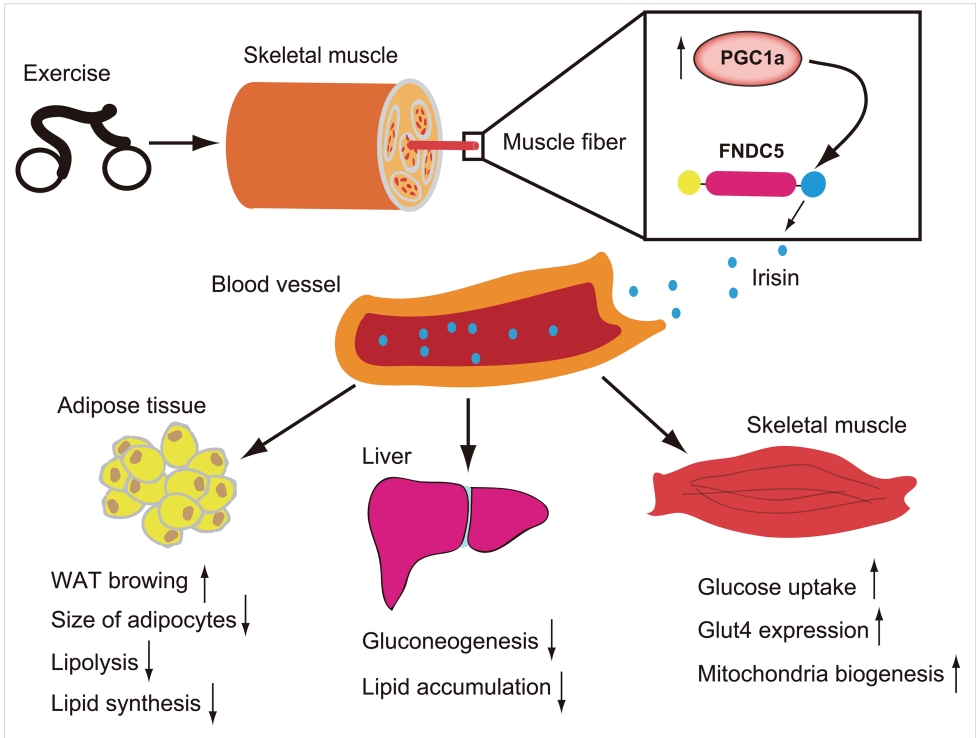

FIGURE 1 | Exercise induces increase of PCG1a expression in skeletal muscle, which in turn drives the production of membrane protein fibronectin type III domain-containing protein 5 (FNDC5). The FNDC5 is cleaved and secretes irisin (blue ball). Irisin enters in the blood circulation and participates in the metabolic regulation of lipid and glucose in some organs such as skeletal muscle, liver, and adipose tissue.

level decreases and remain unchanged as the muscle ATP level remains unchanged (Huh et al., 2012). ATP decline, a state of metabolic demand, may be the initial signal that stimulates irisin secretion to defend muscle ATP homeostasis during exercise (Huh et al., 2012; Bell et al., 2016). A series of studies have shown that acute exercise is a potential stimulus to promote the secretion of irisin (Huh et al., 2015; Fatouros, 2018; Fox et al., 2018). It has been reported that circulating irisin concentrations of young healthy adults increase significantly 30 min after an acute exercise (Huh et al., 2012). Furthermore, a single 40 min of aerobic running can induce minimal increase of serum irisin in both hot (21~25°C) and cold (−5~5°C) environment (Ozbay et al., 2020). Data from animal studies have found that the concentrations of serum irisin increase significantly in hyperthyroidism and hypothyroidism rats after forced swimming with 5% of the weight-bearing body weight for 100 min (Samy et al., 2015). Additionally, one time of high-intensity interval exercise (HIIE), moderate-intensity exercise (CME), or resistance exercise (RE) can significantly increase the circulating irisin concentrations both in healthy people and in patients with metabolic syndrome detected by ELISA kit (Huh et al., 2015). A meta-analysis also shows that the concentrations of circulating irisin in adults increase significantly immediately after an acute bout of exercise, and fitness level is an important factor affecting the effect (Fox et al., 2018).

As everyone knows, resistance training can improve muscle mass more effective than endurance training. Accordingly, it was reported that resistance training promoted irisin secretion more than endurance training or endurance and resistance combined training (Tsuchiya et al., 2015). This finding is consistent with other studies showing that the circulating irisin concentrations are higher after RE than that of HIIE or CME (Huh et al., 2015). On the contrary, it was reported recently that the circulating irisin concentrations in physically active young males did not change after immediate, 30 and 90 min of vigorous-intensity continuous training (VICT, 85%VO_2max) compared with that before exercise (Reycraft et al., 2020). Interestingly, 30 min later, the irisin concentrations are significantly higher than that immediately after exercise (Reycraft et al., 2020). Although the results of this study are not exactly the same as the previous studies from Huh et al. as well as Samy et al. (Huh et al., 2015; Samy et al., 2015), it is believed that exercise at least promotes the secretion of irisin, which is reflected in the significant difference between immediately and 30 min after exercise, in spite of no significant difference in irisin concentrations before and after exercise. Exercise intensity should be an important factor affecting the secretion or the elimination of irisin. Because lower exercise intensity (65% VO_{2max}) does not induce significant increase of circulating irisin concentrations 30 min after exercise compared to immediately after exercise, unlike higher

intensity (85% VO_{2max}) at the same study (Reycraft et al., 2020). This research is consistent with the study of Tsuchiya et al., showing that high-intensity exercise causes greater irisin response compared with low-intensity exercise under similar energy consumption (Tsuchiya et al., 2014).

Acute exercise protocols represent potent stimuli for irisin release if they are characterized by adequate intensity and/or duration (Fatouros, 2018). Of note, the maximum irisin concentration appears immediately after exercise and decreases 1 h later in one study (Huh et al., 2015), while other studies show that the maximum irisin concentration appears 30 min to 1 h after exercise (Tsuchiya et al., 2014; Reycraft et al., 2020). It suggests that time course changes of irisin concentration in response to acute exercise are complex. In addition to the factors such as exercise intensity, duration and subjects' fitness level, a more optimized exercise program that includes a detailed time course is needed to explore the response of irisin to acute exercise in the future.

Chronic Exercise Improves Irisin Metabolic Dynamic and Selectively Increases the Circulating Irisin Concentration

Chronic exercise produces multiple positive effects on overall health in many ways. As a myokine, irisin has received increasing attention in the research related to chronic exercise and overall health. Some studies have reported that chronic exercise increases circulating irisin concentration. For instance, the concentrations of plasma irisin in rodents increase significantly after 3 weeks of free wheel running (Bostrom et al., 2012). In humans, 10 weeks of moderate intensity cycling (65%VO_2max), 20–35 min per day and 4–5 times per week, make a 2-fold rise of irisin concentrations, detected by plasma FNDC5 expression (Bostrom et al., 2012). Similarly, circulating FNDC5 levels in some young male athletes are several times higher than those in middle-aged obese women (Huh et al., 2012), which may be related to higher muscle mass *via* exercise training year after year in athletes. Moreover, the baseline plasma irisin concentration is 3.6 ng/ml in sedentary individuals and significantly increases to 4.3 ng/ml in individuals undergoing 12 weeks of high-intensity aerobic training (Jedrychowski et al., 2015). Furthermore, the circulating irisin concentrations of physically active subjects are higher than that of sedentary, and that of rural are higher than urban subjects (Moreno et al., 2015). This may be due to the fact that rural residents often engage in physical labor, which can exercise muscles similar to physical activity.

However, other studies are inconsistent with the results mentioned above, which has aroused widespread concern and controversy among scholars (Timmons et al., 2012; Hofmann et al., 2014; Spiegelman and Wrann, 2014; Fatouros, 2018). Hecksteden et al. have found that 26 weeks of moderate aerobic exercise (60% HRmax), including walking and running, 45 min per day and three times per week, or strength training with an intensity of 20 repetition maximum (RM), two times of 15 repetitions, three times per week, improves the physical performance of healthy subjects aged 30–60 years (Hecksteden et al., 2013). However, the detection of frozen serum by ELISA kit found that neither aerobic exercise nor strength training caused an increase in the concentration of circulating irisin (Hecksteden et al., 2013). Similarly, Huh also reported that after an 8-week repeated sprint training of moderately trained young male, the circulating irisin concentrations detected by ELISA kit remained unchanged compared to before training (Huh et al., 2012). Additionally, 6 weeks of whole body vibration exercise failed to increase the baseline circulating irisin concentration of healthy untrained females (Huh et al., 2014). Collectively, results from human and animal studies above-mentioned point to different directions, and it seems difficult to draw firm conclusions about the irisin response to chronic exercise.

Interestingly, although the baseline irisin concentrations remained unchanged after 6 weeks of chronic exercise, the circulating irisin levels increased from 9.5 to 18.1% immediately after an acute bout of vibration exercise (Huh et al., 2014). It suggests that chronic exercise promotes the metabolic dynamic and secretion efficiency of irisin. Furthermore, it has been found that 16–20 weeks of moderate intensity exercise (70% HRmax) does not increase circulating irisin concentrations of normal pigs, but increases the circulating irisin concentrations in hypercholesterolemic pigs (Fain et al., 2013). In addition, 6 weeks of intense endurance cycling promoted muscle FNDC5 messenger RNA (mRNA) expression by 30% in elderly subjects, which failed in young subjects (Timmons et al., 2012). It was also reported that the circulating irisin concentrations in older adults increased with the moderate intensity of cardiovascular training program, but not in young (Miyamoto-Mikami et al., 2015; Gmiat et al., 2017). This indicates that the regulation of irisin by chronic exercise may be population-selective. Obese people, the elderly, and patients with metabolic dysfunction may be more sensitive to chronic exercise. In fact, sample fresh or not, reliability of testing methods may be other factors that affect the research results and cause research divergences (Hecksteden et al., 2013; Huh et al., 2015; Albrecht et al., 2020). To fully understand the response of irisin to chronic exercise, the age, gender, health, or disease status of subjects need to be well-controlled in future studies.

IRISIN AND CARDIOVASCULAR HEALTH

Irisin Could Be a Biomarker for Diagnosis of Cardiovascular Diseases

Excessive accumulation of fat, high cholesterol, and metabolism disorders are prone to cause cardiovascular diseases (Angosta et al., 2020). While circulating irisin concentrations are negatively correlated with risk factors of cardiovascular health, such as hyperglycaemia, triglycerides, visceral adiposity, and extramyocellular lipid deposition (Kurdiova et al., 2014). Moreover, it has been reported that serum irisin concentrations are inversely associated with the prevalence of coronary artery calcification (CAC) after adjusting for age and behavioral factors of Japanese men aged 40–79 years (Hisamatsu et al., 2018). Further, after adjustment for cardiometabolic risk factors, the inverse association between serum irisin concentration and

CAC progression still persisted (Hisamatsu et al., 2018). This suggests that circulating irisin concentrations have the potential role to predict occurrence and development of CAC. However, another study has found that under a sedentary lifestyle, rather than active, circulating irisin concentrations are positively correlated with cardiovascular risk factors such as fasting insulin and fasting triglycerides (Moreno et al., 2015). It is possible that in the preclinical stage of some kinds of cardiovascular disease, there may be a compensatory increase in the circulating irisin levels under an inactive lifestyle. Additionally, muscle mass is an important factor affecting circulating irisin concentration (Huh et al., 2012; Sesti et al., 2014; Mai et al., 2020), and the difference in muscle mass among subjects may also be a reason for the divergence of research results. In future studies, it is worth trying to use the ratio of irisin concentration/muscle mass as a biomarker to predict disease and judge pathological processes.

Furthermore, multivariable logistic regression analysis reveals that serum irisin concentration is an independent determinant of the presence of coronary artery disease (CAD; Deng, 2016). Negative correlation between serum irisin concentrations and atherosclerosis index has been found, and the serum irisin concentrations in patients with CAD are significantly lower than that of healthy controls (Deng, 2016). It suggests that those with decreased serum irisin concentrations are more likely to develop coronary atherosclerosis. Similarly, it has been shown that different severity of CAD corresponds to different serum irisin levels in patients with stable angina, suggesting that serum irisin can be used to predict the severity of CAD (Efe et al., 2017). In addition, a meta-analysis based on 741 studies involving 876 patients with CAD and 700 controls, reported that circulating irisin concentration was 18.10 ng/ml lower in patients with CAD or atherosclerosis than those in healthy controls (Guo et al., 2020). Moreover, the serum irisin concentrations of Type-2 diabetic women are significantly lower than that of the normal controls, while the serum irisin concentrations of diabetic patients with atherosclerosis are lower than that of diabetic patients without atherosclerosis (Saadeldin et al., 2018). It indicates that circulating irisin has the potential implication as a diagnostic biomarker for monitoring the progression of cardiovascular disease in diabetic patients. Notably, the irisin concentrations in patients with chronic cardiovascular disease are stable (Panagiotou et al., 2014), whereas the irisin concentrations decrease gradually in saliva and serum within 48 h after acute myocardial infarction (AMI; Aydin et al., 2014). The results above indicate that irisin in body fluids has important clinical value to be used as a diagnostic indicator for the development of AMI.

Effect and Mechanism of Irisin on Cardiovascular Diseases

Atherosclerosis

Atherosclerosis is a common type of cardiovascular disease, characterized by lipids accumulation on the wall, which may lead to arterial rupture and stenosis (Nguyen et al., 2019). It has been demonstrated that circulating irisin concentrations are negatively correlated with the parameters of atherosclerosis, such as coronary atherosclerosis index (CAI) and carotid intima-media thickness (cIMT; Deng, 2016; Icli et al., 2016). In addition, irisin concentrations are significantly lower in type 2 diabetes patients with atherosclerosis and patients with CAD than that of healthy controls (Deng, 2016; Saadeldin et al., 2018). It has been reported that overexpression of PGC1α in the skeletal muscle leads to increased secretion of irisin (Bostrom et al., 2012), and reduces the atherosclerotic plaque area by 40% (Shimba et al., 2019). Further, overexpression of PGC1α in the skeletal muscle decreases the vascular cell adhesion moleculer-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) mRNA and protein expression of aorta in apolipoprotein E-knockout (ApoE-KO) mice (Shimba et al., 2019). Moreover, the treatment of the human umbilical vein endothelial cells (HUVEC) with PGC1a dependent myokines, including irisin and β-aminoisobutyric acid, inhibits the expression of VCAM-1 gene and protein induced by tumor necrosis factor a (TNF-a; Shimba et al., 2019), indicating that irisin is involved in the protective effect of atherosclerosis.

Furthermore, data from animal studies have shown that irisin has a direct therapeutic effect on atherosclerosis (Zhang et al., 2016b; Li et al., 2019). For instance, irisin can ameliorate hyperlipidemia (Li et al., 2019) and promote the proliferation of endothelial cells (ECs) in atherosclerotic mice (Zhang et al., 2016b). In diabetic mice, irisin treatment can significantly ameliorate the endothelial dysfunction and reduce endothelial apoptosis and atherosclerotic plaque area (Lu et al., 2015a). In addition, irisin treatment (0.5 μg/g body weight/day) inhibits the formation of carotid neointima, alleviates aortic inflammation and apoptosis and significantly reduces atherosclerosis in ApoE-deficient mice fed with high cholesterol diet (Zhang et al., 2016a). In the cell model of atherosclerosis, the application of irisin can improve the survival of ECs, promote their migration and tube forming capacity and inhibit apoptosis, proinflammatory cytokine secretion, and reactive oxygen species (ROS) production *via* pAkt/mTOR/nuclear factor E2-related factor-2 (Nrf2) pathway (Zhang et al., 2019).

Myocardial Infarction

Myocardial infarction (MI) is characterized by sudden ischemia, resulting in insufficient oxygen supply and damage or death of cardiomyocytes (Lu et al., 2015b). More than half of the deaths caused by cardiovascular diseases are caused by MI (Pollard, 2000). A large number of studies have demonstrated that exercise has a potential role in reducing the occurrence of MI, and in promoting rehabilitation after MI, which has been a common method to improve cardiovascular health. According to the reports, serum irisin concentrations of MI rats increased after 8 weeks of swimming (Bashar et al., 2018). Further, serum irisin concentrations are positive correlated with QRS duration, total antioxidant status, whereas negative correlated with ST elevation, creatine phosphokinase, collagen deposition and caspase-3 expression (Bashar et al., 2018). This indicates that rising serum irisin concentrations *via* regular exercise or taking irisin as a supplement should improve recovery following MI. Indeed, it has been reported that irisin

treatment for 2 weeks significantly alleviates cardiac dysfunction and ventricular dilation and reduces infarct size as well as fibrosis induced by MI (Liao et al., 2019). Additionally, *vitro* experiments revealed that the molecular mechanism of the effect mentioned above was related to the angiogenesis *via* activating of extracellular signal-related kinase (ERK) signaling pathway in ECs (Liao et al., 2019).

Ischemia and reperfusion (I/R) and hypoxia-reoxygenation (HR) are commonly used models of MI *in vivo* and *in vitro*, respectively. According to the reports, irisin treatment (100 mg/kg) significantly improves ventricular function recovery and reduces the myocardial infarct size after I/R (Wang et al., 2017). Furthermore, HR experiments of H9C2 cardiomyoblasts further reveal that irisin inhibits the opening of mitochondrial permeability transition pore (mPTP), attenuates mitochondrial swelling, and protects mitochondrial functions, which has become an approach of myocardial protection after MI (Wang et al., 2017). It was reported that HR-induced augmented apoptotic ratio of cardiomyocytes under high glucose stress, whereas irisin treatment increased the activity of AMPK pathway, thereby reducing cellular redox stress, maintaining mitochondria potential, and ultimately protecting cardiomyocytes from HR damage (Fan et al., 2020). Moreover, irisin application at a concentration of 50 nmol/L increased metabolism and differentiation of H9C2 cardiomyocytes by activating expression of exercise related genes, including myocardin, follistatin, and nuclear respiratory factor-1 (Xie et al., 2015). In addition, insufficient autophagy flow is an important pathological factor leading to cardiac remodeling and heart failure. It has been illustrated that overexpression of irisin improves mitophagy, autophagy flow and protects cardiomyocytes from myocardial hypertrophy (Li et al., 2018). Recently, both *in vivo* and *in vitro* studies have revealed that optic atrophy 1 (Opa1) expression is downregulated in infarcted heart, whereas irisin treatment upregulated the expression of Opa1 and protects cardiomyocytes from further damage following MI (Xin and Lu, 2020).

Hypertension

It is well-known that hypertension is a severe public health challenge. Data have shown that the prevalence of hypertension ranged from 13 to 41% among 182 countries in the world, accounting for approximately 45% of the global cardiovascular morbidity and mortality (Zeng et al., 2020). Exercise is one of the major non-pharmacological treatments for hypertension, and is broadly recommended by European and American hypertension guidelines (Dimeo et al., 2012). It has been demonstrated that 8 weeks of moderate aerobic exercise training (60–70% VO_2max), 45 min per time and 3 days per week, reduces the carotid-femoral pulse wave velocity (cfPWV) and arterial stiffness in obese rats (Inoue et al., 2019). Further cellular experiments have revealed that the improvement of vascular function produced by exercise is caused by the activation of AMPK-Akt-endothelial nitric oxide synthase (eNOS) signaling pathway *via* irisin (Inoue et al., 2019). Furthermore, irisin application by intravenous injection (0.1, 1, and 10 μg/kg) decreased the blood pressure of spontaneously hypertensive rats (SHRs) in a concentration-dependent manner but failed in normal Wistar-Kyoto rats (Fu et al., 2016). Further, it has been demonstrated that irisin itself has no direct vasodilation effect on the mesenteric artery of SHRs pretreated with phenylephrine, but it increases the phosphorylation of eNOS and the production of NO in ECs by activating AMPK, thus enhancing the relaxation of mesenteric artery in SHRs induced by acetylcholine (Fu et al., 2016). According to another report, irisin stimulates the Ca^{2+} influx through the transient receptor potential vanilloid subtype 4 (TRPV4) channel, the most important Ca^{2+} permeable cation channels in vascular ECs. As a result, Ca^{2+} influx induces endothelium-dependent vasodilation of rat mesenteric artery (Ye et al., 2018). In addition, recent studies have shown that the antihypertensive effect of exercise may be related to the regulation of central nervous system. It has been found that irisin application can activate Nrf2 in the paraventricular nucleus (PVN) of SHRs, thereby reducing oxidative stress, restoring imbalance of neurotransmitters, and ultimately decreasing blood pressure (Huo et al., 2020). Nevertheless, when Nrf2 is knockdown, the protective effects of irisin on hypertension are abolished (Huo et al., 2020).

Vascular endothelial cell homeostasis is essential for maintaining normal blood pressure. A large number of studies have shown that irisin plays a key role in maintaining endothelial cell homeostasis. Endothelial progenitor cells (EPCs) are precursor cells of the vascular endothelial cells (Diaz Del Moral et al., 2020) and play an important role in maintaining endothelial cell homeostasis and repairing vascular injury. According to reports, intraperitoneal injection of irisin in diabetic mice induced an increase in the number of circulating EPCs (Zhu et al., 2016). Further, the co-culture of irisin promoted the proliferation and the migration of EPCs from the bone narrow of diabetic mice (Zhu et al., 2016). The subsequent experiments revealed that the homeostasis of ECs produced by irisin at least partly *via* activating PI3K/Akt pathway and following increased eNOS expression and phosphorylation (Zhu et al., 2016). Moreover, irisin treatment has been found to promote angiogenesis *via* increasing migration and tube formation, and attenuate chemically-induced vessel angiogenic impairment in zebrafish (Wu et al., 2015). The experiments *in vitro* further confirmed that irisin-induced endothelial homeostasis by activating the ERK signaling pathway (Wu et al., 2015). Additionally, other studies have shown that the activation of the ERK signaling pathway in HUVEC inhibits the expression of Bax and Caspase, thereby promoting cell proliferation and reducing cell apoptosis that was induced by high glucose (Song et al., 2014).

Vascular Inflammation

It has been illustrated that physical exercise not only increases the release of irisin, but also inhibits the secretion of pro-inflammatory cytokines and alleviates the inflammatory response of diseases (Mazur-Bialy, 2017). In Chinese obese children, irisin concentrations are significantly lower than that of normal children, and negatively correlated with inflammatory markers of endothelial activation, including high-sensitivity C-reactive protein (hs-CRP), intercellular cell adhesion

molecule-1 (ICAM-1) and E-selectin with irisin levels (Yin et al., 2020). This suggests that lower irisin concentrations may indicate an early state of vascular inflammation in obese children. Additionally, after family-based lifestyle intervention, which mainly including aerobic exercise of 5–7 times per week for at least 30 min and dietary recommendations, irisin concentrations of the obese children were significantly increased and following with improvement of cardiovascular and inflammatory parameters (Yin et al., 2020). Furthermore, lower serum irisin concentrations are also found in children with metabolic syndrome, and the irisin concentrations are negatively correlated with biomarkers of endothelial inflammation and dysfunction, such as ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1; Huerta-Delgado et al., 2020). However, other research have found that there is no correlation between serum irisin concentrations and hs-CRP levels in T2D patients (Elizondo-Montemayor et al., 2019). More than that, there is a positive correlation between serum irisin concentrations and hs-CRP in patients with severe inflammation (Buscemi et al., 2020). Given divergences of the correlation between irisin and inflammatory cytokines, some scholars have hypothesized that there is an irisin "pro-inflammatory/anti-inflammatory" axis in the body (Elizondo-Montemayor et al., 2019), which may be indirectly confirmed by other studies. According to the reports of Mazur-Bialy et al. (2017), irisin treatment induced a decrease in the expression of inflammatory cytokines, such as tumor necrosis factor-a (TNF-a), IL-1β, and MCP-1, in murine macrophages. On the other hand, inflammatory factors such as TNF-a and IL-1β can in turn inhibit the expression of FNDC5 in murine myotubes, thereby reducing the secretion of irisin (Matsuo et al., 2015). Therefore, we speculate that when mild inflammation occurs, the secretion of irisin will increase to resist inflammation, thereby inhibiting the level of inflammatory cytokines, which in turn induces a weakened suppression of FNDC5 expression, and further increases the secretion of irisin, resulting in circulating irisin concentration increasing. For example, the circulating irisin concentrations are higher in some obese people who are considered to have low-grade chronic inflammation (Crujeiras et al., 2014; Pardo et al., 2014), and are positively correlated with BMI or body fat percentage (Perakakis et al., 2017; Abulmeaty et al., 2020). Alternatively, when inflammation gets worse, inflammatory cytokines will directly inhibit the expression of FNDC5, resulting in the decrease of circulating irisin concentration, such as in some other obese, diabetic, or severely inflammatory patients (Shoukry et al., 2016; Elizondo-Montemayor et al., 2019; Yin et al., 2020).

As described above, it has been confirmed that irisin is involved in the regulation of inflammation. Since the molecular mechanism has not been fully elucidated, many studies have been conducted to reveal the anti-inflammatory mechanism of irisin. It has been found that high-fat diet (HFD) can induce over-accumulation of perivascular adipose tissue (PVAT), and severely damage the vascular endothelial function. While, irisin treatment significantly alleviated vascular endothelial injury, promoted the production of NO in PVAT, and inhibited the expression of TNF-a (Hou et al., 2017). Furthermore, irisin treatment significantly ameliorated atherosclerosis in ApoE-deficient mice fed on high cholesterol diet and reduced inflammation of the aortic tissue (Zhang et al., 2016b). Moreover, irisin has been found to reverse oxidative stress and inflammation induced by advanced glycation end products (AGEs) in a *vitro* experiment (Deng et al., 2018). Meanwhile, other studies have found that the anti-inflammatory effect of irisin is related to the activation of AMPK-Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt-eNOS pathway (Lu et al., 2015a), or the inhibition of the ROS/p38 MAPK/NF-κB pathway in HUVECs (Zhang et al., 2016a). Additionally, it was demonstrated that FNDC5, the irisin precursor, induced the decrease of M1 polarization in macrophages and thus suppressed the pro-inflammatory cytokine expression (Xiong et al., 2018). Furthermore, irisin was also reported to convert adipose tissue macrophage polarization from M1 pro-inflammatory phenotype to M2 anti-inflammatory phenotype (Dong et al., 2016). Irisin has also been found to reduce the overproduction of reactive oxygen species (ROS) in a concentration-dependent manner, thereby regulating the activity and phagocytosis of macrophages, and exerting potential anti-inflammatory properties (Mazur-Bialy et al., 2017).

Taken together, studies both *in vivo* and *in vitro* have confirmed that irisin participates in the regulation of cardiovascular health. A series of molecular mechanisms, such as NO production, autophagy, angiogenesis, and inflammation in cardiomyocytes or vascular ECs, have been involved in the regulation of irisin in cardiovascular diseases (**Figure 2**). Based on the theoretical basis of irisin, formulating scientific exercise prescriptions is of great significance to the prevention of cardiovascular disease. Clinically, the injection of irisin in the treatment of cardiovascular disease will have a good prospect.

CONCLUSION AND PROSPECTS

As a hormone mainly secreted by skeletal muscle, irisin is regulated by exercise. Acute exercise can increase the concentration of circulating irisin, and chronic exercise can improve irisin metabolic dynamic and selectively increase the circulating irisin concentration of subjects. Given the significant role in the browning of white fat and energy metabolism, the research of irisin mainly focuses on metabolic diseases. Recently, an emerging number of animal and clinical trials have confirmed that irisin is involved in the regulation of cardiovascular health and has potential therapeutic effects (**Figure 3**). However, there are inconsistencies in the research and literature reports, and the detailed mechanism of irisin in promoting cardiovascular health under pathological conditions has not been fully elucidated. Therefore, a lot of work needs to be done in the future to solve the problems.

First, as described previously, exercise intensity is an important factor affecting the metabolic dynamics and concentrations of circulating irisin. We could not help wondering that what is the intensity threshold? Are there different intensity thresholds for different exercise types, for example, aerobic exercise and

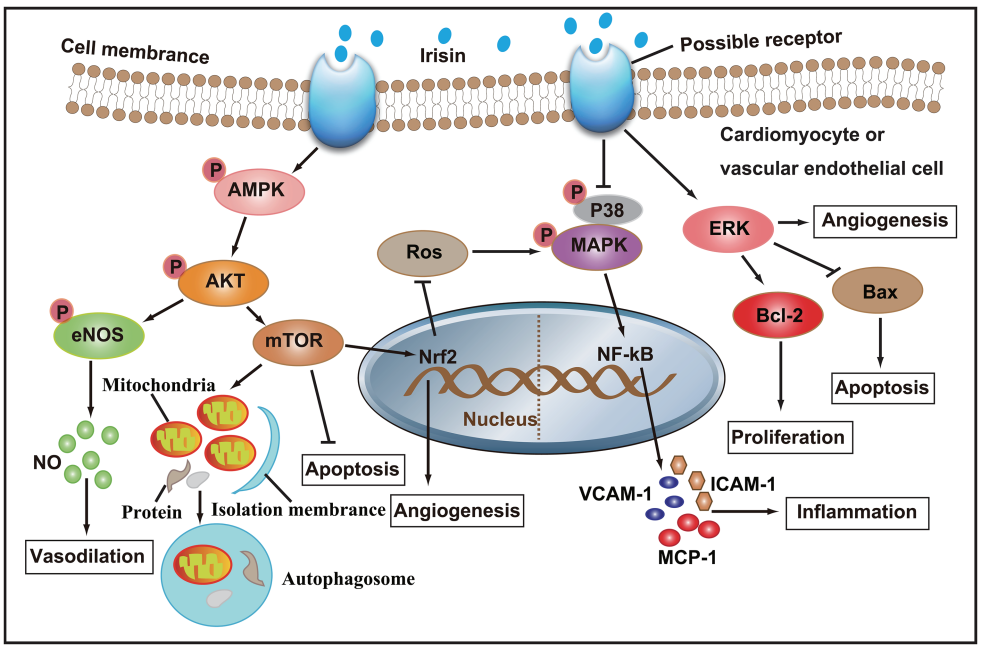

FIGURE 2 | After irisin binds to membrane receptors, AMPK phosphorylation increase, and its downstream Akt/eNOS and Akt/mTOR signaling cascades are activated, thereby promoting NO secretion, autophagy, and angiogenesis, and inhibiting apoptosis and ROS production. On the other hand, irisin can also inhibit the secretion of pro-inflammatory factors by inhibiting p38 MAPK signaling pathway, and promote angiogenesis and cell proliferation, and inhibit apoptosis by activating ERK signaling pathway.

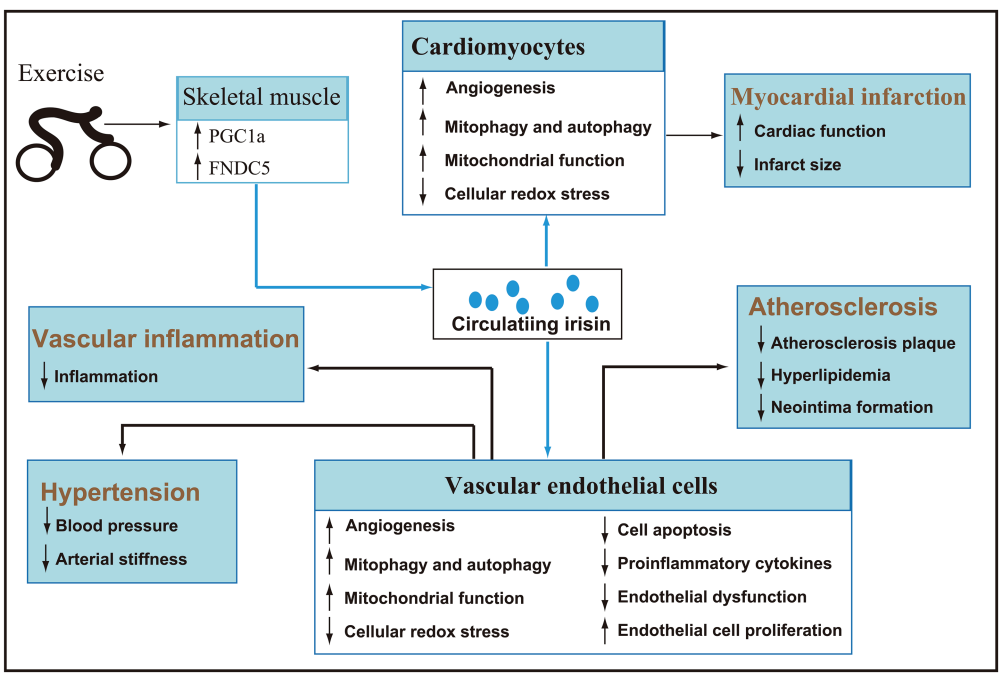

FIGURE 3 | After secreted by skeletal muscle during exercise, irisin reaches cardiomyocytes and vascular endothelial cells *via* blood circulation (blue line). Irisin can promote angiogenesis, autophagy, and endothelial cell proliferation, improve mitochondrial function, inhibit cellular redox stress, apoptosis, inflammation, and endothelial dysfunction, and then play a key role in improving hypertension, myocardial infarction, atherosclerosis, and vascular inflammation.

resistance exercise? Will chronic exercise improve irisin sensitivity the same way as improve insulin sensitivity, so that the baseline irisin concentrations of people who exercise regularly will not increase? Answering the above questions will help us to formulate scientific exercise prescriptions for rehabilitation treatment and cardiovascular diseases.

Second, it is urgent to develop more accurate clinical testing methods. It has been reported that the difference between the test results of the two most commonly used ELISA kits is about 10 times (Huh et al., 2015), and the available commercial polyclonal antibodies produce multiple immune bands due to poor specificity, whereas these immune bands cannot be irisin (Albrecht et al., 2015, 2020). Reliable assays and precise design will help us to realize the practice of irisin's clinical diagnosis of cardiovascular health in the future.

Third, it has been confirmed that αVβ5 integrin is the irisin receptor of osteocytes, adipocytes and enterocytes (Kim et al., 2019; Bi et al., 2020). Given the effective regulation of irisin on the functions of cardiomyocytes and vascular ECs, whether αVβ5 integrin is also the irisin receptor of these cells is the direction of future research. The further identification of irisin receptors in cardiomyocytes and vascular ECs will open up new approaches for the treatment of cardiovascular diseases.

AUTHOR CONTRIBUTIONS

CM and HD prepared the first draft and wrote the final version of the manuscript. YD and HL were involved in literature search. XX and YY critically revised the manuscript and gave constructive opinions on the article. All authors contributed to the article and approved the submitted version.

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Omega 3 Fatty Acid and Skin Diseases

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Humans are exposed to various external environmental factors. Food intake is one of the most influential factors impacting daily lifestyle. Among nutrients obtained from foods, omega-3 polyunsaturated fatty acids (PUFAs) have various beneficial effects on inflammatory diseases. Furthermore, omega-3 PUFA metabolites, including resolvins, are known to demonstrate strong anti-inflammatory effects during allergic and inflammatory diseases; however, little is known regarding the actual impact of these metabolites on skin diseases. In this review, we focused on metabolites that have strong anti-inflammatory actions in various inflammatory diseases, as well as those that present antitumor actions in malignancies, in addition to the actual effect of omega-3 PUFA metabolites on various cells.

Keywords: ω3 polyunsaturated fatty acids, resolvin, protectin, maresin, metabolites

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INTRODUCTION

The environment is fundamental for humans to live on earth and influences various physiological and pathological functions in the human body (1, 2). Food intake is an essential task for animals to drive their body and allows us to reconstruct body structure from nutrients (3). Among nutrients derived from foods, fatty acids are a component of cells and are known to control various cellular functions (4, 5). Omega-3 polyunsaturated fatty acids (PUFAs) are composed of 18 or more carbon chains, with a double bond three atoms away from the terminal methyl group, and are mainly classified into three representative lipids: α-linoleic acid (ALA), docosahexaenoic acids (DHA), and eicosapentaenoic acid (EPA). ALA is enzymatically converted to EPA and subsequently converted into DHA (6). These conversions primarily occur in the liver and are extremely limited owing to the enzyme concentration in the human body (7–9). Therefore, it is reasonable to derive DHA and EPA directly from foods and/or dietary supplements enriched in fish oils.

Omega-3 PUFAs have been known to demonstrate anti-inflammatory actions in various inflammatory diseases, including psoriasis, inflammatory bowel disease, asthma, and rheumatoid arthritis (10–12). In recent studies, omega-3 PUFA metabolites, such as resolvins (Rvs) and maresins, have revealed potent anti-inflammatory actions. Protectins (PD) and D-series Rvs are converted from DHA by 15-lipoxygenase, whereas E-series Rvs are produced from EPA by the cytochrome P450 pathways or acetylated cyclooxygenase-2. These metabolites have strong anti-inflammatory actions in various inflammatory diseases, such as animal models of asthma (13) and colitis (14), as well as antitumor actions in malignancies; however, little is known regarding their role in skin diseases. This review focused on the therapeutic potential of omega-3 PUFA metabolites for inflammatory skin diseases, as well as antitumor actions against cutaneous tumors.

ANTI-INFLAMMATORY ACTION ON IMMUNE CELLS AND EPITHELIAL CELLS

Reportedly, omega-3 PUFA metabolites have demonstrated various actions on immune and epithelial cells. In this section, we first reviewed the influence of omega-3 PUFA metabolites on each cell type, including dendritic cells (DCs)/macrophages, T cells/regulatory T cells (Tregs)/B cells, neutrophils, and epithelial cells, which are known to be present in the skin (**Table 1**).

DCs/Macrophages

DCs and macrophages play central roles in the acquired immune system to determine the direction of immune responses. As antigen-presenting cells, they take up antigens *via* phagocytic mechanisms to present antigens to T cells, to determine the direction of the immune response.

Phagocytosis is promoted by resolvin E1 (RvE1) (15–20), resolvin D1 (RvD1) (20, 34–37), resolvin D2 (RvD2) (20, 64), resolvin D3 (RvD3) (67, 68), resolvin D5 (RvD5) (70), protectin D1 (PD1) (15), and maresin 1 (MaR1) (82). For apoptotic cells, the macrophage phagocytosis is enhanced by RvE1 (15), PD1 (15), and RvD1 (34). Phagocyte-dependent bacterial clearance is enhanced by maresin 1 (MaR1) (82), RvD2 (64), RvD3 (67), and RvD5 (70).

Inflammatory cytokine production is negatively regulated by RvE1 (16, 20, 23), RvD1 (20, 38, 40–42), RvD2 (20, 38), RvD3 (71), and MaR1 (38, 78–80). RvE1, RvD1, and RvD2 suppress chemokine (C-C motif) ligand 4 (CCL4), CCL5, interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α induced by human monocyte-derived macrophages co-cultured with tumor cell debris (20), IL-12 by DCs stimulated with pathogen extract (23), and superoxide production by macrophages stimulated with cigarette smoke (16). In macrophages stimulated with lipopolysaccharide (LPS), RvD1, RvD2, and MaR1 suppress IL-1β and IL-6 (38, 78, 79). In contrast, MaR1 promotes the production of the inhibitory cytokine IL-10 (81).

DCs/macrophages play central roles in antigen presentation, as well as migration into antigen-presenting sites for T cells to initiate their active forms. RvD1 suppresses antigen presentation by suppressing major histocompatibility complex (MHC) class II and CD40 expression (39). Additionally, DC migration and infiltration into inflamed tissues are negatively regulated by RvE1 (22, 23) and RvD1 (43). RvE1 suppresses the migration of DCs in the skin and into draining lymph nodes, attenuating the acquired immune response (22), while RvD1 attenuates the increased infiltration of M1 macrophages (43).

Owing to the characteristics of antigen-presenting cells as a control tower in the immune response, the polarization of macrophages determines the direction of inflammatory responses. M1 macrophages are induced by interferon (IFN)-γ, a microbial component. M1 macrophages produce inflammatory cytokines and induce effector cells in polarized Th1 responses. M1 polarization is reportedly suppressed by RvD1 (44) and RvD2 (44). M2 macrophages are generated during enhanced Th2 reactions,

TABLE 1 | Cell specific action of SPMs.

	DC/Mφ	T cell/Treg/B cell	Neutrophil	Epithelial cell
RvE1	Phagocyte↑ (15–20) M2 polarization↑ (21) Migration↓ (22) (23) TNFα, IL-6, IL-8, CCL4, and CCL5↓ (20), IL-12↓ (23), Superoxide production↓ (16)	Th1/Th17 cells infiltration↓ (24) T-cell trafficking ↓ (21)	Infiltration↓ (17, 25) CXCR1/4, CCR2/6↓ (21) ROS↑ (26, 27) Phagocytosis↑ (26, 28) Apoptosis↑ (29)	Migration↑ (30, 31)
RvE3	No report	No report	Migration↓ (32, 33)	No report
RvD1	Phagocytosis ↑ (20, 34–37) COX-2 and IL-1β, IL-6↓ (38), TNFα, IL-6, IL-8, CCL4, and CCL5↓ (20) MHC II, CD40, and IL-12 ↓ (39), IL-1β, CCXL10, TNF, IL-6, CCR7↓ (40), Tnf-α, IL-6, IL-1β↓ (41), IL-6 and IL-8↓ (42) Infiltration of M1 macrophages and expression of inflammatory cytokines ↓ (43) M1↓/M2 polarization↑ (44) M2 polarization↑ (45–50)	TNF-α, IFN-γ, IL-17↓ (51) Migration↓ (52) Treg↑ (34, 34, 51, 53) B cell IgM and IgG production↑ (54) B-cell IgE production↓ (55)	Apoptosis↑ (50) Infiltration↓ (36, 52, 56–61) CXCR4↓ (62) Phagocytosis↓ (63)	Epithelial barrier integrity (57)
RvD2	Phagocytosis.↑ (20, 64) TNFα, IL-6, IL-8, CCL4, and CCL5↓ (20), COX-2 and IL-1β, IL-6↓ (38) M1↓/M2 polarization↑ (44)	TNF-α, IFN-γ, IL-17↓ (51) Treg↑ (51)	Infiltration↓ (64, 65) Enhancedneutrophil access to the dermis, but prevented neutrophil-mediated damage (66)	No report
RvD3	Phagocytosis↑ (67, 68)		Migration↓ (68) Phagocytosis, ROS↑ (67)	Proliferation↑ (69)
RvD5	Phagocytosis↑ (70)			
PD1	Phagocytosis↑ (15) Inflammatory cytokine suppression (71)	Leukocyte infiltration↓ (72).	Infiltration↓ (73–75) Apoptosis↑ (74)	Anti-apoptosis (76) Anti-apoptosis (77)
MaR1	COX-2, IL-1β↓ (38), *iNos*, *IL-1b*, *IL-6*,*TNFa*↓ (78), ROS, IL-1β, TNF-α, IL-6, and INF-γ ↓ (79), ROS↓, apoptosis↑ (80) M2 polarization, *Il-10*↑ (81) Phagocytosis↑ (82)	TNF-α, IFN-γ, IL-17↓ (51) Treg↑ (51, 83)	Infiltration↓ (84, 85) Apoptosis↑ (86)	IL-6, IL-8, TNF-α, CXCL1↓ (87)

promoting parasite killing (88), tissue repair (89), and immunoregulatory functions (90). M2 polarization is positively regulated by RvD1 (44–50), RvD2 (44), and MaR1 (91).

T Cells/Tregs/B Cells

T cells can respond to pathogens by direct contact with the antigen derived from the pathogen and need to migrate to sites in the presence of the antigen. Reportedly, T cell migration is regulated by RvE1 (21, 24), RvD1 (52), and PD1 (72). RvE1 decreases the infiltration of Th1 and Th17 cells (24). RvE1 suppresses T cell infiltration by decreasing the production of RANTES in vascular smooth muscle cells (21).

After the initiation of immune responses by antigen-presenting cells, naïve T cells develop T cell responses as an appropriate direction of inflammatory immune responses. RvD1, RvD2, and PD1 suppress inflammatory cytokine production (51). RvD1, RvD2, and PD1 reduce Th1 and Th17 cytokine production (51).

Tregs are promising cells for the maintenance of immune homeostasis and tolerance (92). T cell-mediated autoimmune diseases and allergies are closely related to their deficiency or dysfunction. RvD1, RvD2, and PD1 are known to contribute to the inhibition of immune reactions by increasing Tregs (51).

B cells play an important role in the adaptive immune system. The activation of B cells is induced following antigen recognition, differentiating to form plasma cells for antibody secretion. RvD1 influences immunoglobulin production by B cells and increases IgM and IgG production (54), as well as reduces IgE production (55).

Neutrophils

Neutrophils have various functions, including phagocytosis and antimicrobial peptide production (93). Reportedly, neutrophil phagocytosis is positively regulated by RvE1 (26, 28), RvD1 (63), and RvD3 (67). In phagocytes, NADPH oxidase is essential for neutrophil microbicidal activity (94). Furthermore, reactive oxygen species (ROS) generation in neutrophils is promoted by RvE1 (26, 27) and RvD3 (67). In addition, neutrophil migration is suppressed by RvE1 (17, 25), RvE3 (32, 33), RvD1 (36, 52, 56–61), RvD2 (64, 65), RvD3 (68), PD1 (73–75), and MaR1 (84, 85). RvE1 decreases chemokine receptor expression, including C-C chemokine receptor type 2 (CCR2), CCR6, chemokine (C-X-C motif) receptor 1 (CXCR1), and CXCR4 (21), as well as chemotaxis (26). RvD1 decreases CXCR4 expression (62). Moreover, RvD2 allows neutrophils to enhance their access to the dermis; however, RvD2 demonstrates a protective role against neutrophil-mediated damage (66).

Apoptotic neutrophils progress toward secondary necrosis mediated by the release of caspase 3-processed danger signals (93). Neutrophil apoptosis is reportedly enhanced by RvE1 (29), RvD1 (50), PD1 (74), and MaR1 (86). These metabolites enhance apoptotic cell phagocytosis of macrophages, suppressing secondary necrosis-mediated inflammation.

Epithelial Cells

Epithelial cells act as the first line of defense against the external environment. As the outermost organ layer, epithelial cells induce inflammatory cytokine and chemokine production to amplify the response to external stimuli. Inflammatory cytokine production is negatively regulated by MaR1 (87). Furthermore, MaR1 suppresses the production of CXCL1, IL-6, IL-8, and TNF-α by bronchial epithelial cells (87).

As the outer organ layer, epithelial cells exhibit migration and proliferation to shield the defect of the first line of defense against external stimuli. RvE1 enhances epithelial cell migration (30, 31), RvD3 promotes lung epithelial cell proliferation (69), while RvD1 and PD1 exhibit protective effects toward epithelial cells. Furthermore, RvD1 promotes epithelial barrier integrity (57). PD1 is shown to afford protection against repetitive oxidative stress-induced apoptosis (76, 77).

DIFFERENT FUNCTIONS OF PUFA METABOLITES ON IMMUNE CELLS AND EPITHELIAL CELLS

There are some different effects of PUFA metabolites in immune cells and epithelial cells. RvE1 suppresses immune cell migration (22, 23) while it promotes epithelial cell migration (30, 31). In addition, PD1 promotes apoptosis of neutrophils (74); however, it enhances anti-apoptosis in epithelial cells (76, 77). Although the detailed mechanism remains unclear, a possible other point of action might exist in each different type of cell, because they are located in different body sites and surface layers, which are given the appropriate role in the body.

THE ANTI-INFLAMMATORY ACTION OF PUFA METABOLITES ON SKIN DISEASES

Several reports have highlighted the anti-inflammatory actions of omega-3 PUFA metabolites on inflammatory skin diseases, including psoriasis, atopic dermatitis, contact hypersensitivity, and ultraviolet (UV) radiation. Furthermore, the antitumor effects of PUFA metabolites on squamous cell carcinoma and melanoma have been reported (**Table 2**).

TABLE 2 | Anti-inflammatory and anti-tumor action of Omega-3 PUFA metabolites in skin diseases.

Inflammatory skin disease	Omega-3 PUFA metabolites
Psoriasis	MaR1 (95)
	RvE1 (96)
	RvD1 (97)
Atopic dermatitis	RvE1 (98)
Contact dermatitis	RvE1 (22)
UV radiation	MaR1 (99)
Wound healing	PD1 (48)
	RvD1 (100)
Malignancy	
Squamous cell carcinoma	RvD2 (101)
Malignant melanoma	RvD1 (20)
	RvD2 (20)

Psoriasis and PUFA Metabolites

Psoriasis is a representative inflammatory skin disease characterized by scaly erythematous plaques with epidermal hyperplasia (102). Although the underlying mechanism of psoriasis remains unclear, recent studies have revealed some predominant pathways underlying pathological conditions, as well as the contribution of the TNF/IL-23/IL-17 axis (103). Current biologics targeting IL-17, IL-23, and TNF-α play critical roles in the pathogenesis of psoriasis (104).

Reportedly, MaR1 impairs imiquimod-induced psoriasis-like skin inflammation and IL-23 subcutaneous injection-induced skin inflammation (95). MaR1 decreases lymphocyte and neutrophil infiltration, dermal edema, and epithelial hyperplasia. MaR1 inhibits the production of IL-17A by $CD4^+$ and $\gamma\delta TCR^{mid+}$ cells. Consequently, MaR1 attenuates IL-23 receptor expression on $CD4^+$ and $\gamma\delta TCR^{mid+}$ cells by inhibiting retinoic acid-related orphan receptor gamma t (RORγt) in clathrin-dependent IL-23 receptor internalization.

RvE1 impairs imiquimod-induced psoriatic dermatitis (96). Furthermore, IL-17-producing cells and neutrophils are reduced in the skin following RvE1 treatment. Reportedly, IL-23 and IL-17 are downregulated by RvE1. IL-23 production by DCs, as well as the migration of DCs and IL-17 producing cells, is suppressed by RvE1.

RvD1 reduces acanthosis and hyperkeratosis induced by imiquimod (97). Inflammatory cell infiltration into the dermis is reduced following treatment with RvD1. Consistently, IL-17, IL-23, and TNF-α are decreased by RvD1.

Atopic Dermatitis (AD) and PUFA Metabolites

AD is a representative Th2-mediated chronic inflammatory skin disease characterized by inflamed and irritative itchy skin inflammation (105). Several factors are involved in the various environmental factors that drive Th2 dominant skin inflammation, including skin barrier disruption (106, 107), pathogens (108), and prostanoids (109). In NC/Nga mice, RvE1 demonstrates anti-inflammatory actions in repeated hapten application-induced AD-like skin inflammation (98). RvE1 suppresses IL-4 and IFN-γ production by T cells, as well as serum IgE levels, and reduces the infiltration of eosinophils, mast cells, and T cells in skin lesions.

Contact Dermatitis and PUFA Metabolites

Contact dermatitis is a common cutaneous allergic reaction that depends on the acquired immune response (110, 111). RvE1 impairs the inflammatory response in contact hypersensitivity during the sensitization and elicitation phases (22). During the sensitization phase, RvE1-treated mice exhibit significantly reduced DC migration into draining lymph nodes, subsequently reducing the number of central and effector memory T cells. Consistently, antigen-specific T cell proliferation and IFN-γ production are reduced by RvE1. In the elicitation phase, RvE1 impairs DC cluster formation, which is essential for the development of elicitation phase inflammation, subsequently suppressing the number of IFN-γ–producing $CD8^+$ T cells in the skin.

UV Radiation and MaR1

UV radiation is a representative environment-related skin inflammation that causes acute inflammation characterized by skin inflammation after sun exposure (112). MaR1 suppresses skin swelling as well as macrophage infiltration induced by UVB irradiation (99). Furthermore, MaR1 inhibits UVB irradiation-induced keratinocyte apoptosis, production of inflammatory cytokines, IL-1β and TNFα, and oxidative stress.

Wound Healing and PUFA Metabolites

Damage induced by external factors destroys the skin surface, resulting in skin defects that appear as a wound. As the skin acts as a barrier against the external environment, wounds allow outside pathogens and irritants to infiltrate the body and cause skin inflammation (113). PD1 and RvD1 promote skin wound healing (48, 100). In diabetic wounds, RvD1 enhances macrophage phagocytosis, promoting wound closure owing to the reduced number of apoptotic cells (48). Furthermore, PD1 promotes wound closure (100). Skin wounds promote the synthesis of PD1 in the skin; however, in diabetes, the skin suppresses PD1 production. Macrophages are one of the main sources of PD1 in skin wounds and are known to contribute to the development of inflammation and oxidative stress reactions during acute inflammation in diabetic wounds.

Antitumor Effects of PUFA Metabolites

Squamous cell carcinoma is a keratinocyte-derived malignancy, and the advanced clinical stage of this malignancy remains refractory to current systemic treatments (114). RvD2 suppresses squamous cell carcinoma development (101) and decreases inflammatory chemokines and cytokines, including CXCL10, IL-6, monocyte chemoattractant protein-1 (MCP-1), and TNF-α, by cancer cells. In squamous cell carcinoma, RvD2 decreases the infiltration of neutrophils and suppresses myeloperoxidase (MPO) activity. Additionally, RvD2 enhances the M2 macrophage population and their efferocytosis.

Malignant melanoma is a malignancy derived from melanocytes with severe life-threatening clinical behavior owing to a lack of radical treatment (115). RvD1 and RvD2 suppress melanoma development (20). Furthermore, RvD1 or RvD2 inhibit lung metastasis of melanoma cells and regulate the production of chemokines and cytokines, including CCL4, CCL5, IL-6, IL-8, and TNF-α, by human macrophages, providing antitumor immunity.

CLINICAL TRIAL OF OMEGA3 PUFA FOR CUTANEOUS SKIN DISEASES

Finally, we reviewed the clinical trials of omega 3 PUFAs for cutaneous skin diseases. There are several trials for atopic dermatitis and psoriasis.

The intake of omega-3 supplement improves the Scoring in Atopic Dermatitis (SCORAD) score (116). A double-blind, randomized, placebo-controlled trial showed AD patients who received daily ω3 fatty acid supplementation (fish oil, 10%; 200 mL) show high serum EPA concentration and a decreased disease severity of AD (117).

Psoriasis patients with obesity received energy-restricted foods enriched of ω3 PUFAs (average 2.6 g/d), and these patients showed impaired Psoriasis Area Score Index (PASI)

score and Dermatological Life Quality Index (118). A double-blind, randomized study in multicenter trials showed the group of intaking daily an ω3 fatty acid (Omegavenous; 200 ml/day with 4.2 gm of both EPA and DHA) decreased total PASI score without serious side effects (119).

Although there have been no clinical trials of cutaneous malignancies, head and neck squamous cell carcinoma has been reported. Supplementation of daily 2 g EPA intakes impairs the production of serum pro-inflammatory cytokines, reduction of body weight and lean body mass, and increases quality of life in patients with squamous cell carcinoma in head and neck (120).

CONCLUSION

There are a limited number of research and clinical trials for investigations of the effects of ω3 PUFA in skin diseases. According to previous studies assessing immune cells, various beneficial effects are expected in skin diseases. Furthermore, there are few reports available on keratinocytes, which are a major component of cells present in the epidermis. The detailed action of keratinocytes in the skin needs to be clarified in future research. Because the incidences of inflammatory skin diseases and malignancies are currently increasing, future basic research and clinical trials for ω3 PUFAs in dermatology fields are expected give us a beneficial information for the daily clinical treatment for skin diseases.

AUTHOR CONTRIBUTIONS

YS and MN wrote this manuscript, and NS-S conducted a critical review of this paper. All authors contributed to the article and approved the submitted version.

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Breastfeeding Rate, Food Supplementation, Dietary Diversity Among Children Aged 6–59 Months, and Associated Factors in Papua New Guinea

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Background: Along the socioeconomic changes in the past decades, Papua New Guinea (PNG) has undergone significant food transition. Little is known about the influence of household and maternal socioeconomic demographic factors on dietary intake and diversity among children under 5 years of age (CU5).

Objective: This study aimed to examine breastfeeding rate, food supplementation, dietary intake, and diversity among children aged 6–59 months and to identify associations with household and maternal socioeconomic demographic factors in PNG.

Method: Data from 2,943 children were extracted from the Comprehensive Health and Epidemiological Surveillance System database, operated by the PNG Institute of Medical Research and used to estimate breastfeeding rate, food supplementation, and dietary intake of CU5 in a typical week. Dietary diversity score (DDS) was used as a proxy indicator to measure nutrient adequacy. Associations of DDS with household and maternal socioeconomic and demographic factors were examined using multivariate logistic regression analysis.

Result: The breastfeeding rate among children aged 6–8 months was 85% (70% in urban and 90% in rural sectors), and 50% of children of this age group were fed with supplementary foods. Twenty percent of children aged 6–23 months were currently breastfed and received solid, semisolid, and soft foods three times or more per day. Forty-eight percent of children aged 6–59 months had a total DDS below the average level (23 scores). Place of residence, mother's education, and household wealth were associated with dietary diversity among studied children. Children in urban areas are 10% more likely to have a lower level of total DDS than those in rural areas (OR: 1.11 [0.79–1.56]; *p*-value: 0.5). Children whose mothers had a primary education level were 1.6-fold more likely to have a lower level of total DDS than children whose mothers had vocational training or college education (OR: 1.63 [0.68–3.92]; *p*-value: 0.28). Children from the poorest households were 1.2-fold more likely to have a lower DDS than those from the richest households (OR: 1.22 [0.79–1.87]; *p*-value: 0.37).

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Discussion: A range of factors has been identified, contributing to the eating behaviors among CU5 in PNG, in which mother's education and household wealth are among the most important determinants of childhood dietary diversity as they have a direct effect on accessibility to and affordability of a variety of foods at the household level.

Conclusion: Evidence-based integrated and comprehensive approaches are needed to improve women education and household wealth, contributing to the improvement of food diversity among young children in PNG.

Keywords: food supplementary, dietary diversity, CHESS, Papua New Guinea, breastfeeding

INTRODUCTION

About one-third of the children do not achieve their full health and developmental potential due to inadequate dietary intake, and this has major health implications throughout life course (1, 2). According to the World Health Organization (WHO), malnutrition refers to deficiencies, excesses, or imbalances in a person's intake of energy and/or nutrients. Malnutrition can lead to two health conditions: (i) undernutrition, which includes stunting (low height for age), wasting (low weight for height), underweight (low weight for age), and micronutrient deficiencies or insufficiencies (a lack of important vitamins and minerals), and (ii) overweight and obesity, which are related to non-communicable diseases such as heart disease, stroke, diabetes, and cancer (3). Approximately 6.3 million children under 5 years of age (CU5) die every year globally, and about one-third of child deaths are particularly linked to diet-related health conditions (1, 4–6).

The World Health Assembly targets for 2025 and the United Nations' Sustainable Development Goals (SDGs) for 2030 have set out indicators of child malnutrition and for countries to monitor the food and nutrition status among CU5 (2, 4, 5). Nutrition is a cross-cutting issue and is vital for child health and development, but it is often neglected in the implementation of public health programs in low-middle-income countries (LMICs) (6). The recent changes in socioeconomic development in these countries are known to have greatly increased impacts on food choices and dietary habits among their populations (2, 7). In the Pacific Island Countries (PICs), including Papua New Guinea (PNG), food transition has been characterized by a move from traditional low-calorie diets to the consumption of processed high-calorie diets (8). At the same time, these countries show an emerging face of lifestyle diseases (9), with diet-related non-communicable diseases (NCDs) seen as an emerging public health problem, particularly among the adult population in urban areas (10, 11). By contrast, the problem of poor dietary intakes among child population continues to be a significant impediment in child health and development in these countries (12).

PNG is located just south of the equator and 160 km north of mainland Australia, in the South Pacific region, and consists of 22 provinces with a population of ∼8.7 million in 2019. PNG has experienced dramatic social and economic changes following its opening to the outside world since the second half of the nineteenth century. The majority of the population (85%) lives in rural areas and is involved in subsistence-based agriculture (13). PNG has great potential to improve its socioeconomic development status through the economic development of its natural resources: land, agriculture, forestry, and fisheries (14). The mining and energy sectors contribute ∼80% of the total export revenue of the country. The development of mining and extraction industries has led to significant social and economic changes across the country in recent years. With 38.2% of the population being under the age of 15 years, PNG has a young population (15).

Early childhood is a special developmental period offering great opportunity but also great vulnerability for individual health and development. Previous studies show that parental food choices, eating habits, and feeding strategies are the most important determinants affecting their children's eating behavior and nutritional diet, particularly at the early childhood (2, 16–20). Poor dietary intake, including breastfeeding and food supplementation constitutes larger risks contributing to childhood malnutrition in PNG (21–23), but there are few studies on diet-related child health issues in the food transition in PNG (24, 25).

We hypothesize that the food and nutrition transition occurring in PNG posed increased health risks, particularly for child breastfeeding, food feeding, and dietary behaviors. **Figure 1** showed the analytical conceptual framework for analyzing food transition in PNG, which may have been effected by macro environmental factors as well as micro household and maternal sociodemographic factors.

Macro environmental factors including extreme natural events such as drought, flood, earthquake, and landslide have direct impacts on agriculture production and distribution, hence affecting the transition at a large scale. Discussion of these factors will be covered in a separate paper. Urbanization processes redistribution of the population and social classes, contributing to socioeconomic development. The urbanization process is one of the important factors influencing availability and access to foods, especially in urban areas, hence contributing to the dietary diversity of urban children. Geographical location determines the local climate and culture, defining the food habits of a population. The four geographical regions of PNG with typical climate and ecosystems have various impacts on agriculture production, food processing, and eating behavior of the population, hence contributing to the nutritional status of children.

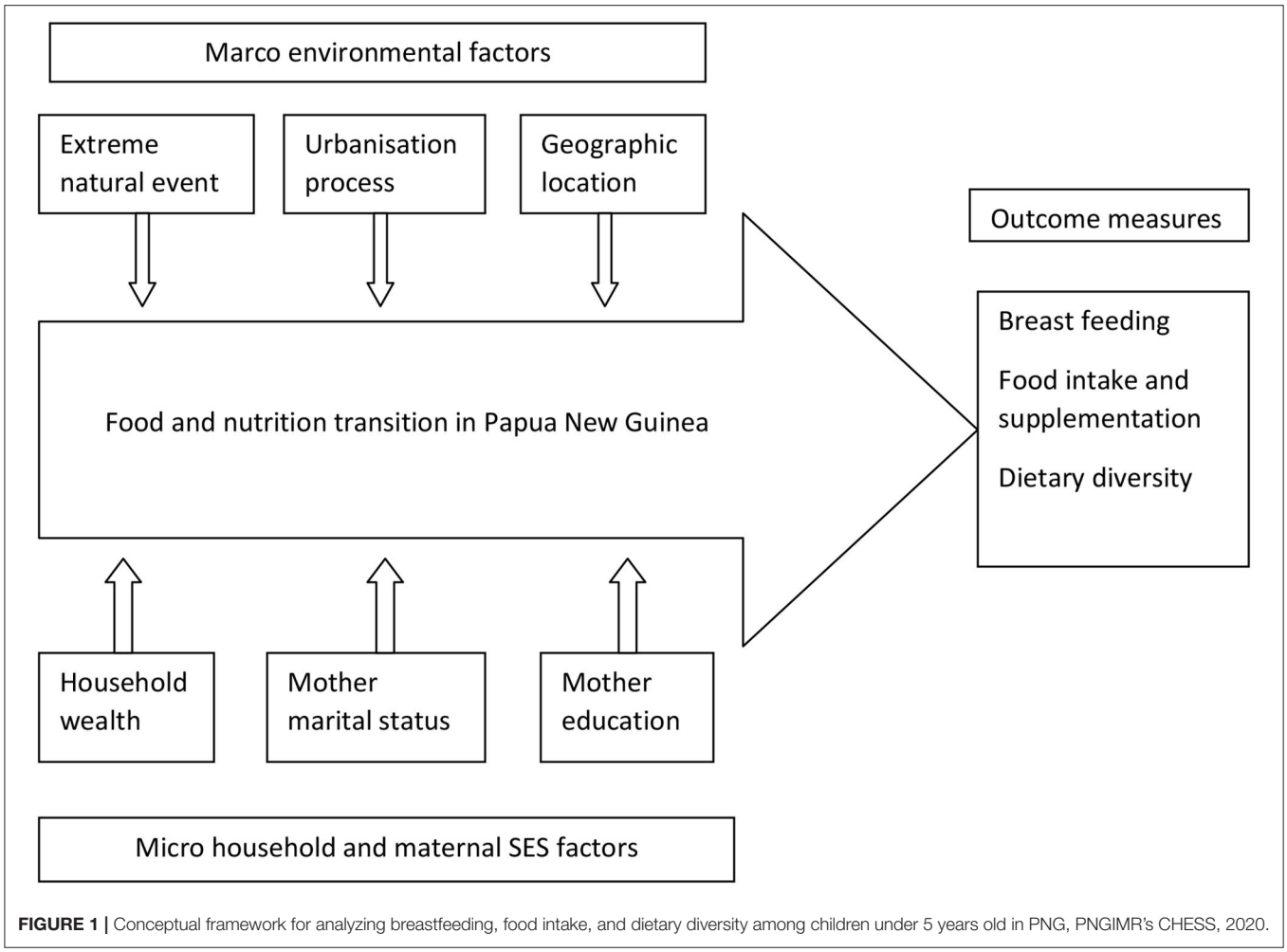

FIGURE 1 | Conceptual framework for analyzing breastfeeding, food intake, and dietary diversity among children under 5 years old in PNG, PNGIMR's CHESS, 2020.

Micro environmental factors at the household and individual levels such as household socioeconomic status have a direct effect on a household's choice and consumption of food. Household wealth has direct influences on the food choice of household members and of children in particular. On the other hand, maternal demographic characteristics such as marital status and education could be also factors influencing breastfeeding practice, food supplementation, and dietary intake of children. Those children who are from households with better-off socioeconomic status and whose mothers have better education could have better dietary diversity and food intake than children from households with a poor socioeconomic status. The outcome of these processes is breastfeeding practice among women and food intake among children. All these contribute to the dietary diversity of the children, especially among CU5.

In this study, we attempted to report key indicators on eating behaviors among children aged 6–59 months, including breastfeeding, food supplementation, and dietary intake (16). We also explored the household and maternal sociodemographic factors influencing the children's dietary diversity in PNG.

METHODS

Study Design, Location, and Population

We used the data from the Comprehensive Health and Epidemiology Surveillance System (CHESS), operated by the Papua New Guinea Institute of Medical Research (PNGIMR) since 2010 (13, 17, 18). CHESS is designed as a cohort longitudinal follow-up study, and the CHESS methodology has been published elsewhere (19, 20). **Figure 2** shows the five CHESS surveillance sites, located in four provinces representing the four geographic regions of PNG: Hiri and Port Moresby (POM) sites in Central Province for the southern region, Asaro and Goroka sites in Eastern Highlands Province (EHP) for the highlands region, the Newtown site in Madang Province for the Momase region, and Baining and Kokopo sites in East New Britain Province (ENB) for the islands region. These sites cover the population living in both urban and rural sectors, which are defined based on the PNG National Statistics Office's classifications and standards (26). Household interviews with parents and caregivers of child participants were conducted between July and December 2018 by CHESS national scientific

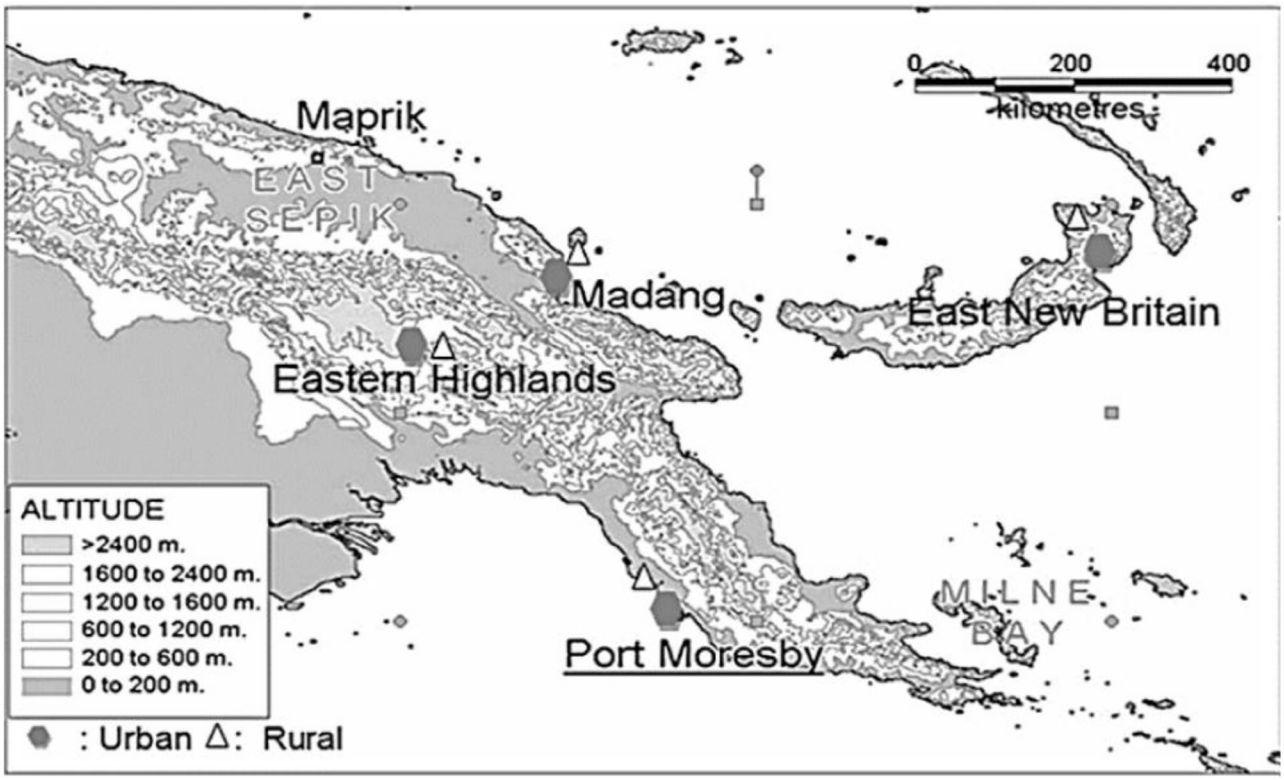

FIGURE 2 | Geographic coverage and surveillance sites of PNGIMR's CHESS, 2020.

officers, using a paper-based questionnaire, which is specifically designed for collecting information and data of CU5 who live in the surveillance sites.

To understand the dietary intake of children aged 6–59 months, parents or caregivers were asked questions on how many days they fed their children with a particular type of food in the typical week, meaning a normal week when the child diet was not affected by cultural, religious, or other social events. Mothers'/caregivers' responses were recorded with a score from 0 to 7, representing 0–7 days/week. The food items were categorized into nine groups of foods relevant to the PNG context: (i) root vegetables such as potato, sweet potato (kaukau), yam, and taro, providing starchy staples; (ii) green vegetables such as aibika and beans; (iii) fresh fruits such as papaw, orange, mango, and avocado; (iv) fresh meat such as chicken, pork, and fish; (v) tinned meat, fish, pork or chicken; (vi) fried foods which were cooked in the house such as fried flour and fried rice; (vii) fried foods purchased from shops such as flour balls, chips, and lamp flaps, providing a mixture of starch and meat; (viii) salt, providing additional iodine to children (all salt sold in PNG is iodized); and (ix) seasoning food such as Maggi and stock cube, providing additional flavor and taste.

Variables

Variables on child background include age (in months), sex (male and female), residence (urban–rural), and province (surveillance sites). Key variables are derived mostly from the two data modules: (i) breastfeeding and nutrition and (ii) food intake and diet.

The CU5 dataset was linked with selected variables on household and maternal sociodemographic characteristic data, derived from the data component of the CHESS database comprising women of reproductive age, 15–49 years. The maternal variables included the highest educational attainment, employment, and marital status. These variables were linked with CU5 data using the unique household identification codes.

The household wealth index was calculated as an overall marker of household socioeconomic status. The estimates were based on the data on housing characteristics and household assets, extracted from the household data component of the CHESS database, using the factor analysis method. The household wealth indices were then divided into five categories (household wealth quintile), from the first to the fifth, representing the poorest, poorer, middle, richer, and richest groups of households, respectively. The process of calculating household wealth quintiles was discussed and published elsewhere (15).

Data Analysis

Raw datasets were extracted in Microsoft Excel spreadsheet format, converted into Statistical Package of Social Science (SPSS) version 20.0 for data analysis. This process generated

the results, and data were summarized as frequencies and percentages to understand the distributions of breastfeeding, eating behavior, and dietary intake and diversity among children aged 6–59 months.

Figure 3 shows the flow diagram of child participation in the study and child selection for particular analysis. A total of 4,134 children aged 0–57 months were recruited across the surveillance sites, including 1,368 in urban areas (33%) and 2,766 in rural areas (67%). After sorting for children whose information on age and sex were missing, 3,458 children were found eligible and included in the data analyses for different purposes. The household and maternal socioeconomic and demographic characteristics of these children are presented in **Table 1**.

Specifically, 92 children aged 6–8 months were included in the analysis of breastfeeding rate and food supplementation. It is recommended to introduce solid, semisolid, or soft foods to children in the age of 6–8 months as supplementary food to breastfeeding (2). For all children aged 6–8 months, they are recommended to be breastfed (up until 2 years old). These children are also recommended to start having supplementary foods, including soft, semisolid, and solid foods. It means all children in this age group should be both breastfed and fed with supplementary foods. There are three separate indicators to measure nutritional status among these children, namely (i) proportion of children having been currently breastfed (breastfeeding rate); (ii) proportion of children currently receiving supplementary foods; and (iii) proportion of children who are currently both breastfed and fed with supplementary foods. Analysis of drinks such as plain water, infant formula, milk, fruit juice, meat/vegetable water, vitamin/mineral supplements, oral rehydration solution (Oresol), and liquids (tea, soft drinks, and others) is not included in this paper. A chi-square test was used to compare proportions between child groups. Results are shown in **Table 2**.

Furthermore, 687 children aged 6–23 months were included in the data analysis of frequency of meals for food supplementation. For children of the age group 6–23 months, they are recommended to be fed with supplementary foods with *at least three meals per day* for those children who are currently breastfed and *at least four meals per day* for those children who are currently not breastfed (27). Two indicators are calculated for reporting the food supplementation among children of this age group: (i) proportion of children who are currently breastfed and fed with three meals or more per day and (ii) proportion of children who are not currently breastfed but have four meals or more per day. Similar to the above analysis, the consumption of drinks is not analyzed in this paper. Chi-square tests were conducted to provide a significant level (*p*-value). Results are shown in **Table 3**.

Data of 2,943 children aged 6–59 months were analyzed for dietary intake and diversity. Results are shown in **Table 4**. Children aged 0–5 months were not included in the analysis as they should be exclusively breastfed while children aged 60 months and above were also not included because they are not defined as denominators in the measurement of dietary diversity among CU5. Dietary diversity score (DDS) was used as a proxy indicator to measure the variety of foods. This method is commonly used in low-resource settings (28, 29). There are different approaches to calculate DDS such as totaling the number of times when a child is fed with different types of food in a day (29). In this study, one score was given to the child, equivalent to 1 day, when the child was reported as having been fed with a particular type of food. The total DDS was calculated for each child by adding all the numbers of days when the child was fed with any food group mentioned above in a typical week prior to the interview. The mean of total DDS for a week was also calculated for each type of food as well as for all types of foods and disaggregated by province. Analysis of variance (ANOVA) and eta tests were used to compare means of total DDS between provinces. Results are shown in **Table 4**.

Lastly, the data of 1,264 children aged 6–59 months were found eligible and included in statistical modeling for the analysis of the association of household and maternal socioeconomic factors with dietary diversity among these children. Multivariate logistic regression (MLR) analysis was conducted for this purpose. The mean of total DDS was used as the cutoff point to divide children into two groups: children with low total DDS (below the mean) and those with high total DDS (above the mean). A new categorical variable on total DDS level (low and high) was created and included in the model MLR as a dependent variable, with low total DDS level being used as a reference category. Independent variables, including household and maternal socioeconomic and demographic variables, were included in the MLR model as factors to predict the outcome of a low-level total DDS. Non-significant variables were excluded from the models, and only significant factors remained, including resident location (urban–rural sector), province, sex of the child, child age group, mother's highest educational attainment (elementary, primary, lower secondary, upper secondary, and vocational training and above), mother's marital status (currently married/in union, not married/in union, and single mother), child age group (6–11, 12–23, 24–35, 36–47, and 48–59 months), sex of the child (male or female), and household wealth quintile (from poorest to richest), assuming other predictors remain constant. The model of the main effect was selected to produce maximum likelihood estimates of odds ratios (ORs) of having low total DDS. A statistical likelihood test was also selected in the model to provide estimates of 95% CI of OR and significance levels. Results are shown in **Table 5**.

RESULTS

Household, Maternal, and Child Socioeconomic Demographic Characteristics

Table 1 shows the sociodemographic characteristics of all child participants and their mother. About 66.9% of child participants were from the rural areas and 33.1% from the urban areas. Note that all children in the Central Province were from the rural sector, but all children in Madang Province and POM were from the urban sector. The proportion of children increased with the increase in age group, from 5.4% in the age group 0–5 to 23.1% in the age group 48–59 months. About 51.9% were males,

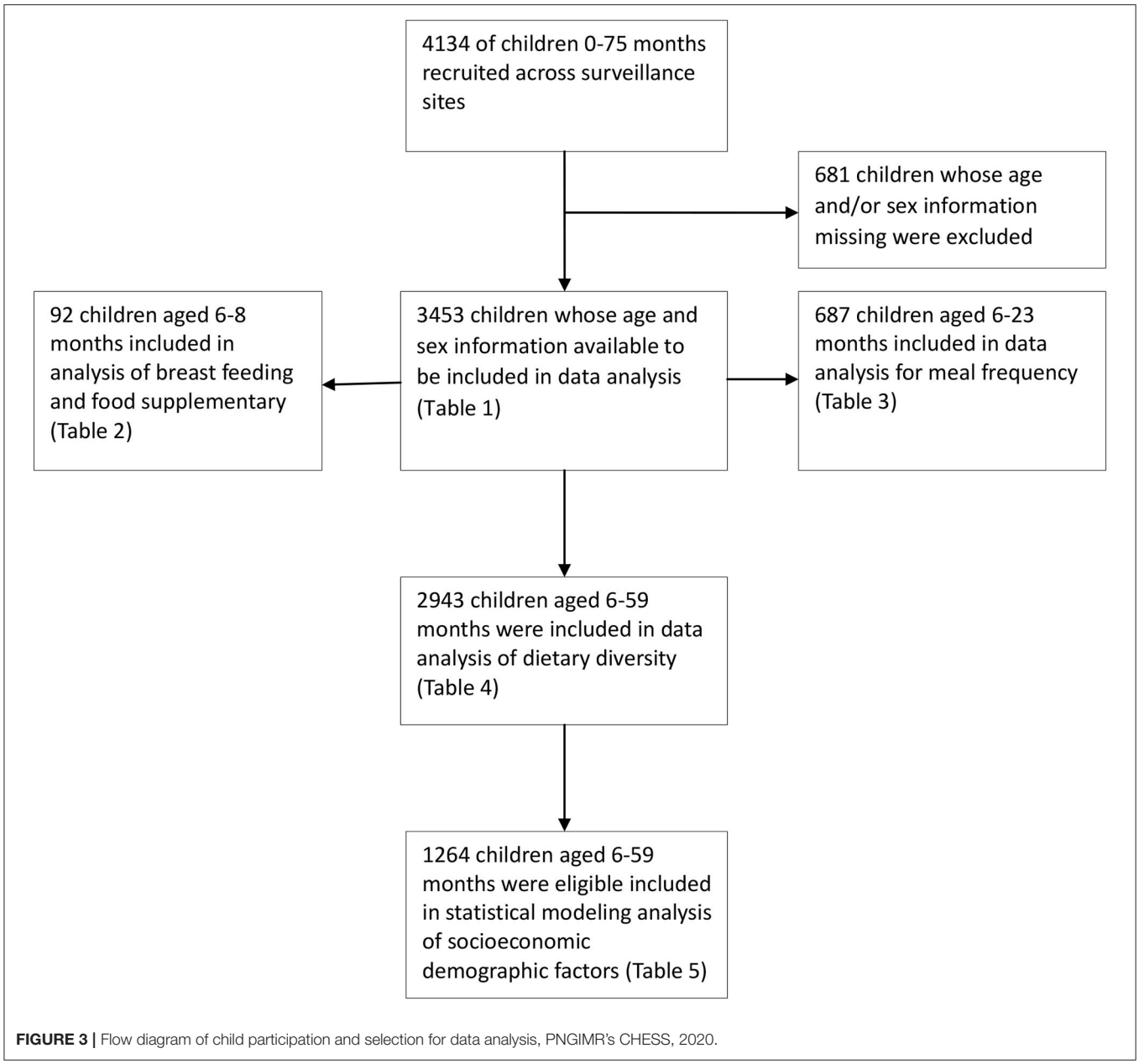

FIGURE 3 | Flow diagram of child participation and selection for data analysis, PNGIMR's CHESS, 2020.

and 48.1% were females. In regard to maternal characteristics, 68.0% of mothers attained primary education, compared to 21.9, 3.4, and 1.9% who attained lower, upper secondary, and vocational/college education, respectively. Marital status shows that 51.7% were married, and 12.5% were in union, but 35.8% reported as not being married or in union.

Breastfeeding and Supplementary Food

As shown in **Table 2**, about 85% children were currently being breastfed, and more than 50% were given solid, semisolid, or soft foods. The proportion of children aged 6–8 months being breastfed and given supplementary food (solid and semisolid foods) was about 42%, with a higher rate in rural areas (47%) than in urban areas (32%). The higher rate of breastfeeding and appropriate food supplementation was reported in EHP and Central Province, about 60%. The proportion of female children being breastfed and given supplementary foods was significantly higher than male counterparts, 50 and 35%, respectively (chi-square test results <0.5).

Meal Frequency

Table 3 shows that above 20% of children aged 6–23 months were currently breastfed, plus received solid, semisolid, and soft foods three times per day or more, with a lower proportion in urban (12%) than in rural areas (24%). EHP recorded the highest proportion (36%) followed by Central Province (21%),

TABLE 1 | Sociodemographic characteristics of all child participants and their mothers by surveillance site, PNGIMR's CHESS, 2020.

		Central province	EHP	Madang province	ENB	POM	All sites
Sector	Urban	0 (0.0%)	397 (34.5%)	331 (100.0%)	272 (31.0%)	372 (100.0%)	1,368 (33.1%)
	Rural	1,401 (100.0%)	755 (65.5%)	0 (0.00%)	606 (69.0%)	0 (0.0%)	2,766 (66.9%)
	Total	1,401 (100.0%)	1,152 (100.0%)	331 (100.0%)	878 (100.0%)	372 (100.00%)	4,134 (100.0%)
Age group in months	0–5	83 (6.8%)	25 (2.6%)	26 (8.4%)	36 (4.8%)	15 (6.9%)	185 (5.4%)
	6–11	70 (5.7%)	25 (2.6%)	27 (8.7%)	63 (8.5%)	16 (7.3%)	201 (5.8%)
	12–23	169 (13.8%)	124 (13.0%)	39 (12.6%)	131 (17.6%)	16 (7.3%)	479 (13.9%)
	24–35	287 (23.4%)	169 (17.7%)	59 (19.1%)	141 (18.9%)	59 (27.1%)	715 (20.7%)
	36–47	274 (22.3%)	197 (20.7%)	63 (20.4%)	147 (19.7%)	49 (22.5%)	730 (21.1%)
	48–59	342 (27.9%)	211 (22.1%)	71 (23.0%)	132 (17.7%)	40 (18.3%)	796 (23.1%)
	60–75	3 (0.2%)	202 (21.2%)	24 (7.8%)	95 (12.8%)	23 (10.6%)	347 (10.0%)
	Total	1,228 (100.0%)	953 (100.0%)	309 (100.0%)	745 (100.0%)	218 (100.0%)	3,453 (100.0%)
Sex of child	Male	629 (51.0%)	503 (52.3%)	158 (50.3%)	394 (52.8%)	111 (54.7%)	1,795 (51.9%)
	Female	604 (49.0%)	459 (47.7%)	156 (49.7%)	352 (47.2%)	92 (45.3%)	1,663 (48.1%)
	Total	1,233 (100.0%)	962 (100.0%)	314 (100.0%)	746 (100.0%)	203 (100.0%)	3,458 (100.0%)
Mother's educational attainment	Elementary	47 (6.2%)	41 (6.4%)	2 (0.6%)	27 (3.3%)	12 (6.3%)	129 (4.7%)
	Primary	584 (76.6%)	444 (69.4%)	114 (35.0%)	576 (70.7%)	142 (74.3%)	1,860 (68.0%)
	Lower secondary	112 (14.7%)	115 (18.0%)	170 (52.1%)	172 (21.1%)	29 (15.2%)	598 (21.9%)
	Upper secondary	12 (1.6%)	26 (4.1%)	26 (8.0%)	27 (3.3%)	3 (1.6%)	94 (3.4%)
	Vocational/college+	7 (0.9%)	14 (2.2%)	14 (4.3%)	13 (1.6%)	5 (2.6%)	53 (1.9%)
	Total	762 (100.0%)	640 (100.0%)	326 (100.0%)	815 (100.0%)	191 (100.0%)	2,734 (100.0%)
Mother marital status	In marriage	705 (56.0%)	483 (50.2%)	153 (46.6%)	386 (46.2%)	196 (58.5%)	1,923 (51.7%)
	In union	124 (9.9%)	176 (18.3%)	54 (16.5%)	84 (10.0%)	27 (8.1%)	465 (12.5%)
	Single mother	429 (34.1%)	303 (31.5%)	121 (36.9%)	366 (43.8%)	112 (33.4%)	1,331 (35.8%)
	Total	1,258 (100.0%)	962 (100.0%)	328 (100.0%)	836 (100.0%)	335 (100.0%)	3,719 (100.0%)
Household wealth quintile	Poorest	336 (23.9%)	404 (35.0%)	0 (0.0%)	27 (3.1%)	90 (24.2%)	857 (20.7%)
	Poorer	354 (25.2%)	259 (22.4%)	0 (0.0%)	139 (15.8%)	88 (23.7%)	840 (20.3%)
	Middle	274 (19.5%)	160 (13.9%)	3 (0.9%)	203 (23.1%)	83 (22.3%)	723 (17.5%)
	Richer	291 (20.7%)	228 (19.8%)	51 (15.4%)	278 (31.6%)	71 (19.1%)	919 (22.2%)
	Richest	148 (10.5%)	103 (8.9%)	277 (83.7%)	232 (26.4%)	40 (10.8%)	800 (19.3%)
	Total	1,403 (100.0%)	1,154 (100.0%)	331 (100.0%)	879 (100.0%)	372 (100.0%)	4,139 (100.0%)

but the lowest proportion was in POM (6%). Among children who were currently not breastfed, only 14 children (2%) received solid, semisolid, and soft foods or milk feeds four times per day or more.

Dietary Intake

As shown in **Table 4**, child participants were reportedly being fed with root vegetables for 3.7 days/week on average, with the highest rate reported in EHP (5.1 days/week), but the lowest in Madang Province (2.8 days/week). Similarly, green vegetables were being fed to children with the highest rate in EHP (5.3 days/week) and the lowest in Central Province (1.8 days/week). Fresh meat or fish were given to children for 2.52 days/week, compared to 3.51 days/week for tinned meat or tinned fish. Children were reportedly being fed with homemade fried foods with the highest rate reported in Madang Province (2.7 days/week) and the lowest rate in EHP (1.7 days/week). In contrast, children were fed with purchased fried foods with the highest rate in EHP (2.08 days/week) and the lowest in Central Province (0.73 days/week). Stock cube directly put on the food was reported, with the highest rate in POM (2.85 days/week) and the lowest in Madang Province (0.39 days/week). Similarly, salt was put directly on the food for children, with the highest rate reported in POM (3.5 days/week) and the lowest rate in Madang Province (1.05 days/week).

Dietary Diversity Score

The mean of total DDS among children was 23.3 (SD: ±11.96) for all children across the sites. The mean DDS varied across the sites, with the most diversity found in POM (26.23), followed by EHP (25.31), ENB (23.06), and Central Province (22.49), and the lowest in Madang Province (19.65). Both ANOVA and eta tests showed that the differences between means of DDS were statistically significant between provinces across different types of foods and for all types of foods.

Furthermore, **Figure 4** represents the distribution of CU5 by total DDS in each province. The data revealed that about 49% of children in all sites had a total DDS below the mean of 23.3 scores. We used the value of 23 scores as the cutoff point to divide child participants into two groups: low DDS (≤23) and high DDS

TABLE 2 | Breastfeeding and supplementary semi-solid and solid foods among infants aged 6–8 months, PNGIMR's CHESS, 2020.

		No. of children aged 6–8 months	Currently being breastfed	Currently being fed with supplementary foods	Currently being breastfed and fed with supplementary foods
Sector	Urban	28	20 (71.4%)	13 (46.4%)	9 (32.1%)
	Rural	64	58 (90.6%)	34 (53.1%)	32 (50.1%)
p-value		0.04	0.003	0.35	0.02
Province	Central	36	33 (91.7%)	23 (63.9%)	22 (61.1%)
	POM	10	5 (50.0%)	4 (40.0%)	2 (20.0%)
	EHP	10	9 (90.0%)	7 (70.0%)	7 (70.0%)
	Madang	10	7 (70.0%)	4 (40.0%)	2 (20.0%)
	ENB	26	24 (92.3%)	9 (34.6%)	8 (30.8%)
p-value		0.20	0.002	0.10	0.01
Sex	Male	48	40 (83.3%)	22 (45.8%)	18 (37.5%)
	Female	44	38 (86.4%)	25 (56.8%)	23 (52.3%)
p-value		0.35	0.59	0.29	0.36
	Total	92	78 (84.8%)	47 (51.1%)	41 (42.4%)

TABLE 3 | Frequency of meals among children aged 6–23 months who received solid, semisolid, and soft foods in the previous day, PNGIMR's CHESS, 2020.

		No. of children aged 6–23 months	Currently breastfeeding and having 3+ meals per day	Currently not breastfeeding but having 4+ meals per day
Sector	Urban	202	25 (12.4%)	8 (4.0%)
	Rural	485	118 (24.3%)	6 (1.2%)
p-value		0.03	0.45	0.92
Province	Central	243	52 (21.4%)	3 (1.2%)
	POM	34	2 (5.9%)	2 (5.9%)
	EHP	150	54 (36.0%)	3 (2.0%)
	Madang	66	8 (12.1%)	6 (9.1%)
	ENB	194	27 (13.9%)	0 (0.0%)
p-value		0.03	0.04	0.54
Sex	Male	362	80 (22.1%)	8 (2.2%)
	Female	325	62 (19.0%)	6 (1.8%)
p-value		0.01	0.54	0.53
	Total	687	142 (20.9%)	14 (2.1%)

(≥24). EHP had the highest proportion of children (49%) with a high total DDS (24–35 points) in a typical week preceding the interview, followed by POM (40%) and Central Province (38%). On the other hand, Madang, Central, and ENB provinces had the highest proportions of children with a low total DDS (12–23 points), ranging 38–34%.

Household, Maternal, and Child Sociodemographic Factors Associated With DDS

In this section, we looked at the socioeconomic determinants of total DDS among children aged 6–59 months. **Table 5** presents the estimated OR with 95% CI and *p*-value, using the MLR model (no data of children in Madang Province were included because of either missing or ineligible values in mother's education, marital status, or household wealth quintile). Results showed that children in urban areas are more likely to have a low level of total DDS than children in rural areas (OR: 1.11 [0.79–1.56]; *p*-value: 0.5). Similarly, children in EHP are more likely to have a low DDS than children in POM (OR: 1.16 [0.67–2.02]; *p*-vale: 0.28). No significant difference was observed between male and female children and across child age groups (except children of age group 24–35 months).

Among maternal sociodemographic variables, mother's highest educational level attainment has significant associations with the total DDS across all educational levels. Specifically, children of women with lower secondary education were more likely to have a low total DDS than children from mothers with vocational training or college education and above (OR: 2.00 [0.8–4.98]; *p*-value: 0.14). Children whose mothers lived in union were less likely to have a low DDS than children of single mothers (OR: 0.88 [0.62–1.25]; *p*-value: 0.47). Noticeably, children from the poorest quintile were more likely to have a low total DDS than those from the richest quintile (OR: 1.22 [0.79–1.87]; *p*-value: 0.37).

TABLE 4 | Dietary intake and dietary diversity score among children aged 6–59 months for a typical week by surveillance site, PNGIMR's CHESS, 2020.

Type of foods	Dietary diversity score	Central	EHP	Madang	ENB	POM	All sites	ANOVA *p*-value	Eta *p*-value
Root vegetable	0	99 (8.6%)	20 (2.7%)	36 (13.7%)	74 (12.0%)	24 (13.0%)	253 (8.6%)		
	1	58 (5.1%)	5 (0.7%)	15 (5.7%)	64 (10.4%)	14 (7.6%)	156 (5.3%)		
	2	317 (27.6%)	50 (6.8%)	46 (17.6%)	93 (15.1%)	23 (12.4%)	529 (18.0%)		
	3	284 (24.8%)	98 (13.4%)	80 (30.5%)	88 (14.3%)	44 (23.8%)	594 (20.2%)		
	4	162 (14.1%)	118 (16.1%)	39 (14.9%)	62 (10.0%)	15 (8.1%)	396 (13.5%)		
	5	93 (8.1%)	62 (8.5%)	13 (5.0%)	50 (8.1%)	8 (4.3%)	226 (7.7%)		
	6	40 (3.5%)	53 (7.2%)	8 (3.1%)	7 (1.1%)	4 (2.2%)	112 (3.8%)		
	7	94 (8.2%)	323 (44.1%)	25 (9.5%)	149 (24.1%)	48 (25.9%)	639 (21.7%)		
	DK	0 (0.0%)	3 (0.4%)	0 (0.0%)	30 (4.9%)	5 (2.7%)	38 (1.3%)		
Mean DDS (SD)		2.89 (1.9)	5.1 (2.0)	2.8 (2.0)	3.5 (2.5)	3.5 (2.5)	3.7 (2.3)	<0.001	0.38
Green vegetable	0	232 (20.2%)	24 (3.3%)	25 (9.5%)	53 (8.6%)	31 (16.8%)	365 (12.4%)		
	1	213 (18.6%)	7 (1.0%)	6 (2.3%)	29 (4.7%)	17 (9.2%)	272 (9.2%)		
	2	338 (29.5%)	40 (5.5%)	40 (15.3%)	64 (10.4%)	19 (10.3%)	501 (17.0%)		
	3	196 (17.1%)	78 (10.7%)	49 (18.7%)	101 (16.4%)	41 (22.2%)	465 (15.8%)		
	4	90 (7.8%)	86 (11.7%)	31 (11.8%)	50 (8.1%)	15 (8.1%)	272 (9.2%)		
	5	43 (3.7%)	106 (14.5%)	14 (5.3%)	48 (7.8%)	13 (7.0%)	224 (7.6%)		
	6	8 (0.7%)	78 (10.7%)	27 (10.3%)	20 (3.2%)	5 (2.7%)	138 (4.7%)		
	7	25 (2.2%)	309 (42.2%)	70 (26.7%)	216 (35.0%)	38 (20.5%)	658 (22.4%)		
	DK	2 (0.2%)	4 (0.5%)	0 (0.0%)	36 (5.8%)	6 (3.2%)	48 (1.6%)		
Mean DDS (SD)		1.86 (1.6)	5.3 (2.0)	3.7 (2.5)	4.3 (2.5)	3.2 (2.5)	3.58 (2.5)	<0.001	0.56
Fruit	0	130 (11.3%)	34 (4.6%)	21 (8.0%)	32 (5.2%)	22 (11.9%)	239 (8.1%)		
	1	185 (16.1%)	81 (11.1%)	52 (19.8%)	65 (10.5%)	27 (14.6%)	410 (13.9%)		
	2	372 (32.4%)	202 (27.6%)	70 (26.7%)	108 (17.5%)	40 (21.6%)	792 (26.9%)		
	3	158 (13.8%)	151 (20.6%)	33 (12.6%)	149 (24.1%)	39 (21.1%)	530 (18.0%)		
	4	109 (9.5%)	103 (14.1%)	12 (4.6%)	61 (9.9%)	19 (10.3%)	304 (10.3%)		
	5	87 (7.6%)	32 (4.4%)	18 (6.9%)	56 (9.1%)	10 (5.4%)	203 (6.9%)		
	6	39 (3.4%)	25 (3.4%)	6 (2.3%)	19 (3.1%)	4 (2.2%)	93 (3.2%)		
	7	66 (5.8%)	94 (12.8%)	50 (19.1%)	95 (15.4%)	20 (10.8%)	325 (11.0%)		
	DK	1 (0.1%)	10 (1.4%)	0 (0.0%)	32 (5.2%)	4 (2.2%)	47 (1.6%)		
Mean DDS (SD)		2.42 (1.9)	3.25 (2.0)	2.84 (2.3)	3.36 (2.1)	2.63 (2.1)	2.90 (2.1)	<0.001	0.17
Fresh meat	0	56 (4.9%)	110 (15.0%)	29 (11.1%)	126 (20.4%)	17 (9.2%)	338 (11.5%)		
	1	60 (5.2%)	241 (32.9%)	90 (34.4%)	159 (25.8%)	20 (10.8%)	570 (19.4%)		
	2	191 (16.7%)	214 (29.2%)	33 (12.6%)	106 (17.2%)	19 (10.3%)	563 (19.1%)		
	3	229 (20.0%)	98 (13.4%)	45 (17.2%)	81 (13.1%)	48 (25.9%)	501 (17.0%)		
	4	248 (21.6%)	30 (4.1%)	26 (9.9%)	45 (7.3%)	23 (12.4%)	372 (12.6%)		
	5	165 (14.4%)	9 (1.2%)	12 (4.6%)	18 (2.9%)	27 (14.6%)	231 (7.8%)		
	6	51 (4.4%)	1 (0.1%)	2 (0.8%)	10 (1.6%)	3 (1.6%)	67 (2.3%)		
	7	144 (12.6%)	11 (1.5%)	24 (9.2%)	20 (3.2%)	23 (12.4%)	222 (7.5%)		
	DK	3 (0.3%)	18 (2.5%)	1 (0.4%)	52 (8.4%)	5 (2.7%)	79 (2.7%)		
Mean DDS (SD)		3.47 (2.0)	1.68 (1.3)	2.22 (2.0)	1.92 (1.8)	3.13 (2.1)	2.52 (2.0)	<0.001	0.46
Tinned meat	0	61 (5.3%)	53 (7.2%)	32 (12.2%)	75 (12.2%)	20 (10.8%)	241 (8.2%)		
	1	49 (4.3%)	105 (14.3%)	71 (27.1%)	86 (13.9%)	9 (4.9%)	320 (10.9%)		
	2	146 (12.7%)	150 (20.5%)	21 (8.0%)	79 (12.8%)	15 (8.1%)	411 (14.0%)		
	3	149 (13.0%)	156 (21.3%)	15 (5.7%)	100 (16.2%)	30 (16.2%)	450 (15.3%)		
	4	156 (13.6%)	102 (13.9%)	20 (7.6%)	80 (13.0%)	15 (8.1%)	373 (12.7%)		
	5	177 (15.4%)	75 (10.2%)	20 (7.6%)	67 (10.9%)	15 (8.1%)	354 (12.0%)		
	6	96 (8.4%)	31 (4.2%)	16 (6.1%)	24 (3.9%)	3 (1.6%)	170 (5.8%)		
	7	311 (27.1%)	55 (7.5%)	67 (25.6%)	54 (8.8%)	71 (38.4%	558 (19.0%)		
	DK	2 (0.2%)	5 (0.7%)	0 (0.0%)	52 (8.4%)	7 (3.8%)	66 (2.2%)		

(Continued)

TABLE 4 | Continued

Type of foods	Dietary diversity score	Central	EHP	Madang	ENB	POM	All sites	ANOVA *p*-value	Eta *p*-value
Mean DDS (SD)		4.11 (2.3)	3.05 (1.9)	3.14 (2.7)	3.08 (2.2)	4.11 (2.6)	3.51 (2.3)	<0.001	0.29
Home-made fried foods	0	130 (11.3%)	210 (28.7%)	54 (20.6%)	138 (22.4%)	54 (29.2%)	586 (19.9%)		
	1	122 (10.6%)	137 (18.7%)	23 (8.8%)	135 (21.9%)	24 (13.0%)	441 (15.0%)		
	2	287 (25.0%)	144 (19.7%)	60 (22.9%)	78 (12.6%)	26 (14.1%)	595 (20.2%)		
	3	301 (26.2%)	117 (16.0%)	37 (14.1%)	77 (12.5%)	29 (15.7%)	561 (19.1%)		
	4	179 (15.6%)	46 (6.3%)	21 (8.0%)	47 (7.6%)	13 (7.0%)	306 (10.4%)		
	5	86 (7.5%)	30 (4.1%)	12 (4.6%)	40 (6.5%)	8 (4.3%)	176 (6.0%)		
	6	28 (2.4%)	7 (1.0%)	9 (3.4%)	15 (2.4%)	3 (1.6%)	62 (2.1%)		
	7	10 (0.9%)	8 (1.1%)	44 (16.8%)	23 (3.7%)	22 (11.9%)	107 (3.6%)		
	DK	4 (0.3%)	33 (4.5%)	2 (0.8%)	64 (10.4%)	6 (3.2%)	109 (3.7%)		
Mean DDS (SD)		2.44 (1.6)	1.72 (1.6)	2.71 (2.4)	2.18 (2.2)	2. 28 (2.3)	2.20 (1.8)	<0.001	0.22
Shop-purchased fried foods	0	594 (51.8%)	142 (19.4%)	157 (59.9%)	220 (35.7%)	96 (51.9%)	1,209 (41.1%)		
	1	286 (24.9%)	134 (18.3%)	60 (22.9%)	185 (30.0%)	29 (15.7%)	694 (23.6%)		
	2	200 (17.4%)	165 (22.5%)	19 (7.3%)	60 (9.7%)	22 (11.9%)	466 (15.8%)		
	3	55 (4.8%)	120 (16.4%)	8 (3.1%)	42 (6.8%)	9 (4.9%)	234 (8.0%)		
	4	8 (0.7%)	87 (11.9%)	6 (2.3%)	16 (2.6%)	5 (2.7%)	122 (4.1%)		
	5	2 (0.2%)	33 (4.5%)	4 (1.5%)	6 (1.0%)	3 (1.6%)	48 (1.6%)		
	6	1 (0.1%)	11 (1.5%)	0 (0.0%)	1 (0.2%)	0 (0.0%)	13 (0.4%)		
	7	0 (0.0%)	17 (2.3%)	8 (3.1%)	4 (0.6%)	9 (4.9%)	38 (1.3%)		
	DK	1 (0.1%)	23 (3.1%)	0 (0.0%)	83 (13.5%)	12 (6.5%)	119 (4.0%)		
Mean DDS (SD)		0.73 (1.0)	2.08 (1.7)	0.78 (1.4)	1.07 (1.3)	1.04 (1.8)	1.20 (1.4)	<0.001	0.38
Stock cube**	0	295 (25.7%)	275 (37.6%)	230 (87.8%)	288 (46.7%)	71 (38.4%)	1,159 (39.4%)		
	1	180 (15.7%)	100 (13.7%)	9 (3.4%)	91 (14.7%)	2 (1.1%)	382 (13.0%)		
	2	165 (14.4%)	138 (18.9%)	5 (1.9%)	31 (5.0%)	8 (4.3%)	347 (11.8%)		
	3	166 (14.5%)	82 (11.2%)	5 (1.9%)	37 (6.0%)	16 (8.6%)	306 (10.4%)		
	4	148 (12.9%)	50 (6.8%)	0 (0.0%)	18 (2.9%)	8 (4.3%)	224 (7.6%)		
	5	95 (8.3%)	31 (4.2%)	4 (1.5%)	13 (2.1%)	15 (8.1%)	158 (5.4%)		
	6	18 (1.6%)	9 (1.2%)	1 (0.4%)	19 (3.1%)	1 (0.5%)	48 (1.6%)		
	7	70 (6.1%)	14 (1.9%)	8 (3.1%)	65 (10.5%)	49 (26.5%)	206 (7.0%)		
	DK	10 (0.9%)	33 (4.5%)	0 (0.0%)	55 (8.9%)	15 (8.1%)	113 (3.8%)		
Mean DDS (SD)		2.19 (2.1)	1.6 (1.7)	0.39 (1.3)	1.81 (2.5)	2.85 (3.0)	1.82 (2.2)	<0.001	0.28
Salt*	0	357 (31.1%)	304 (41.5%)	205 (78.2%)	273 (44.2%)	51 (27.6%)	1,190 (40.4%)		
	1	200 (17.4%)	78 (10.7%)	7 (2.7%)	61 (9.9%)	9 (4.9%)	355 (12.1%)		
	2	164 (14.3%)	85 (11.6%)	4 (1.5%)	16 (2.6%)	11 (5.9%)	280 (9.5%)		
	3	68 (5.9%)	49 (6.7%)	1 (0.4%)	22 (3.6%)	11 (5.9%)	151 (5.1%)		
	4	52 (4.5%)	71 (9.7%)	0 (0.0%)	5 (0.8%)	9 (4.9%)	137 (4.7%)		
	5	42 (3.7%)	35 (4.8%)	3 (1.1%)	14 (2.3%)	7 (3.8%)	101 (3.4%)		
	6	20 (1.7%)	21 (2.9%)	1 (0.4%)	7 (1.1%)	0 (0.0%)	49 (1.7%)		
	7	225 (19.6%)	41 (5.6%)	39 (14.9%)	168 (27.2%)	78 (42.2%)	551 (18.7%)		
	DK	19 (1.7%)	48 (6.6%)	2 (0.8%)	51 (8.3%)	9 (4.9%)	129 (4.4%)		
Mean DDS (SD)		2.35 (2.6)	1.96 (2.2)	1.05 (2.4)	2.64 (3.1)	3.58 (3.1)	2.26 (2.7)	<0.001	0.22
Total of children		1,147 (100.0%)	732 (100.0%)	262 (100.0%)	617 (100.0%)	185 (100.0%)	2,943 (100.0%)		
Mean total DDS (SD)		22.49 (11.45)	25.31 (9.3)	19.65 (12.59)	23.06 (14.42)	26.23 (13.56)	23.30 (11.96)	<0.001	0.14

**Any type of salt, including iodized salt was put directly on any food fed to the child participants.*

***Refers to food seasoning that adds tastes and flavor and put directly on any food fed to child participants.*

DK, do not know.

TABLE 5 | Odds ratio of having low level of total dietary diversity scores among children aged 6–59 months by household and maternal socioeconomic demographic factors in PNG, PNGIMR's CHESS, 2020.

Factor	Category	No. of children	%	Odds ratio	Lower bound	Upper bound	*p*-value
Sector	Urban	300	23.7%	1.11	0.79	1.56	0.54
	Rural	964	76.3%	Ref.			
Province	Central	556	44.0%	0.72	0.40	1.31	0.28
	EHP	384	30.4%	1.16	0.67	2.02	0.59
	ENB	246	19.5%	0.59	0.33	1.05	0.07
	POM	78	6.2%	Ref.			
Sex of child	Male	668	52.8%	0.99	0.79	1.23	0.90
	Female	596	47.2%	Ref.			
Child age group	6–11	91	7.2%	1.11	0.69	1.79	0.66
	12–23	196	15.5%	1.07	0.75	1.53	0.70
	24–35	312	24.7%	0.89	0.65	1.21	0.47
	36–47	305	24.1%	0.98	0.72	1.35	0.92
	48–59	360	28.5%	Ref.			
Mother education	Elementary	77	6.1%	2.30	0.85	6.20	0.10
	Primary	942	74.5%	1.63	0.68	3.92	0.28
	Lower secondary	196	15.5%	2.00	0.80	4.98	0.14
	Upper secondary	27	2.1%	2.80	0.86	9.14	0.09
	Vocational/college+	22	1.7%	Ref.			
Mother marital status	Married	543	43.0%	0.94	0.74	1.20	0.62
	In union	170	13.4%	0.88	0.62	1.25	0.47
	Single mother	551	43.6%	Ref.			
HH wealth quintile	Poorest	251	19.9%	1.22	0.79	1.87	0.37
	Poorer	285	22.5%	0.97	0.64	1.45	0.87
	Middle	264	20.9%	1.07	0.71	1.62	0.74
	Richer	308	24.4%	1.06	0.71	1.57	0.77
	Richest	156	12.3%	Ref.			
Total of children		1,264	100.0%				

Multivariate logistic regression model with a dependent variable of total DDS level with low total DDS (below the mean) as a reference category. HH and maternal factors included in the model were urban–rural sector; province; sex and age group of child; mother's age group, educational attainment, occupation, and marital status; and household wealth quintile.

DISCUSSION

This study has provided large data collected from both rural and urban areas in five main provinces representing four geographic regions of PNG and covered a range of information on eating behaviors among children aged 6–59 months. Household and maternal socioeconomic demographic data were collected, facilitating analyses of factors associated with the choice of food in PNG.

Eating Behavior and Associated Factors Among CU5 in PNG

More than half of the breastfed children aged 6–8 months were given supplementary foods, but with appropriate introduction of solid and semisolid foods in less than half of the children. For children aged 6–23 months, 20% of currently breastfed children meet the minimum meal frequency requirement, compared to only 2% of currently non-breastfed children. These proportions are lower than those found in a previous study (27), but this suggests that there is a need to further strengthen the nutrition education for mothers on the recommended child feeding practices.

Most children aged 6–59 months were fed with vegetables and fruits, with dietary variations. Child participants in the urban sites of POM and Madang Province, with diet low in vegetables and fruits, not only reflect limited subsistence farming and limited availability of and accessibility to these foods but also family socioeconomic factors and dietary choices in the urban areas. High consumption of vegetables in the EHP reflects subsistence farming and availability of and accessibility of these foods through its own production or market. The households in EHP may have limited access to these foods in dry season, often in the third quarter, but also due to social instability associated with the national election in 2017.

Children of this age group were more likely to be fed fresh and tinned meat or fish, especially in POM, which may be due to the availability of and accessibility to this food, but also the food preferences of the parents living in the urban areas. This is likely associated with household affordability and convenience. Fresh meat is more expensive than tinned meat, but tinned meat can be kept for a long time without a fridge or freezer. This is particularly

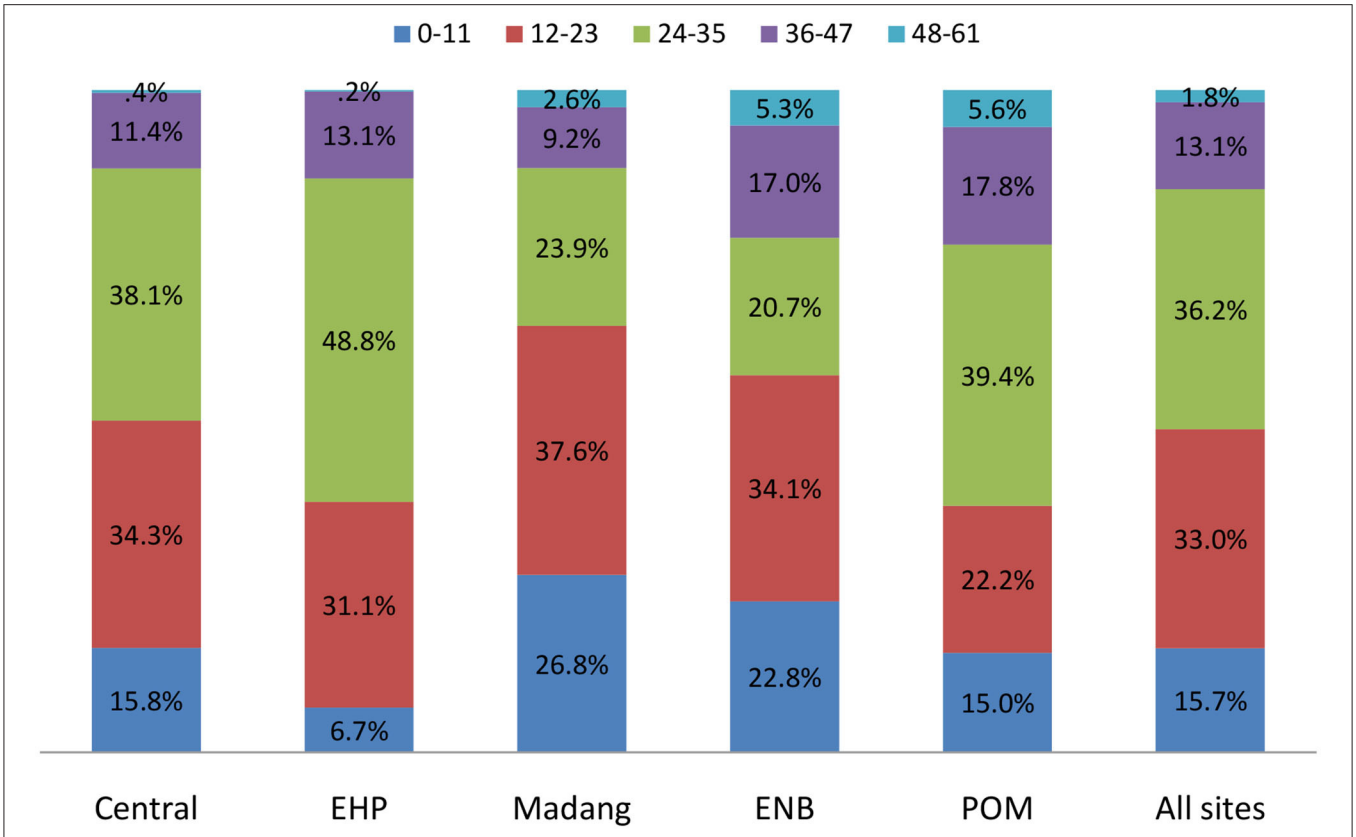

FIGURE 4 | Distribution of child participants by total dietary diversity scores by province, PNGIMR's CHESS, 2020.

true in the context of PNG, where 20% of households had access to electricity and 5% owned a fridge or freezer (15). Tinned meat, fish, rice, and flour, which are either locally made or imported, are available in village food stores all over the country, while fresh fish are more likely available in coastal areas such as Madang Province and ENB. Access to these foods is therefore more related to household socioeconomic status (30). Fresh and tinned meat and fish are much higher in protein, zinc, and energy than the local root vegetables such as sweet potatoes, yam, and taros. Dietary effects on the linear growth of children were discussed by Smith et al. (31). A previous study in PNG suggests that high consumption of local root vegetables (with the exception of potatoes) was correlated with reduced growth of children and that protein and/or zinc limitation might be a major component of slow growth in PNG, though growth is usually limited by multiple, simultaneous deficiencies (32). Unusually for PNG, the protein content of bush meat is an important part of the diets and exceeds the recommended Food and Agriculture Organization (FAO)/WHO levels (33).

These children were more likely to be fed homemade rather than purchased fried food, especially in Central Province, POM, and Madang Province, indicating the common practice in urban settings and rural areas which have undergone an urbanization process. Mothers from better-off families could be more concerned about the quality of fried food sold in the shop, so they make it at home for their children. But the higher proportion of children in EHP who consumed purchased fried foods may suggest their availability and affordability and the food choices and preferences by adults with children. Cash income from subsistence cash cropping, particularly from coffee after July when the data collection was conducted, may explain the situation (34). The consumption of fried foods with high fat intake raises child health issue and also public health concern as individuals may be at a greater risk of developing heart disease, diabetes, obesity, and cancer (35, 36). Access to and uptake of iodized salt can alleviate iodine deficiency and improve health outcomes (37). Mothers from poorer households are likely to prefer salt, while mothers from better-off households are more likely to prefer stock cubes. That is because, in PNG, salt is cheaper and can be used for more days than stock cubes. However, the higher proportion of children in Central Province, POM, and ENB consuming both salt and stock cubes raises child health concern and also poses great health risks for adults. There is a relationship between increased salt consumption and subsequent risk of cardiovascular diseases. Children with high salt levels are at risk of raised blood pressure and become adult with elevated blood pressure, which increases the risk of kidney disease, heart disease, heart attack, and stroke. High salt intake also promotes overconsumption of fatty foods, which increases the risk of obesity. By contrast, lower sodium intake could also have negative impact on child health and development (38).

Policy and Program Implications

Our study shows that children from wealthier households have better dietary diversity than children from poor households. This could be because mothers from better-off households often purchase more foods and feed their children with more variety of foods (34, 36, 39). In PNG, food supply relies more on agricultural production at the household level rather than the food chain systems (40). Previous studies in Ethiopia suggest that household livelihood strategies with appropriate family farming interventions can improve household income and productivity. Furthermore, each additional food group produced by the household increases the food quantity and diversity for children (27, 39, 41). Recent analyses in Bangladesh found household income to be a significant determinant of household dietary diversity (28, 37). In PNG, household wealth can also be improved by increasing the transportation and circulation of agricultural products across provinces and geographical regions. PNG women's participation in agriculture works and other livelihood activities likely constrained their time for child care, especially for single mothers. Hence, PNG men are encouraged to support women in taking care of children so that women could optimally breastfeed and provide food supplements for their children (37).

Our study revealed that mother's education is a significant factor contributing to the higher level of dietary diversity among children. This finding is consistent with a previous study suggesting that women's higher education is a protective factor against childhood malnutrition. More highly educated mothers are more likely to have better knowledge and practices of nutritious food habits. Nutritional education programs can improve breastfeeding practices and dietary diversity. These results are supported by findings from other developing countries (42). However, the translation of education to improved dietary practices and subsequent improvements in child nutritional status could be a lengthy process in PNG.

Building human resource capacity to address nutritional issues is crucial in PNG, requiring strategy, consistent financing, and leadership to successfully implement the National Nutritional Strategy 2015–2020 (33). Stand-alone nutrition education programs have been carried out in the Pacific region and PNG in particular (8, 40). Nutritional education should be integrated into postnatal care services and become standard practice at primary health facilities in PNG. Health workers should be trained in delivering messages on how to improve nutrition outcomes among children. Research findings are needed to support interventions to improve healthy eating behavior, food choices among household members and young women in particular, to ensure that nutritional education reaches population living in rural and remote areas. Home-based nutrition counseling would improve child dietary intake, in both quantity and quality, contributing to the improvement of nutrition status of PNG children in the long term.

Data Limitation

Data used in this study have some limitations. First, the study was conducted with CU5 participants living within the surveillance sites of PNG. Hence, the data are not nationally representative. Second, parents and caregivers reported on breastfeeding and food feeding in a typical week, but the provided information might have resulted in recall biases, and it may not be representative; for instance, food diversity varies across seasons in a year. Third, the DDS method is among the few research methods available to measure food and dietary diversity at the population level. This method appears to have some limitations when we applied it to our study. The same scoring system was applied to all child age groups and across all types of foods with one score being given to a child whenever the child was reported to be fed with a type of food regardless of the amount of food being given. In the local context of PNG, a variety of foods are given to children that may be not necessarily be limited to the nine groups, as defined in this study. The DDS method is for first time applied to assess food diversity among CU5 in PNG; hence, a comparison between our findings and data from previous studies in PNG or similar settings is relatively limited.

CONCLUSION

Our study confirms that the urban–rural sector, household wealth, and maternal education attainment are the key socioeconomic determinants of dietary diversity and intake among CU5 in PNG. Evidence-based interventions are needed to improve dietary diversity and intake among young children. That could reduce the burden of diet-related morbidity and mortality in PNG in the future. Any solution aiming at improving health and nutrition among PNG children needs to address the fundamental economic issues at the household level. There is a need of an evidence-based and location-specific approach, considering the differentials in household and maternal socioeconomic characteristics. Monitoring and reporting breastfeeding, food supplementation, and dietary intake not only provide information about the health status of children but also inform policy for effectively programming interventions at the national and local levels. We call for an integrated comprehensive approach to improving household wealth, contributing to the improvement of the nutritional status of children in PNG.

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 CHESS was granted ethics approvals from Internal Review Board of PNG Institute of Medical Research (IRB's Approval no. 18.05) and the Medical Research Advisory Committee of Papua New Guinea (MRAC's Approval no. 18.06). These approvals covered all the data components under the CHESS, including data of children under 5 years of age, which

were used in this manuscript. Informed consent was sought from self-identified household heads and woman participants. Women were informed about their right to withdraw from the study at any stage. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BP designed the CHESS, conceptualized the paper, analyzed and interpreted the data, drafted, revised, finalized, and submitted the manuscript. VS supervised the fieldwork, collected data, and provided the inputs. AO reviewed and commented and edited the manuscript. WP oversaw the CHESS and approved the submission of the manuscript. All authors contributed to the article and approved the submitted version.

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Measuring Wasting and Stunting Prevalence Among Children Under 5 Years of Age and Associated Risk Factors in Papua New Guinea: New Evidence From the Comprehensive Health and Epidemiological Surveillance System

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Background: Papua New Guinea (PNG) has undergone a significant health transition, with the prevalence of non-communicable diseases increasing. Many children under 5 years of age suffer from the burden of malnutrition. While wasting and stunting still remain high, children who are overweight and obese are reportedly increasing.

Objective: This study reports the prevalence of wasting, stunting, underweight, and overweight children under five in PNG and explores potential household and maternal socioeconomic factors associated with malnutrition.

Method: Data were drawn from the Comprehensive Health and Epidemiological Surveillance System (CHESS) in PNG. Height and weight were directly measured, and wasting, stunting, overweight, and underweight statistics were determined using the 2006 WHO Standard Growth Standards. Household and maternal factors were assessed with parent interviews conducted by trained data collectors. Multivariate logistic regression analyses were conducted to report associations between selected socioeconomic correlates and child malnutrition outcomes.

Result: The prevalence of wasting, stunting, underweight, and overweight children was 13.8, 46.5, 18.2, and 18%, respectively. Children from households with food shortage were more likely to be wasted than those from households without such an experience [OR: 1.43 (95% CI: 0.93–2.21)]. Children from the poor quintile were more likely to be stunted than those from the richest quintile [OR: 1.2 (95% CI: 0.79–1.82)]. Other factors associated with wasting included living in an urban vs. rural area [OR: 1.36 (0.77–2.4)], middle household wealth quintile vs. richest quintile [OR: 0.77 (0.38–1.55)], mothers in union with a man vs. mother unmarried or live in union [OR: 0.76 (0.4–1.42)], and male children vs. female [OR: 0.77 (0.53–1.11)]. Factors associated with stunting included residing in urban vs. rural areas [OR: 1.13 (0.8–1.6)], mother in union vs. single mother

[OR: 0.86 (0.59–1.24)], and mothers with preparatory/elementary vs. mothers with vocational/college education [OR: 0.15 (0.02–1.01)].

Conclusion: An integrated approach is needed to comprehensively address the household socioeconomic factors at the household level, contributing to the improvement of child health and development in PNG.

Keywords: wasting, stunting, children under 5 years of age, CHESS, Papua New Guinea

INTRODUCTION

Nutrition at Global Level

Approximately 6.3 million children under 5 years of age (CU5) die every year, globally. About one-third of these deaths are associated with malnutrition conditions (1–3). While malnutrition among CU5 continues to be a significant population health issue in low and middle income countries (LMICs), new challenges have emerged alongside socioeconomic transition (4). This includes a shift from traditional food choices and low calorie diets to greater access to a variety of high calorie, processed foods.

The second Sustainable Development Goal (SDG) and its associated targets and indicators highlight the importance of monitoring the prevalence of stunting, wasting, and other forms of malnutrition among children under 5 years of age.

Factors Associated With Child Nutrition in PNG

Malnutrition contributed to 36% of child deaths in hospitals in Papua New Guinea (PNG) in 2012 (5). Despite recent economic growth, malnutrition remains a child health issue in PNG (6, 7). Recent social changes have impacted food choices and eating habits among the population and CU5 in particular (8, 9). The interactions between disease episodes and child growth are well known in PNG. Infectious diseases are important risk factor for stunting among children (10). Associations between malaria and reduced growth in children have been reported previously, especially among children under 2 years of age. The higher incidence of respiratory tract infections may explain the impaired growth among children in the highlands (11). A similar negative effect on growth was found in association with intestinal helminthiasis in children in urban and rural areas. Diarrhea is well-documented in the literature as a contributor to stunting and is associated with the mal-absorption of nutrients, especially zinc (12).

Socioeconomic development is a further contextual factor, in which poverty is the major risk contributor to poor child health and development (13). Previous studies on nutrition-associated risk factors in PNG suggested that general economic development has not led to improved nutritional outcomes among children (14). Low intake of protein has been reported among children who lived in families working in traditional subsistent agriculture, and the impact of geographical location on child growth, especially for children in Eastern Highland Province (EHP) and Madang Province, has been highlighted (15).

Most socioeconomic factors associated with child growth were related to household socioeconomic status. This suggests that the availability and accessibility to a variety of foods at the household level could be key determinants of child growth in PNG. Parents' socioeconomic demographic characteristics such as mother's education, marital status, and occupation are also important predictors of the nutrition status of their children (16).

Rationale of the Study

Data on malnutrition including wasting, stunting, underweight, and overweight CU5 remain limited in PNG (9). Very few studies have examined these indicators in relation to the country's current health and epidemiological transition where both under-nutrition and over-nutrition co-exist (17). Analysis of the 1982 National Nutrition Survey (NNS) provided an estimation of nutrition indicators based on the local definition of child growth standards, stunting, wasting, or underweight statistics were defined as >1 SD below the national mean of child participants (12). Hence the data generated were not available for comparison with international data. Most recent data reported by the 24-h surveillance pilot study conducted by PNGIMR among 4 year olds in the Goroka and Daulo districts in EHP in 2018 showed that about 75% of child participants were of normal weight, around 5% were underweight, and over 20% were overweight or obese (18). These data suggest that considerable shifts in child nutrition are occurring in PNG. Furthermore, little is known about risk factors associated with wasting and stunting in PNG.

In this study, we report on two key SDG indicators: the prevalence of stunting and wasting among CU5 (aged 0–59 months) and also the estimated prevalence of underweight and overweight children. We also examined associations between household socioeconomic status and mothers' socio-demographic characteristics and wasting and stunting. Our hypothesis was that household socio-economic status and maternal socioeconomic demographic characteristics were associated with malnutrition outcomes in CU5.

METHOD

Data Source

In this paper, we used data from the Comprehensive Health and Epidemiology Surveillance System (CHESS), operated by the Papua New Guinea Institute of Medical Research (PNGIMR). CHESS is a new generation population-based surveillance system with an electronic population database consisting of six components. (i) Household socioeconomic demographic data; (ii) children under 5 years of age; (iii) women of reproductive

age, 15–49 years; (iv) men of working age, 15–64 years; (v) morbidity of patients seeking healthcare services at primary health facilities; and (vi) mortality of deceased persons who died in the communities. These data components are interlinked via unique household and individual identification codes, following the PNG national coding system issued by the National Statistics Office. The design of CHESS has been described elsewhere (19).

Data Collection and Management

Data were collected from five major provinces of PNG: Port Moresby (POM, the National Capital), Central, Eastern Highlands (EHP), Madang, and East New Britain (ENB), covering children living in both urban and rural sectors in the period July–December 2018. Interviews were conducted in *Tok-Pisin*, the most common local language in PNG, by village-based data collectors, who were trained in interview skills and research methods before visiting households to interview parents/child caregivers, using the CU5 questionnaire, which was specifically designed to collect child health data.

The CU5 data component comprised eight modules, including a module on anthropometry, in which all eligible children's height and weight were measured, using a portable electronic scale and stadiometer in accordance with WHO anthropometric protocol (20). Length was measured in children younger than 24 months. All the anthropometric records were quality checked before being entered into the CHESS database, using a standard data entry template, developed on the MySQL/Process Maker platform.

Dataset and Variables

Raw data sets were extracted from the CHESS database as Microsoft Excel spreadsheets and converted into SPSS (version 20.0) for analysis. CU5 data included variables on resident location (rural-urban sector), date of birth and date of interview, age (in months) and sex, and anthropometry (height and weight). Data on maternal socioeconomic demographic characteristics include marital status, highest education attainment, and maternal age. Household wealth index was calculated using selected household socioeconomic variables on housing characteristics, water and sanitation, and household assets, using the principal component analysis method (multi-dimension factor analysis). Household wealth index was then grouped into five quintiles from the 1st to the 5th quintile, in which each household was designated a corresponding category, representing the poorest, poor, middle, richer, and the richest. Details of this analysis have been discussed elsewhere (21).

Definitions

In this study, we used the 2006 WHO Child Growth Standards as the reference population, which is expressed in standard deviations (SD) (or z-scores). Anthropometric data (height and weight), together with individual data on age (in month) and sex were converted into age- and sex-specific Z-scores, using the WHO Anthro software (20). According to this method, children who had missing data on either age, sex, weight, or height were not included in the conversion. Three new variables: weight-for-height, height-for-age, and weight-for-age z-scores were then created in the dataset.

Wasting

Wasting children were defined as those whose weight-for-height was >2 SDs below the median of the reference population. Moderately wasting was between −2 and −3 SDs while those who were >3 SDs below the median were classified as severely wasting.

Stunting

Children whose height-for-age was >2 SDs below the median of the reference population were classified as moderately or severely stunted. Those whose height-for-age was >3 SDs below the median were classified as severely stunted.

Underweight

Children whose weight-for-age was >2 SDs below the median of the reference population were considered as moderately underweight and those who were >3 SDs below the median were classified as severely underweight.

Overweight

Given the recent epidemiological shift in child contrition in PNG, we also calculated overweight prevalence among CU5. Overweight was defined as a weight-for-age between 2 and 3 SDs units above the median of the reference population (moderate) and severely overweight (obese) was defined as a weight-for-age >3 SDs above the median of the reference population.

These indicators were then stratified by urban-rural sector, province, child age group and sex, maternal education and marital status, household wealth quintile, and household food shortage experience (**Table 2**).

Logistic Regression Analysis

To identify risk factors of child health and development, we conducted multinomial logistic regression analysis, in which stunting and wasting were treated as dependent variables for outcome measures of child health (the association of overweight and risk factors will be addressed in another paper). Socioeconomic demographic variables including child age groups (in months), sex of children, province, rural-urban sector, household wealth quintile and household experience of food shortage, mother's marital status, and mother's highest educational level attainment were used as dependent factors. Odds ratios (OR) were estimated for each category of these variables, with the last category being used as a referral group regarding the two outcome measures: wasting and stunting. Next, 95% confidence intervals (CIs) and *p*-values were calculated for every OR estimates to confirm the level of significance of association (**Table 3**). These analyses were conducted to address the question of whether household and maternal socioeconomic characteristics had an impact on the stunting and wasting status of children. Hence, the analyses provide a better understanding of child health and development in the broader context of socioeconomic determinants in PNG.

RESULTS

Data from 3,132 CU5 were included in the analysis, comprising 1,231 in Central Province, 758 in EHP, 289 in Madang, 654 in ENB, and 200 in POM.

Household and Maternal Socioeconomic Demographic Characteristics of Child Participants

Table 1 shows household and maternal socioeconomic demographic characteristics of child participants. About 70% of children were from rural areas and 30% were from urban areas. The child distribution increased across age groups, from 6.0% in the youngest age group (0–5 months) to 27% in the oldest age group (48–59 months). More than 50% of the total children were male.

More children of younger mothers than those of older mothers participated in the study, the highest proportion of mothers (about 25%) was in the youngest age group (15–19 years) and <10% were in the oldest age group (45–49 years). Most mothers (67%) reported obtaining a primary educational. Regarding current marital status, more than 50% of mothers reported that they were married, about 10% had partners, with more than one third (36%) reporting that they were single. Around 50% of the child participants in Central, EHP, and POM were from the poor and poorest households, compared to 20% in ENB, and zero in Madang. Nearly 20% of the households admitted food shortage in the past 12 months, with the highest proportion in Central (38%), followed by POM (19%) and EHP (15%), with negligible proportions in Madang and ENB.

Prevalence, Trend, and Variation

Table 2 presents the estimated prevalence of wasting, stunting, overweight, and underweight child participants by selected socioeconomic demographic characteristics.

Wasting prevalence was 13.8%, including 8% severe and 5.8% moderate. Wasting prevalence was slightly higher in the urban sector than the rural sector, 15.4 and 13.3%, respectively. Children in POM had the highest prevalence of wasting and children in ENB had the lowest. The difference between male and female children in moderate wasting was significant. There was no clear pattern of wasting across the child age groups. However, wasting was significantly higher among children from poor households than those from better-off households. For example, 15.2% were in the poorest and 17.2% in the poor compared to 11.3% in the richer and 11.2% in the richest household wealth quintile. Household food shortage was an important factor contributing to increased wasting. Seventeen percent of children who reported lack of food in the past 12 months were wasting, compared to 12.7% of children from households without food shortages.

Stunting prevalence was 46.5%, with a non-significant difference between urban and rural children. Severe stunting varied widely across provinces, with the highest prevalence in ENB (46.8%), followed by EHP (34.1%), and the lowest in POM (20.5%). Two different patterns in stunting by age groups were observed; severe stunting was likely to increase with increasing age whereas moderate stunting tended to decrease as age increased (**Figure 1**).

Underweight prevalence among CU5 was estimated at 18.2%, with a non-significant difference between urban and rural children. A pattern of increased severely underweight children by age group was observed, with the lowest level (1.6%) in 6–11 months and the highest level (7.8%) in 36–47 months. By contrast, moderately underweight children fluctuated around 10–11% across age groups. The trend of decreased underweight children by household wealth quintiles was clear, with the highest prevalence of severe and moderate (7 and 12.8%) among children from the poorest households, and the lowest among children from the richest households (4.8 and 9.6%) (**Figure 2**). Comparing underweight statistics between children from households that experienced food shortage in the past 12 month and those without such an experience, the number of severely underweight children was similar between the two groups, but the number of moderately underweight children was significantly higher in the former compared with the latter, 14.3 vs. 10.8%, respectively.

Overweight prevalence was 29% with similar results between urban and rural sectors. The number of overweight children was noticeably highest in ENB (above 50%), followed by EHP (above 35%), and lowest in Central and POM (17–19%). The number of overweight children was similar between male and female children, 29.5 and 29.1%, respectively. There was no clear trend of moderately overweight children, but obesity increased by household wealth quintiles, from the lowest level of 14.6% in the poorest to 18.6% in middle, and 22% in the richest. The amount of overweight children was significantly higher among children whose families reported no food shortage in the last 12 months (30%) than those who reported a lack of food (20%).

Associated Risk Factors for Wasting and Stunting

Associations between household maternal socioeconomic demographic factors and child wasting and stunting are shown in **Table 3**.

Children in urban areas were more likely to be wasting than those in rural areas [OR: 1.36 (95% CI 0.77–2.4)]. Children in ENB were less likely to be wasting than those from POM [OR: 0.20 (95% CI 0.07–0.58)]. Children from households that experienced food shortage in the past 12 months were more likely to be wasting than those from households without such an experience [OR: 1.43 (95% CI 0.93–2.21)]. Children from households of middle wealth quintile were less likely to be wasting than those from the richest wealth quintile [OR: 0.77 (95% CI 0.38–1.55)]. Sex of the child was an important factor associated with wasting among children, with male children less likely to be wasting than their female counterparts [OR: 0.77 (95% CI 0.53–1.11)].

Children from Central, EHP, and ENB were all more likely to be stunted than those from POM, with ORs of 1.43 (0.77–2.67), 1.75 (0.99–3.10), and 3.29 (1.80–6.01), respectively. Children from a poor household wealth quintile were more likely to be

TABLE 1 | Household socioeconomic and maternal demographic characteristics of children under 5 years of age by province, PNGIMR's CHESS, 2020.

			Central	EHP	Madang	ENB	POM	All sites
Sector	Urban	*N*	–	261	289	225	200	974
		%	–	34.4%	100.0%	34.4%	100.0%	31.1%
	Rural	*N*	1,231	497	–	429	–	2158
		%	100.0%	65.6%	–	65.6%	–	68.9%
Total		*N*	1,231	758	289	654	200	3,132
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Age group (month)	0–5	*N*	84	26	27	37	15	189
		%	6.8%	3.4%	9.3%	5.7%	7.5%	6.0%
	6–11	*N*	72	26	27	63	18	206
		%	5.8%	3.4%	9.3%	9.6%	9.0%	6.6%
	12–23	*N*	171	124	39	131	16	481
		%	13.9%	16.4%	13.5%	20.0%	8.0%	15.4%
	24–35	*N*	287	169	60	143	59	718
		%	23.3%	22.3%	20.8%	21.9%	29.5%	22.9%
	36–47	*N*	275	199	65	147	50	736
		%	22.3%	26.3%	22.5%	22.5%	25.0%	23.5%
	48–59	*N*	342	214	71	133	42	802
		%	27.8%	28.2%	24.6%	20.3%	21.0%	25.6%
Total		*N*	1,231	758	289	654	200	3,132
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Sex of child	Male	*N*	628	394	142	335	110	1609
		%	51.2%	52.4%	49.1%	51.8%	56.1%	51.7%
	Female	*N*	599	358	147	312	86	1502
		%	48.8%	47.6%	50.9%	48.2%	43.9%	48.3%
Total		*N*	1,227	752	289	647	196	3,111
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Age group of women	15–19	*N*	209	112	55	161	30	567
		%	25.3%	18.2%	19.2%	25.8%	22.7%	22.8%
	20–24	*N*	123	85	45	105	20	378
		%	14.9%	13.8%	15.7%	16.9%	15.2%	15.2%
	25–29	*N*	115	90	42	92	18	357
		%	13.9%	14.6%	14.7%	14.8%	13.6%	14.4%
	30–34	*N*	125	95	34	84	27	365
		%	15.1%	15.4%	11.9%	13.5%	20.5%	14.7%
	35–39	*N*	85	75	41	70	10	281
		%	10.3%	12.2%	14.3%	11.2%	7.6%	11.3%
	40–44	*N*	102	103	40	77	16	338
		%	12.3%	16.7%	14.0%	12.4%	12.1%	13.6%
	45–49	*N*	68	56	29	34	11	198
		%	8.2%	9.1%	10.1%	5.5%	8.3%	8.0%
Total		*N*	827	616	286	623	132	2,484
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Mother educational level	Preparatory/elementary	*N*	43	33	2	22	6	106
		%	6.5%	8.0%	0.7%	3.6%	5.9%	5.1%
	Primary	*N*	506	273	100	423	80	1382
		%	76.0%	66.4%	35.1%	69.2%	79.2%	66.6%
	Lower secondary	*N*	100	81	146	138	13	478
		%	15.0%	19.7%	51.2%	22.6%	12.9%	23.0%

(Continued)

TABLE 1 | Continued

			Central	EHP	Madang	ENB	POM	All sites
	Upper secondary	*N*	11	16	25	16	1	69
		%	1.7%	3.9%	8.8%	2.6%	1.0%	3.3%
	Vocational/ college+	*N*	6	8	12	12	1	39
		%	0.9%	1.9%	4.2%	2.0%	1.0%	1.9%
Total		*N*	666	411	285	611	101	2,074
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Mother marital status	Married	*N*	611	327	131	285	104	1,458
		%	55.3%	52.8%	45.8%	45.6%	56.5%	51.7%
	With partner	*N*	109	106	45	69	20	349
		%	9.9%	17.1%	15.7%	11.0%	10.9%	12.4%
	Single	*N*	385	186	110	271	60	1012
		%	34.8%	30.0%	38.5%	43.4%	32.6%	35.9%
Total		*N*	1105	619	286	625	184	2,819
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Household wealth quintile	Poorest	*N*	294	255	–	20	54	623
		%	23.9%	33.6%	–	3.1%	27.0%	19.9%
	Poor	*N*	313	169	–	102	45	629
		%	25.4%	22.3%	–	15.6%	22.5%	20.1%
	Middle	*N*	245	114	3	151	43	556
		%	19.9%	15.0%	1.0%	23.1%	21.5%	17.8%
	Richer	*N*	259	147	49	202	36	693
		%	21.0%	19.4%	17.0%	30.9%	18.0%	22.1%
	Richest	*N*	120	73	237	179	22	631
		%	9.7%	9.6%	82.0%	27.4%	11.0%	20.1%
Total		*N*	1,231	758	289	654	200	3,132
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
HH food shortage in the past 12 months	Yes	*N*	464	109	–	–	37	612
		%	37.8%	14.5%	–	–	19.0%	19.7%
	No	*N*	765	643	287	651	158	2,502
		%	62.2%	85.5%	100.0%	100.0%	81.0%	80.3%
Total		*N*	1,229	752	287	651	195	3,114
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

stunted than children from the richest quintile [OR: 1.2 (0.79–1.82)]. Children whose mothers lived with partners were less likely to be stunted than those whose mothers were currently not married or did not have partners [OR: 0.86 (0.59–1.24)].

DISCUSSION

Malnutrition encompasses wasting, stunting, being underweight, and even overweight in some cases, and can have both short and long-term impacts on child health and development (4). These impacts are well known to be associated with poor educational performance in childhood, lost productivity in adulthood, and increased risks of non-communicable diseases later in life (22).

As shown in **Table 4**, the 2005 PNG National Nutrition Survey (NNS) reported the prevalence of wasting among children aged 6–59 months at 4.5%, stunting at 43.9%, and being underweight at 18.1% (23). The 2010 HIES estimated wasting among CU5 at 15.8%, stunting at 46%, and being underweight at 25% (7). More recently, the National Health Information System (NHIS) reported that the prevalence of being underweight among CU5, who attended maternal and child health clinics, was 20% in 2018 (24). Our estimates support these figures, with the current numbers of wasting among CU5 at 16%, stunting at 46%, and being underweight at 18%. The data trend suggest that wasting is likely to have increased by more than 10% and there has been little improvement in stunting and being underweight among CU5 over the period 2005–2020, if the estimates of the 2005 NNS data are considered as reliable. It is noticeable that our study indicates a wasting prevalence (16%) higher than that reported by the 2005 NNS (4.5%), but it is similar to the level detected by the 2010 HIES. These figures suggest that wasting

TABLE 2 | Wasting, stunting, overweight and underweight prevalence among children under 5 years of age according to WHO Child Growth Standards by household socioeconomic and maternal demographic characteristics, PNGIMR's CHESS, 2020.

			Weight for height						Height for age						Weight for age					
			Wasting			Overweight			Stunted						Underweight					
			Severe (< −3 SD)	Moderate (−3 to −2 SD)	Normal (−2 to +2 SD)	Moderate (2 to 3 SD)	Obese (>3 SD)	No. of CU5	Severe (< −3 SD)	Moderate (−3 to −2 SD)	Normal (−2 to +2 SD)	2 to 3 SD	>3 SD	No. of CU5	Severe (< −3 SD)	Moderate (−3 to −2 SD)	Normal (−2 to +2 SD)	2 to 3 SD	>3 SD	No. of CU5
Sector	Urban	*N*	65	46	405	86	117	719	229	125	346	17	21	738	57	86	558	13	20	734
		%	9.0%	6.4%	56.3%	12.0%	16.3%	100.0%	31.0%	16.9%	46.9%	2.3%	2.8%	100.0%	7.8%	11.7%	76.0%	1.8%	2.7%	100.0%
	Rural	*N*	157	114	1,172	227	370	2,040	608	365	1,045	28	70	2,116	130	240	1,590	68	57	2,085
		%	7.7%	5.6%	57.5%	11.1%	18.1%	100.0%	28.7%	17.2%	49.4%	1.3%	3.3%	100.0%	6.2%	11.5%	76.3%	3.3%	2.7%	100.0%
Total		*N*	222	160	1,577	313	487	2,759	837	490	1,391	45	91	2,854	187	326	2,148	81	77	2,819
		%	8.0%	5.8%	57.2%	11.3%	17.7%	100.0%	29.3%	17.2%	48.7%	1.6%	3.2%	100.0%	6.6%	11.6%	76.2%	2.9%	2.7%	100.0%
Province*	Central	*N*	104	74	739	107	114	1,138	262	205	657	19	43	1,186	63	150	913	21	15	1,162
		%	9.1%	6.5%	64.9%	9.4%	10.0%	100.0%	22.1%	17.3%	55.4%	1.6%	3.6%	100.0%	5.4%	12.9%	78.6%	1.8%	1.3%	100.0%
	EHP	*N*	81	51	498	98	243	971	338	153	450	19	31	991	86	119	685	43	55	988
		%	8.3%	5.3%	51.3%	10.1%	25.0%	100.0%	34.1%	15.4%	45.4%	1.9%	3.1%	100.0%	8.7%	12.0%	69.3%	4.4%	5.6%	100.0%
	ENB	*N*	8	5	159	82	107	361	175	66	120	4	9	374	19	21	313	15	4	372
		%	2.2%	1.4%	44.0%	22.7%	29.6%	100.0%	46.8%	17.6%	32.1%	1.1%	2.4%	100.0%	5.1%	5.6%	84.1%	4.0%	1.1%	100.0%
	POM	*N*	29	30	181	26	23	289	62	66	164	3	8	303	19	36	237	2	3	297
		%	10.0%	10.4%	62.6%	9.0%	8.0%	100.0%	20.5%	21.8%	54.1%	1.0%	2.6%	100.0%	6.4%	12.1%	79.8%	0.7%	1.0%	100.0%
Total		*N*	222	160	1,577	313	487	2,759	837	490	1,391	45	91	2,854	187	326	2,148	81	77	2,819
		%	8.0%	5.8%	57.2%	11.3%	17.7%	100.0%	29.3%	17.2%	48.7%	1.6%	3.2%	100.0%	6.6%	11.6%	76.2%	2.9%	2.7%	100.0%
Sex	Male	*N*	84	61	683	140	207	1,175	366	200	583	19	42	1,210	74	134	921	32	40	1,201
		%	7.1%	5.2%	58.1%	11.9%	17.6%	100.0%	30.2%	16.5%	48.2%	1.6%	3.5%	100.0%	6.2%	11.2%	76.7%	2.7%	3.3%	100.0%
	Female	*N*	97	64	609	120	197	1,087	318	189	561	23	34	1,125	81	138	829	37	28	1,113
		%	8.9%	5.9%	56.0%	11.0%	18.1%	100.0%	28.3%	16.8%	49.9%	2.0%	3.0%	100.0%	7.3%	12.4%	74.5%	3.3%	2.5%	100.0%
Total		*N*	181	125	1,292	260	404	2,262	684	389	1,144	42	76	2,335	155	272	1,750	69	68	2,314
		%	8.0%	5.5%	57.1%	11.5%	17.9%	100.0%	29.3%	16.7%	49.0%	1.8%	3.3%	100.0%	6.7%	11.8%	75.6%	3.0%	2.9%	100.0%
Age group (month)	0–5	*N*	11	6	69	9	18	113	24	26	61	3	1	115	5	11	93	2	3	114
		%	9.7%	5.3%	61.1%	8.0%	15.9%	100.0%	20.9%	22.6%	53.0%	2.6%	0.9%	100.0%	4.4%	9.6%	81.6%	1.8%	2.6%	100.0%
	6–11	*N*	8	9	70	13	23	123	29	27	66	1	2	125	2	18	100	2	3	125
		%	6.5%	7.3%	56.9%	10.6%	18.7%	100.0%	23.2%	21.6%	52.8%	0.8%	1.6%	100.0%	1.6%	14.4%	80.0%	1.6%	2.4%	100.0%
	12–23	*N*	21	13	175	34	48	291	87	54	139	8	11	299	17	43	217	9	12	298
		%	7.2%	4.5%	60.1%	11.7%	16.5%	100.0%	29.1%	18.1%	46.5%	2.7%	3.7%	100.0%	5.7%	14.4%	72.8%	3.0%	4.0%	100.0%

(Continued)

TABLE 2 | Continued

			Weight for height						Height for age						Weight for age					
			Wasting			Overweight			Stunted						Underweight					
			Severe (< −3 SD)	Moderate (−3 to −2 SD)	Normal (−2 to +2 SD)	Moderate (2 to 3 SD)	Obese (>3 SD)	No. of CU5	Severe (< −3 SD)	Moderate (−3 to −2 SD)	Normal (−2 to +2 SD)	2 to 3 SD	>3 SD	No. of CU5	Severe (< −3 SD)	Moderate (−3 to −2 SD)	Normal (−2 to +2 SD)	2 to 3 SD	>3 SD	No. of CU5
	24–35	*N*	42	36	286	60	74	498	136	87	266	11	14	514	32	53	403	11	11	510
		%	8.4%	7.2%	57.4%	12.0%	14.9%	100.0%	26.5%	16.9%	51.8%	2.1%	2.7%	100.0%	6.3%	10.4%	79.0%	2.2%	2.2%	100.0%
	36–47	*N*	41	22	284	54	87	488	159	85	226	10	24	504	39	59	378	12	9	497
		%	8.4%	4.5%	58.2%	11.1%	17.8%	100.0%	31.5%	16.9%	44.8%	2.0%	4.8%	100.0%	7.8%	11.9%	76.1%	2.4%	1.8%	100.0%
	48–59	*N*	38	34	311	58	94	535	160	81	297	5	16	559	34	61	419	17	18	549
		%	7.1%	6.4%	58.1%	10.8%	17.6%	100.0%	28.6%	14.5%	53.1%	0.9%	2.9%	100.0%	6.2%	11.1%	76.3%	3.1%	3.3%	100.0%
Total		*N*	161	120	1,195	228	344	2,048	595	360	1,055	38	68	2,116	129	245	1,610	53	56	2,093
		%	7.9%	5.9%	58.3%	11.1%	16.8%	100.0%	28.1%	17.0%	49.9%	1.8%	3.2%	100.0%	6.2%	11.7%	76.9%	2.5%	2.7%	100.0%
HH wealth quintile	Poorest	*N*	43	34	311	46	74	508	121	87	296	5	14	523	36	66	383	14	17	516
		%	8.5%	6.7%	61.2%	9.1%	14.6%	100.0%	23.1%	16.6%	56.6%	1.0%	2.7%	100.0%	7.0%	12.8%	74.2%	2.7%	3.3%	100.0%
	Poor	*N*	49	33	268	54	73	477	144	82	244	9	16	495	31	71	364	9	12	487
		%	10.3%	6.9%	56.2%	11.3%	15.3%	100.0%	29.1%	16.6%	49.3%	1.8%	3.2%	100.0%	6.4%	14.6%	74.7%	1.8%	2.5%	100.0%
	Middle	*N*	24	23	229	48	74	398	124	71	197	10	13	415	26	39	317	13	13	408
		%	6.0%	5.8%	57.5%	12.1%	18.6%	100.0%	29.9%	17.1%	47.5%	2.4%	3.1%	100.0%	6.4%	9.6%	77.7%	3.2%	3.2%	100.0%
	Richer	*N*	31	19	261	57	74	442	141	78	208	9	19	455	25	47	365	9	8	454
		%	7.0%	4.3%	59.0%	12.9%	16.7%	100.0%	31.0%	17.1%	45.7%	2.0%	4.2%	100.0%	5.5%	10.4%	80.4%	2.0%	1.8%	100.0%
	Richest	*N*	14	11	126	23	49	223	65	42	110	5	6	228	11	22	181	8	6	228
		%	6.3%	4.9%	56.5%	10.3%	22.0%	100.0%	28.5%	18.4%	48.2%	2.2%	2.6%	100.0%	4.8%	9.6%	79.4%	3.5%	2.6%	100.0%
Total		*N*	161	120	1,195	228	344	2,048	595	360	1,055	38	68	2,116	129	245	1,610	53	56	2,093
		%	7.9%	5.9%	58.3%	11.1%	16.8%	100.0%	28.1%	17.0%	49.9%	1.8%	3.2%	100.0%	6.2%	11.7%	76.9%	2.5%	2.7%	100.0%
HH food shortage	Yes	*N*	52	33	314	41	59	499	116	86	290	6	20	518	32	74	383	10	17	516
		%	10.4%	6.6%	62.9%	8.2%	11.8%	100.0%	22.4%	16.6%	56.0%	1.2%	3.9%	100.0%	6.2%	14.3%	74.2%	1.9%	3.3%	100.0%
	No	*N*	109	86	874	187	283	1,539	476	272	759	32	48	1,587	97	169	1,219	43	39	1,567
		%	7.1%	5.6%	56.8%	12.2%	18.4%	100.0%	30.0%	17.1%	47.8%	2.0%	3.0%	100.0%	6.2%	10.8%	77.8%	2.7%	2.5%	100.0%
Total		*N*	161	119	1,188	228	342	2,038	592	358	1,049	38	68	2,105	129	243	1,602	53	56	2,083
		%	7.9%	5.8%	58.3%	11.2%	16.8%	100.0%	28.1%	17.0%	49.8%	1.8%	3.2%	100.0%	6.2%	11.7%	76.9%	2.5%	2.7%	100.0%

**No Z-scores were generated for children in Madang by the WHO Anthrop software program because the anthropometric data were either missing or ineligible.*

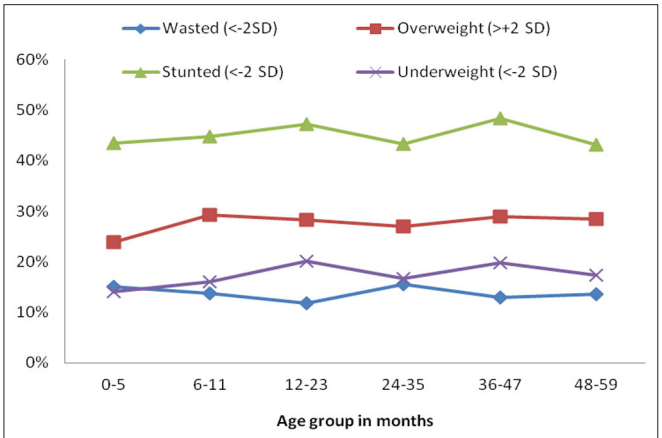

FIGURE 1 | Percentage of children under 5 years of age, who are undernourished by age group in PNG, PNGIMR's CHESS 2020.

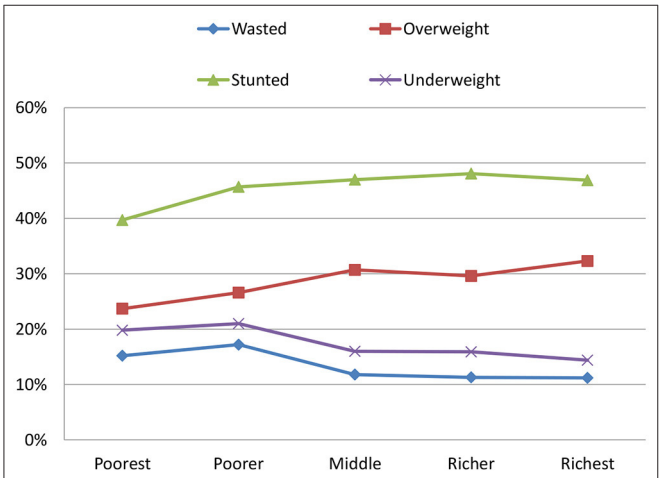

FIGURE 2 | Percentage of children under 5 years of age, who are undernourished by household wealth quintile in PNG, PNGIMR's CHESS 2020.

prevalence among CU5 had increased most likely in the period 2005–2010, but no further increase in the period 2010–2020. According to our knowledge and fieldwork experience, there is no clear socioeconomic or technical reason possibly explaining these trends. Furthermore, we have estimated that 28% of CU5 were overweight, for which the three other national data sources failed to report. This indicator is particularly important in the current health and epidemiological transition in PNG (25, 26).

For the first time, we measure and report all four key nutritional indicators of PNG children against the 2006 WHO Child Growth Standards. Our data uncover an alarming level of wasting as well as overweight CU5 in PNG. We call for urgent action to improve the nutrition status among PNG children and to achieve the SDGs by 2030 as part of PNG's government commitments. Compared to other Pacific countries, our estimates of stunting among CU5 in 2020 (46%) are higher than that of the Solomon Islands in 1989 (34%), Indonesia in 2007 (40%), but lower than Timor-Leste in 2009 (58%) (27).

Our data show a complicated picture of nutrition among CU5 in PNG, in which multiple factors interact to cause child malnutrition. Examining possible associations with various household, maternal, and individual socioeconomic demographic factors, we found that wasting and stunting are distinct phenomena with different correlates. These two issues could share the same common socioeconomic determinants, but maternal socioeconomic demographic factors appear to be different in the development of wasting and stunting.

Wasting may exhibit significant seasonal shifts associated with recent changes in the availability and/or accessibility of food and/or presence of illnesses. We found that children in urban areas are more likely to be wasting than children in rural areas. The recent social changes and urbanization in PNG could explain this. The urban population has increased at an annual rate of 0.2% over the last decade, from 13.0% in 2010 to 13.25% in 2020 (28). The proportion of urban children in our study is higher (31%) because we included data from two urban sites in Madang and POM. Noticeably, the migration rates in our urban surveillance sites are also higher than the country average and many children in these sites reside in new resettlements (29). This could have been an important explanation for the high estimation of wasting among urban children. Earlier studies reported poor nutritional status among children in resettlement areas (8, 17).

Wasting among urban children is unlikely to be due to the lack of access to good quality foods, but more likely associated with poor breastfeeding practice and inappropriate food supplementation to CU5 (16). Most urban children aged 6–23 months are breastfed and feeding with supplementary foods is less than one half. This rate is even lower in POM and Madang. There are a number of reasons for suboptimal feeding practices among PNG women. Some are possibly culturally linked, for example, PNG men are advised not to have sex with their partners who are breastfeeding (12, 16). These men could have stopped their partners from breastfeeding their infants, contributing to the high level of wasting among CU5 in urban areas (8). Health education is therefore needed with interventions integrated into antenatal care and post-natal care and focused in urban areas to improve the knowledge of women and men about breastfeeding and food supplementation for their children, especially in the first 1,000 days of their life.

Household wealth is a good predictor of wasting. Cash crops are the main source of household income to contribute to and sustain household wealth in rural areas. Previous studies have found that wasting increases before the harvest and improves after major cash crops such as coffee, cocoa, coconut, and oil palm (30). We suggest that rural household wealth with the effects of cash crops are linked with consumption of good quality foods and both are important contributors to lowering the current wasting prevalence among CU5 in rural areas.

Cash crops are heavily dependent on seasonal effects. In PNG, climatic events such as drought and social conflicts such as tribal fights, possibly lead to acute food shortage at the household level (21). In this event, food supply is interrupted and reduces the quantity and quality of food, posing a threat to food security at

TABLE 3 | Household socioeconomic and maternal demographic factors associated with wasting and stunting among children under 5 in PNG, PNGIMR's CHESS 2020.

		Wasting status (< −2 SD)						Stunting status (< −2SD)					
		N	Percentage	*P*-value	Odds ratio	Lower bound	Upper bound	*N*	Percentage	*P*-value	Odds ratio	Lower bound	Upper bound
Sector	Urban	274	23.0%	0.29	1.36	0.77	2.40	281	22.9%	0.49	1.13	0.80	1.60
	Rural	919	77.0%		Ref.			944	77.1%		Ref.		
Province	Central	537	45.0%	0.76	0.87	0.35	2.15	552	45.1%	0.26	1.43	0.77	2.67
	EHP	345	28.9%	0.90	1.05	0.48	2.30	349	28.5%	0.05	1.75	0.99	3.10
	ENB	237	19.9%	0.00	0.20	0.07	0.58	248	20.2%	0.00	3.29	1.80	6.01
	POM	74	6.2%		Ref.			76	6.2%		Ref.		
Household wealth quintile	Poorest	229	19.2%	0.67	1.16	0.59	2.28	233	19.0%	0.97	0.99	0.64	1.54
	Poor	274	23.0%	0.51	1.25	0.65	2.37	281	22.9%	0.39	1.20	0.79	1.82
	Middle	252	21.1%	0.46	0.77	0.38	1.55	260	21.2%	0.53	1.14	0.75	1.74
	Richer	286	24.0%	0.90	0.96	0.50	1.85	296	24.2%	0.70	1.08	0.72	1.63
	Richest	152	12.7%		Ref.			155	12.7%		Ref.		
Food shortage	Yes	259	21.7%	0.11	1.43	0.93	2.21	265	21.6%	0.51	0.90	0.67	1.23
	No	934	78.3%		Ref.			960	78.4%		Ref.		
Mother marital status	Married	536	44.9%	0.26	1.25	0.85	1.86	549	44.8%	0.89	1.02	0.79	1.31
	With partner	156	13.1%	0.39	0.76	0.40	1.42	162	13.2%	0.42	0.86	0.59	1.24
	Single	501	42.0%		Ref.			514	42.0%		Ref.		
Mother education attainment	Preparatory	23	1.9%	0.24	0.17	0.01	3.29	25	2.0%	0.05	0.15	0.02	1.01
	Elementary	52	4.4%	0.26	0.24	0.02	2.91	52	4.2%	0.24	0.35	0.06	2.01
	Primary	883	74.0%	0.67	0.62	0.07	5.53	901	73.6%	0.17	0.31	0.06	1.66
	Lower secondary	196	16.4%	0.78	0.73	0.08	6.67	203	16.6%	0.16	0.30	0.06	1.62
	Upper secondary	24	2.0%	0.95	0.93	0.08	10.43	27	2.2%	0.62	0.63	0.10	3.94
	Vocational/College+	15	1.3%		Ref			17	1.4%		Ref.		
Child age group in month	0–5	65	5.4%	0.66	0.81	0.32	2.06	65	5.3%	0.94	0.98	0.56	1.71
	06–11	77	6.5%	1.00	1.00	0.43	2.33	79	6.4%	0.97	1.01	0.60	1.69
	12–23	159	13.3%	0.40	1.29	0.71	2.34	165	13.5%	0.86	0.97	0.66	1.42
	24–35	284	23.8%	0.26	1.34	0.81	2.23	290	23.7%	0.89	1.02	0.74	1.43
	36–47	298	25.0%	0.81	0.94	0.54	1.61	307	25.1%	0.03	1.44	1.04	1.99
	48–59	310	26.0%		Ref.			319	26.0%		Ref.		
Sex of child	Male	620	52.0%	0.16	0.77	0.53	1.11	635	51.8%	0.63	0.95	0.75	1.19
	Female	573	48.0%		Ref.			590	48.2%		Ref.		
Total valid no. of children		1193	100.0%					1225	100.0%				
Subpopulation		808*						827**					

**The dependent variable has only one value observed in 756 (93.6%) subpopulations.*

***The dependent variable has only one value observed in 684 (82.7%) subpopulations.*

the household level. In response to malnutrition, children will stop growing and lose body weight, including lean and fat tissues (31). This is another important seasonal aspect of PNG child wasting revealed in our data. Food insecurity in households is a key seasonal factor attributing to an increase in wasting among CU5 in PNG.

Household food shortage plays a large role in wasting among urban children in PNG. Around one-fifth of households experienced food shortage in the past 12 months. This is likely connected with social instabilities occurring in POM during the national election in 2017. Shops were closed, road traffic and transportation was blocked, and food supply chain in POM was broken. Job cuts led to a decline in households' affordability for foods. Serious droughts in the second half of 2017 resulted in the lost of subsistence farming throughout the country, but the most hard hit were those in the southern region. About 40% of households in Central Province reported food shortage in our data. Food security at the household level is an important seasonal factor, amplifying loss of weight-for-height, and contributing to the high wasting prevalence among CU5 in PNG. To effectively prevent wasting, food security must be addressed at the household level.

Previous studies have found that stunting is closely linked to poverty (15). This was also confirmed in our data. Stunting is often unrecognized because short stature is so common in some communities, particularly in the rural areas of PNG (32, 33). Our data show that children in Central Province, EHP, and ENB have a consistently higher risk of stunting than those in POM. Early nutritional surveys showed that a higher prevalence of stunting was related to the low protein and energy content of typical PNG

TABLE 4 | Comparison of health and development indicators among children under 5 years of age across different data sources.

	2005 NNS (published 2011) (23)	2010 HIES (published 2015) (7)	2018 NHIS (published 2019)* (24)	2019 CHESS (published 2020)
Wasting	4.5%	15.8%	N/A	16%
Stunting	43.9%	46%	N/A	46%
Underweight	18.1%	25%	20%	18%
Overweight	N/A	N/A	N/A	28%

**National Health Information System reported underweight among CU5 attended Maternal and Child Health Clinics.*

diets (34, 35). In most of the rural areas of PNG, up to 80% of the total dietary energy comes from root crops such as yams, kaukau, and tapioca, which are high in fiber and moisture (12, 36).

Local factors maybe also contribute to stunting in children. Children in the highlands region, i.e., EHP, are more stunted as they are more involved in subsistence agriculture, which is connected with a higher energy expenditure and therefore with a need for more food. In EHP, infectious diseases such as respiratory tract infections, skin infections, and diarrhea are prevalent during the wet season and may have led to a higher level of stunting in this province (16, 25). By contrast, the frequent rainfall deficit along the coastal areas of the Southern region could have resulted in a persistent shortage of quality foods in Central Province. Lack of parental care during crop season and greater involvement of women in non-agriculture economic sectors such as trading and services might have also contributed to higher stunting among children in this province (21). Yams are one of the main foods in ENB. Studies on traditional food consumption in PNG showed strong associations between eating yams and stunting (12, 15, 30). Unlike other root vegetables, yams are harvested only once a year, often in the dry season, in the second half of the year and can be stored in households for a short period. This dietary factor could have contributed to high stunting among CU5 in ENB as shown in our data.

At the individual level, our data show that maternal socioeconomic status appears to be an important factor in the wasting and stunting of children. Women's marital status is a proxy for generational influence with polygamy widely accepted. Women's marital status could reflect their socioeconomic status in the household. In most rural areas of PNG, men are the ones who clear the bush and establish the gardens while women do gardening and food gathering (21). Single mothers might not be able to do the agriculture work needed to generate enough food to feed their children. Similarly, in urban areas, men make up the main labor force generating income for the household. Children whose fathers have multiple sexual partners might not have been adequately fed or attended to by their parents.

Maternal education appears to be a strong predictor for wasting and stunting among PNG CU5. Children whose mother had primary education were less likely to be wasted or stunted than children whose mothers had college/university level education. Previous study has found that children living in households where the household heads had a higher level of education were less likely to be malnourished (12). Our finding is not contradictory because most household heads are men rather than women regardless of their educational level. Higher educated mothers are more likely to participate in the official labor market and in full-time employment in the public or private sector, rather than doing housework. While the early learning system and childcare centers are not common in PNG, particularly in rural areas, more educated women often hire young women from their villages to take care of their children. However, these young babysitters often do not have adequate knowledge and experience to look after a child. Many infants are reportedly bottle fed (16), which may be explained by the higher education level of their mothers. Nutritional programs should include education intervention targeting child caretakers who work either in households or in childcare centers in order to reduce and prevent wasting and stunting among PNG children.

There are some limitations in this study. Child participants were recruited from CHESS surveillance sites located in the five main provinces, representing the four geographic regions of PNG. Hence, the data are not a nationally representative sample of the CU5 population of PNG. Information of maternal age, education level attainment and marital status, and household experience of food shortage in the past 12 months are self-reported by parents and caregivers, meaning the data might have been biased due to recall process. Furthermore, these covariates might have correlated with each other in the multinomial logistic regression models, resulted in collinearity and over-adjustment of ORs. Anthropometric data provide a profile of child participants' nutritional status, i.e., prevalence of wasting, stunting, being underweight, and overweight in child participants only at the data collection time. The data do not show the duration of such nutritional conditions. Lastly, a large proportion of child participants were excluded from statistical modeling because of missing value data on age and/or sex, and/or anthropometric measurement.

CONCLUSION

This study has provided an update on nutritional status and household and maternal socioeconomic demographic factors associated with wasting and stunting among children under 5 years of age in PNG. There is no unique and clear explanation for the current status of wasting and stunting among PNG children. The best possible explanations in the context of contemporary PNG include the recent socioeconomic development leading to changes in the society, especially in urbanization associated

with household wealth and in maternal education which have resulted in a shift in food habits and eating behaviors among children from a traditional low calorie diet to a diet which includes more high-calorie processed foods. This transition is triggered by various seasonal effects, from extreme climates to food shortage in the household and morbidity of child individuals. This transition is more apparent in urban areas, where children are likely exposed to more risk factors. These findings could be useful to inform policy and interventions in order to improve child nutrition among children in PNG. The study results provide evidence for further interventional studies of child health and development.

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 CHESS was granted ethics approvals from Internal Review Board of PNG Institute of Medical Research (IRB's Approval No. 18.05) and the Medical Research Advisory Committee of Papua New Guinea (MRAC's Approval No. 18.06). These approvals covered all the data components under the CHESS, including data of children under 5 years of age, which were used in this manuscript. Informed consent was sought from self-identified household heads and woman participants. Women were informed about their right to withdraw from the study at any stage. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BP designed the CHESS and analyzed the data, conceptualized the paper, interpreted the data, drafted, revised, finalized, and submitted the manuscript. VS supervised the fieldwork, collected data, and provided the inputs. AO reviewed, commented, and provided inputs, particularly on the English edition. WP oversaw the CHESS and approved the submission of the manuscript. All authors contributed to the article and approved the submitted version.

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Arachidonic Acid Metabolism Controls Macrophage Alternative Activation Through Regulating Oxidative Phosphorylation in PPARγ Dependent Manner

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Macrophage polarization is mainly steered by metabolic reprogramming in the tissue microenvironment, thus leading to distinct outcomes of various diseases. However, the role of lipid metabolism in the regulation of macrophage alternative activation is incompletely understood. Using human THP-1 and mouse bone marrow derived macrophage polarization models, we revealed a pivotal role for arachidonic acid metabolism in determining the phenotype of M2 macrophages. We demonstrated that macrophage M2 polarization was inhibited by arachidonic acid, but inversely facilitated by its derived metabolite prostaglandin E2 (PGE2). Furthermore, PPARγ bridges these two seemingly unrelated processes *via* modulating oxidative phosphorylation (OXPHOS). Through inhibiting PPARγ, PGE2 enhanced OXPHOS, resulting in the alternative activation of macrophages, which was counterweighted by the activation of PPARγ. This connection between PGE2 biosynthesis and macrophage M2 polarization also existed in human and mouse esophageal squamous cell carcinoma. Our results highlight the critical role of arachidonic acid and metabolic PGE2 as immune regulators in modulating tissue homeostasis and pathological process.

Keywords: macrophage alternative activation, arachidonic acid metabolism, peroxisome proliferator-activated receptor gamma (PPARgamma), oxidative phosphorylation (OXPHOS), prostaglandin E2 (PGE2)

INTRODUCTION

Metabolic reprogramming is a hallmark of many pathological processes, such as obesity, cancer and cardiovascular diseases. Energy metabolic homeostasis profoundly impacts immune responses in tissue microenvironment (1). When energy is surplus, immune cells reprogram their metabolic pathway to trigger metaflammation (2). Obesity is a prototypical example of how energy metabolic homeostasis affects immunological function. Lipids depositing in various tissues leads to hypoxia and adipocyte stress thus recruits innate immune cells and promotes chronic activation of survival

pathway (3). In return, phenotype change of immune cells can also function to regulate system or local metabolic state (4).

Macrophages as one of the prominent components of immune system are versatile. They adopt different polarization states depending on the context of tissue microenvironment. Macrophages sensor, integrate and response to stimulus to achieve metabolic homeostasis through initiating inflammation or insulin action (5). In cancer, metabolic shaping of tumor microenvironment (TME) profoundly impacts the functional responses of immune cells (6). Cancer cells release lactate, glutamine, succinate and α-ketoglutarate (α-KG) and thereby prompt T cells and macrophages to polarize towards immunosuppressive phenotype (7–9). In contrast, metabolic reprogramming of the tumor-associated macrophages (TAMs) inhibits tumor progression by allowing the accumulation of T cell receptor engineered T cells (10). Dysfunction of macrophages contributes to systemic inflammation, thus maintaining the normal state of macrophages is critical for health state (11). Based on functional diversity, macrophages are mainly divided into two phenotypes, classically activated macrophages (M1) and alternatively activated macrophages (M2). Metabolic homeostasis especially within adipose and liver tissues has been found closely related to M2 macrophages, which can promote insulin sensitivity (5, 12). However, the metabolic regulation of macrophage polarization is incompletely understood. Emerging evidences have suggested that macrophages use glucose or fatty acids as fuel sources to attain differential activation (13). How these energy metabolism especially lipid metabolism contribute to macrophage polarization remains unclear.

In this study, we aim to elucidate the mechanism underlying metabolic regulation of macrophage polarization. By using integrated analysis of transcriptomic and lipid metabolomic signatures, we showed that arachidonic acid (AA) metabolism determined the polarization of M2 macrophages. Arachidonic acid and metabolic prostaglandin E2 (PGE2) regulated macrophage polarization induced by IL-4/IL-13. Furthermore, activation of PPARγ by the specific agonist rosiglitazone inhibited the induction of M2 polarization by PGE2. Mechanistically, PGE2 enhanced mitochondrial oxidative phosphorylation (OXPHOS) through suppressing PPARγ, resulting in the M2 polarization of macrophages. Our data suggest arachidonic acid and metabolic PGE2 as critical regulators of macrophage alternative activation.

MATERIALS AND METHODS

Reagents and Antibodies

Arachidonic acid (purity > 98.5%), PGE2 and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cytokines (IL-4, IL-13 and IFN-γ) were provided by Peprotech (Cranbury, NJ, USA). Fluorescence labeled antibodies and bead-based multiplex LEGENDplex assay were from Biolegend (San Diego, CA, USA). Specific inhibitors were acquired from MedChemExpress (Monmouth Junction, NJ, USA) and Selleckchem (Houston, TX, USA). Information about key reagents was provided in **Supplementary Table S1**.

Humanized THP-1 Derived Macrophage Polarization Model

Human monocytic THP-1 cells were obtained from American Type Culture Collection (ATCC, VA, USA). THP-1 and THP-1 derived macrophages were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin and 0.05 mM 2-mercaptoethanol at a controlled atmosphere with 37°C, 95% relative humidity, 5% CO_2. To acquire undifferentiated macrophages (M0), THP-1 cells were treated with PMA (25 ng/ml) for 48 h and rest in PMA-free growth medium for 24 h. Interferon-gamma (IFN-γ, 25 ng/ml) and lipopolysaccharide (LPS, 100 ng/ml) were added into M0 for an extra 24 h to obtain M1; Interleukin-4 (IL-4, 20 ng/ml) and Interleukin-13 (IL-13, 20 ng/ml) were added for M2 macrophages. Cells were treated with chemicals as figure captions indicated during the induction of polarization. Anti-CCR7 and anti-CD209 fluorescence labeled antibodies were used to validate M1/M2 macrophages *via* immunofluorescence staining and high content imaging system (HCI, ImageXpress Micro Confocal, Molecular Device, LLC, CA, USA). Cell supernatants were collected for further analysis. The characteristics of M0, M1 and M2 macrophages were validated by morphology, surface markers, gene transcription and functional cytokines (**Supplementary Figures S1**, **S2**).

Mice Bone Marrow Derived Macrophage (BMDM) Polarization Model

Wild type C57BL/6 mice were obtained from Beijing Vital River Laboratory and C57BL/6 PPARγ^{loxP} mice (*Pparg*$^{tm2Rev/J}$) were obtained from the Jackson laboratory. Monocyte-specific PPARγ deletion mice (*Pparg*$^{-/-\Delta Mono}$) were generated by intercrossing *Pparg*$^{tm2Rev/J}$ with Lyz2^{cre} mice. Tibias and femurs were isolated from 12-week-old mice. Bone marrow medium (BMM) were prepared by adding M-CSF (10 ng/ml) into DMEM complete medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. Bone marrow cells were collected by flushing tibias and femurs with BMM. Next, cells were removed for debris or any remnants with strainers, centrifuged, re-suspended in BMM and cultured for 7 days. BMM was refreshed on day 3, day 5. On day 7, cells were collected and validated through flow cytometry. IFN-γ (25 ng/ml) and LPS (100 ng/ml) were used for M1 polarization. IL-4 (10 ng/ml) and IL-13 (10 ng/ml) were used for M2 polarization. After 48 h, macrophages markers (CD206 for M2, CD69 for M1) were detected by HCI.

Esophageal Squamous Cancer Carcinoma (ESCC) Mice Model

ESCC mice was established as previously described (14). Briefly, N-Nitrosodimethylamine (NMBA) was administered to C57BL/

6 mice by gavage at the dose of 0.25 mg/kg BW, twice a week for 5 weeks. The control group (CT) was given the solvent carboxyl methyl cellulose [CMC, 1% (v/v)] with equivalent volume. All mice were housed in controlled atmosphere with 12 h/12 h light/dark cycle and fed standard chow diets. After gavage, mice were maintained for extra 20 weeks. At the endpoint of experiment, mice were sacrificed and forestomachs were collected and flash frozen in liquid nitrogen for RNA-seq. Transcriptomics data are available in GEO database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE134067.

Live-Cell High Content Imaging

For surface marker staining, cells in black wall 96-well-plate were washed with PBS and blocked with FcR blocking buffer (FcX block, Biolegend, San Diego, CA, USA) at room temperature for 10 min. Afterwards, cells were incubated with antibodies and Hoechst 33342 in cell staining buffer for 30 min at room temperature. Next, PBS was used for washing and FluoroBrite™ DMEM (Gibco, Grand Island, NY, USA) was used for reducing background fluorescence. For intracellular protein staining, cells were fixed with fix/perm buffer (BD Bioscience) for 30 min and washed with Perm/Wash buffer (BD Bioscience) for twice. Then cells were incubated with primary antibodies at room temperature for 30 min. After washing with Perm/Wash buffer, cells were incubated with fluorescence conjugated secondary antibodies for 30 min. Finally, cells were counterstained for nucleic with Hoechst 33342 and subjected to analysis with ImageXpress software (Molecular Device, LLC, San Jose, CA, USA).

RNA-Sequencing

Total RNA was extracted from cells or tissues with RNeasy kit (QIAGEN) or TRIzol. RNA quality and quantity was detected with NanoDrop and Agilent 2100 Bioanalyzer. After that, mRNA was enriched with Oligo (dT) magnetic beads and broke into short fragments for cDNA synthesis. The cleaved RNA fragments were reversely transcribed into first strand cDNA using random hexamers, following by second strand cDNA synthesis using DNA Polymerase I and RNase H. The double-stranded cDNA was purified, added A tail and connected with a sequencing adapter. Then, PCR amplification was performed on ABI StepOnePlus Real-Time PCR System and the constructed sequencing library was sequenced at Illumina HiSeq. Raw RNA sequencing data is available through the National Center for Biotechnology Information Gene Expression Omnibus (NCBI–GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE159112, GSE159120.

Raw data was filtered and clean reads were aligned with reference genome (hg19) using HISAT. Total mapped reads of all samples are higher than 95%. Reads were reconstructed into transcripts and their abundance was estimated and expressed as Fragments per kilo base per million mapped reads (FPKM). DEseq2 was used to determine differentially expressed genes (DEGs).Fold change≥ 2 or ≤ 0.5 and adjust p value ≤ 0.05 were set for DEGs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed with R phyper. Heatmaps were generated with online tools (http://www.ehbio.com/ImageGP/index.php/Home/Index).

Gene Set Enrichment Analysis (GSEA)

All FPKM values of identified genes from RNA-sequencing were input into GSEA software 4.0.3 for enrichment analysis (15). Data were normalized first and then a ranked gene list were generated. Database was downloaded from Molecular Signatures Database (MSigDB) gene sets (http://software.broadinstitute.org/gsea/index.jsp).

Lipid Metabolomics Analysis

M0/M1/M2 macrophages were collected and immediately stored at liquid nitrogen until analysis. Samples were thawed on ice and added 800 μl pre-chilled dichloromethane/methanol (3: 1) buffer, then precipitated in refrigerator at -20°C for 2 h. Then samples were centrifuged at 25,000 g, 4°C for 15 min. The supernatants (650 μL/each) were transferred to new tubes and centrifuged again. Then the supernatants (600 μL/each) were frozen-dry and reconstituted by lipid reconstituted solution (isopropanol: acetonitrile: water = 2:1:1, 600 μL/each). After centrifuging, the supernatants (60 μL/each) were detected on the LC-MS system. Quality control (QC) was obtained by mixing the supernatants from 3 samples (20 μL/each) and detected under same condition.

Raw data from mass spectrometer were firstly preprocessed (noise filtering, peak matching and extraction), and corrected based on the quality control-based robust LOESS signal correction (QC-RSC). Human Metabolome Database (HMDB) and LipidMaps database were used for peak alignment. Secondly, multivariate analysis principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were introduced to test for difference. Metabolites with fold change ≥ 1.2 or ≤ 0.8333 and q-value < 0.05 were selected as differential metabolites. Finally, the differential metabolites identification were performed with Progenesis QI (version 2.2) software. Pathway analysis was based on KEGG database.

Metabolite Set Enrichment Analysis (MSEA) and Joint Pathway Analysis

Differential lipid metabolites from positive or negative ion mode were input for Metabolite Set Enrichment Analysis with online tools (MetaboAnalyst, https://www.metaboanalyst.ca) as previous study introduced (16).

Differential lipid metabolites and DEGs from RNA-sequencing were input simultaneously to conduct joint pathway analysis on MetaboAnalyst. Integrated metabolic pathway database from current KEGG version was chosen for enrichment. Parameter listed as follow: hyper geometric test for enrichment analysis, closeness centrality for topology measure and overall combine p value for integration method.

Macrophages Cytokines Determination

After induction of polarization, culture medium was refreshed and 24 h later, cell supernatants were collected for cytokines determination. The concentration of cytokines was measured

with LEGENDplex™ Human macrophage panel using flow cytometry following manufacturer's instruction. Data were analyzed with Legendplex software (v8.0).

Correlation Analysis

The mRNA expression data used for correlation analysis in this study is available in the Genomic Data Commons (https://portal.gdc.cancer.gov/). Briefly, a total of 90 cases of ESCC were included, and clinical characteristics had been described in previous study (17). FPKM values were log transformed (log_2(X+1), X= raw FPKM) for analysis. Pearson correlation coefficients and liner regression were analyzed with Graphpad Prism 6.

Statistical Analysis

All quantitative experimental values were presented as mean ± SEM. Data were processed and visualized with Graphpad Prism 6. Unpaired t test or ANOVA analysis were applied to determine statistical significance within different treatments. $P < 0.05$ was set for significance.

RESULTS

Macrophage M2 Polarization Is Tightly Associated with Lipid Metabolism

To investigate the role of lipid metabolism in the regulation of macrophage polarization, we analyzed the transcriptomic changes with THP-1 derived macrophage polarization model. M1/M2 macrophages showed divergent features of energy metabolism when compared with M0 macrophages (**Figure 1** and **Supplementary Figure S3**). The expression of genes controlling fatty acid biosynthesis (FAS) (**Figure 1A**) and OXPHOS (**Figure 1B**) was particularly enhanced in M2 macrophages. To validate whether these pathways contribute to M2 polarization, cells were treated with series of inhibitors in the induction of M2 polarization. FAS inhibition induced by FASN-IN-4 tosylate (FAI), Fatostatin (FATO), FT113 (FT) dose-dependently decreased CD209 expression (**Figures 1C–E**), suggesting that M2 polarization could be promoted by the activation of FAS pathway. Similarly, blockade of OXPHOS by

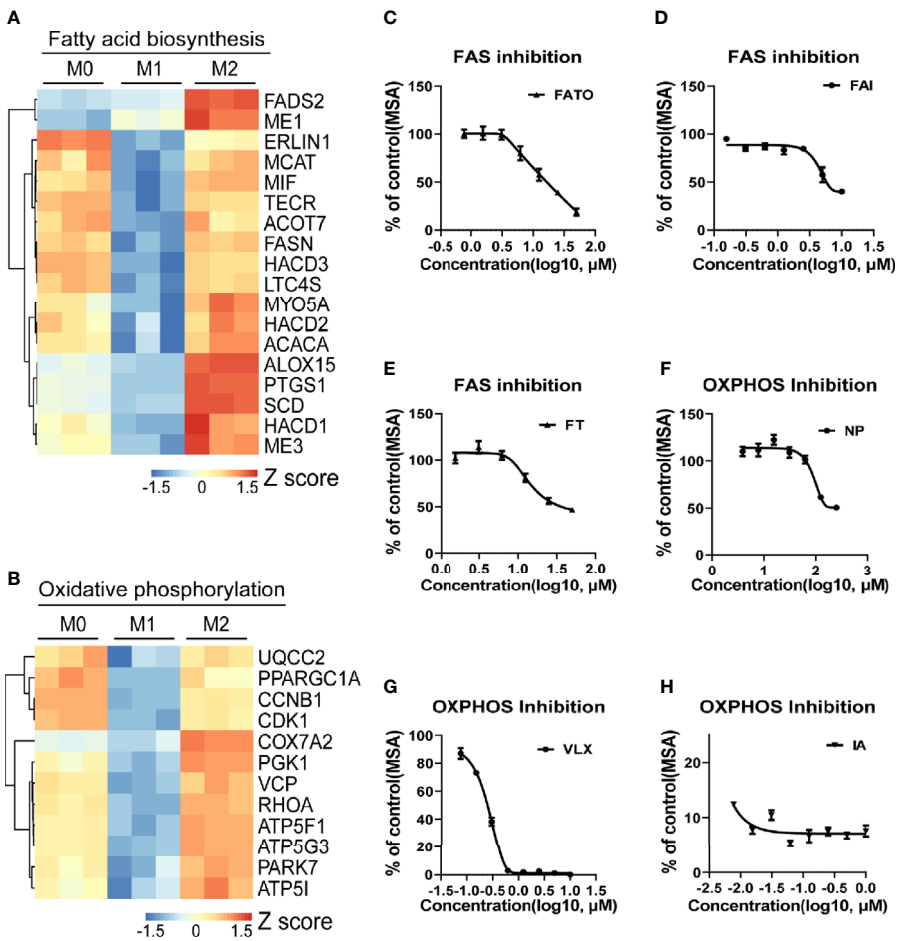


FIGURE 1 | Macrophage M2 polarization is tightly associated with lipid metabolism. **(A, B)** Gene expression in THP-1 derived differentially activated macrophages related to fatty acid biosynthesis (FAS) or oxidative phosphorylation (OXPHOS), respectively. **(C–E)** CD209 expression curves of THP-1 derived M2 macrophages *via* high content imaging (HCI) with specific FAS inhibitors treated as indicated for 48 h. **(F–H)** CD209 expression curves of THP-1 derived M2 macrophages *via* HCI with specific OXPHOS inhibitors treated as indicated for 48 h. Error bars represent the mean ± SEM from 3 biological replicates. MSA, mean stain area.

3-Nitropropanoic acid (NP), VLX600 (VLX) and IACS-10759 (IA) (**Figures 1F–H**) also attenuated macrophage M2 polarization in a dose-dependent manner. Other processes associated with lipid utilization including lipolysis, fatty acid transport (FAT) and fatty acid oxidation (FAO) did not show featured alternation during the M2 polarization (**Supplementary Figures S3A–C**). However, inhibition of these processes also affected M2 polarization (**Supplementary Figures S3D–F**). Inhibition of FAT and FAO significantly suppressed M2 polarization (**Supplementary Figures S3D, E**). This is in line with the results from blockade of FAS and OXPHOS. In contrast, inhibition of lipolysis dramatically promoted macrophage M2 polarization (**Supplementary Figure S3F**). These data suggested that fatty acid biosynthesis and utilization were crucial for macrophage M2 polarization.

Arachidonic Acid Metabolism Is Enhanced in M2 Macrophages

Next, we aimed to identify the key lipid metabolic regulator in M2 polarization by the transcriptomic and metabolomic analysis. Firstly, among all DEGs between M1 and M2 macrophages, 1645 genes were up-regulated in M2 macrophages (**Figure 2A**). Analysis of the expression profiles against the hallmark gene sets available from MSigDB suggested an enrichment of arachidonic acid metabolism in M2 macrophages (**Figure 2B**). Expression level of genes associated with arachidonic acid metabolism were significantly higher in M2 macrophages (**Figure 2C**). Elevated expression of these genes was largely related to prostaglandins and leukotrienes production (**Figure 2C**). In accordance, the expression of key metabolic enzymes that utilize arachidonic acid as a substrate for the synthesis of eicosanoids, including 15-lipoxygenase (15-LO, encoded by ALOX15) and cyclooxygenases (COX-1/COX-2, encoded by PTGS1/PTGS2) were significantly elevated in M2 macrophages (**Figure 2D**).

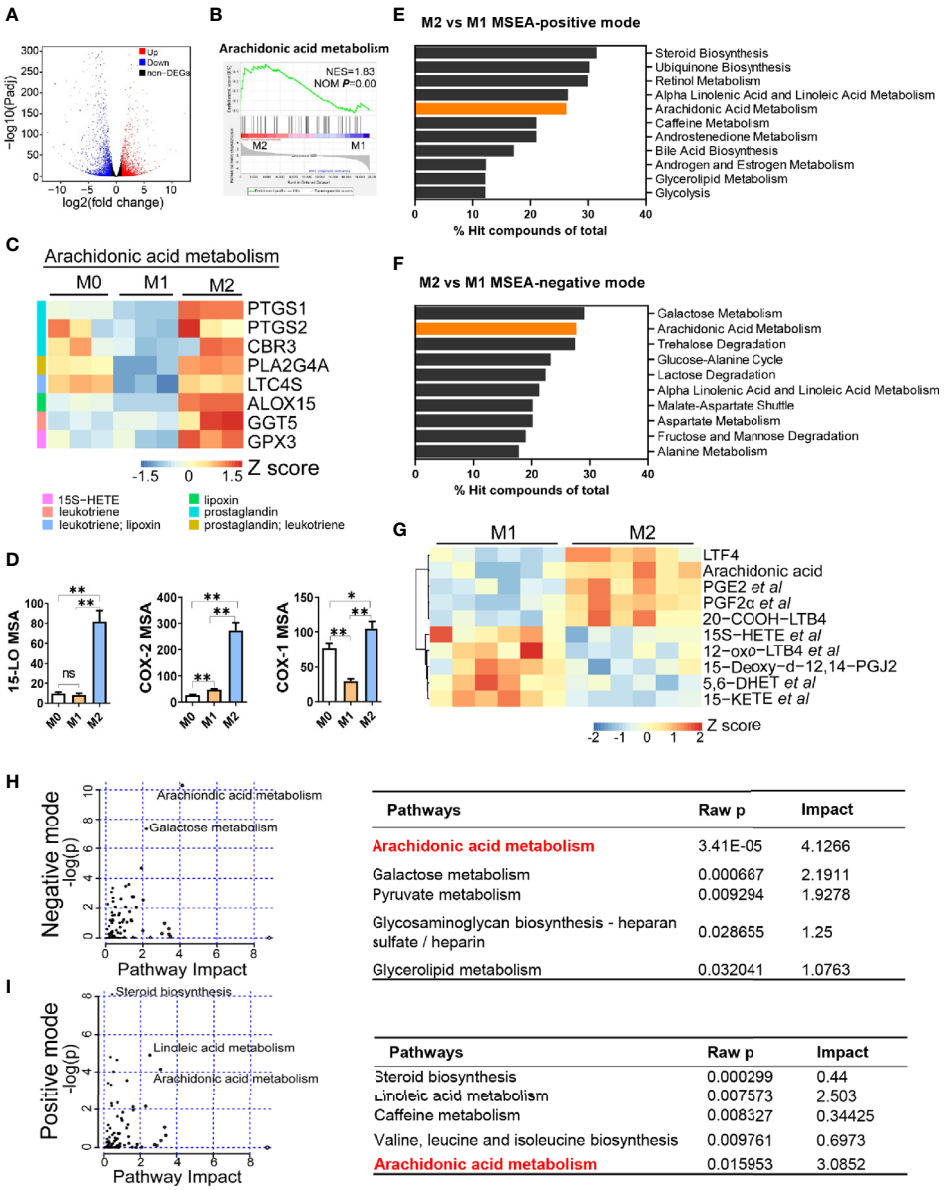
To decipher the lipid metabolic signature for macrophage polarization, we further analyzed metabolomic difference between M1 and M2 macrophages. PCA and PLS-DA analysis revealed metabolic disparity of M1 and M2 macrophages (**Supplementary Figures S4A, B**). A total of 3652 and 2328 differential ions (identified as 808 and 510 differential metabolites) were obtained in positive and negative mode, respectively (**Supplementary Figure S4C**). We next performed MSEA to these differential metabolites. Top 10 enriched pathways showed that arachidonic acid metabolism was the only two pathways that included in both modes, ranking 5th and 2nd respectively (**Figures 2E, F**). Another pathway, alpha linoleic acid and linoleic acid metabolism, was also included in top 10 pathways (**Figures 2E, F**). This is possibly due to that it shares some common enzymes with arachidonic acid metabolism. Next, we compared differential metabolites associated with arachidonic acid metabolism. Metabolites profiles of M1 and M2 were significantly different (**Figure 2G**, **Supplementary Table S2**). Arachidonic acid, and prostaglandins (PGE2, PGF2α et al.) and leukotrienes (LTF4, 20-COOH-LTB4) were enriched in M2 macrophages. Integrated analysis of transcriptomics and metabolomics demonstrated that arachidonic acid metabolism was the most remarkable pathway with highest pathway impact in both modes (**Figures 2H, I**). Other metabolic pathways such as linoleic acid pathway and galactose metabolism were also significantly changed but with lower pathway impact (**Figures 2H, I**). Together, these data revealed that arachidonic acid metabolism was the most remarkable lipid metabolism disparity between M1 and M2 macrophages. Enhanced arachidonic acid metabolism could be a hallmark of M2 macrophages.

Arachidonic Acid and PGE2 Inversely Regulate M2 Polarization

Next, we aimed to investigate the impact of arachidonic acid metabolism on M2 polarization *in vitro*. By treating cells with arachidonic acid during polarization, we found that both surface markers (CD209 for THP-1 model, CD206 for BMDM model) and functional cytokines (IL-4, TARC) had been decreased by arachidonic acid, indicating that macrophage M2 polarization was suppressed (**Figures 3A–D**). In addition, the key enzymes associated with arachidonic acid metabolism are generally constitutively expressed and determine what eicosanoids a cell can synthesize. Our data revealed relative expression of lipoxygenases and cyclooxygenases in M2 macrophages (**Figure 2**), thus we tested how these enzymes link to M2 polarization. We found that inhibition of lipoxygenases (by PD146176 and MK886) decreased M2 polarization in a dose-dependent manner (**Supplementary Figures S5A, B**). Consistently, inhibition of cyclooxygenases by indomethacin (INDO) decreased M2 polarization, which was indicated by lower expression of IL-4, TARC and CD209, CD206 (**Figures 3E–H**). This suggested that metabolites of arachidonic acid may favor M2 polarization. Thus we assessed the expression of markers in the presence or absence of corresponding arachidonic acid metabolites. We tested several lipoxygenases related metabolites lipoxin A4 (LXA4), 15-hydroxyeicosatetraenoic acid (15S-HETE) and leukotriene B4 (LTB4). Neither of them affected macrophage M2 polarization in THP-1 model or BMDM model (**Supplementary Figures S5C, D**). However, the presence of an cyclooxygenases associated metabolite, PGE2, significantly promoted M2 polarization, as increasingly expressed M2 markers (IL-1RA, CD209, CD206) suggested (**Figures 3I–L**). In addition, all these metabolites inhibited M1 polarization (indicated by CCR7 expression) in THP-1 model (**Supplementary Figure S5E**) while only PGE2 inhibited M1 polarization (indicated by CD69 expression) in BMDM model (**Supplementary Figure S5F**), suggesting that PGE2 may determine the polarization of M1/M2 polarization. Collectively, these data indicated a critical role for arachidonic acid and its metabolic PGE2 in optimal M2 polarization of macrophages induced by IL-4/IL-13.

PGE2 Facilitates Macrophage M2 Polarization Through PPARγ Suppression

Previous studies had demonstrated that PPARγ were essential for M2 polarization (5), therefore we investigated the involvement of



Pathways	Raw p	Impact
Arachidonic acid metabolism	3.41E-05	4.1266
Galactose metabolism	0.000667	2.1911
Pyruvate metabolism	0.009294	1.9278
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	0.028655	1.25
Glycerolipid metabolism	0.032041	1.0763

Pathways	Raw p	Impact
Steroid biosynthesis	0.000299	0.44
Linoleic acid metabolism	0.007573	2.503
Caffeine metabolism	0.008327	0.34425
Valine, leucine and isoleucine biosynthesis	0.009761	0.6973
Arachidonic acid metabolism	0.015953	3.0852

FIGURE 2 | Arachidonic acid metabolism is enhanced in M2 macrophages. **(A)** Differentially expressed genes (DEGs) in M2 macrophages; fold changes are in comparison with M1 macrophages. Genes with fold change ≥ 2 or ≤ 0.5 and Padj ≤ 0.05 were seen as DEGs. **(B)** Enrichment plot for arachidonic acid metabolism in THP-1 derived M2 macrophages from Gene Set Enrichment Analysis (GSEA). **(C)** Heatmap of DEGs matching "arachidonic acid metabolism" expression signature according to KEGG Pathway Analysis of RNA-sequencing data from M0, M1, M2 macrophages with three biological replicates. **(D)** Protein expression of key metabolic enzymes for arachidonic acid *via* HCl in M0, M1, M2 macrophages. *P < 0.05; **P < 0.01. Error bars represent the mean ± SEM from three biological replicates. **(E, F)** Top 10 pathways of positive or negative ion mode from Metabolites Set Enrichment Analysis (MSEA). **(G)** Heatmap of differentially expressed metabolites matching "arachidonic acid metabolism" expression signature according to KEGG Pathway Analysis of lipidomics data from M1,M2 macrophages with six biological replicates. **(H, I)** Enrichment pathways from integrated transcriptomics and lipidomics data by Joint Pathway Analysis. Left panel: Enrichment plots. Right panel: corresponding information for left plots.

PPARγ in the molecular mechanism of macrophage polarization induced by arachidonic acid and PGE2. When treated with specific agonist for PPARγ, rosiglitazone (R), M2 marker (CD209) was decreased dose-dependently (**Figure 4A**). In contrast, CD209 was significantly enhanced by the inverse agonist T0070907 (T) dose-dependently (**Figure 4A**), suggesting that human macrophage M2 polarization might be closely associated with PPARγ de-activation. Functional cytokines secreted by M2 macrophages (IL-1RA and TARC) were correspondingly reduced by PPARγ activation and TARC was increased by PPARγ de-activation (**Figure 4B**). In consistent with THP-1 model, PPARγ activation by R inhibited

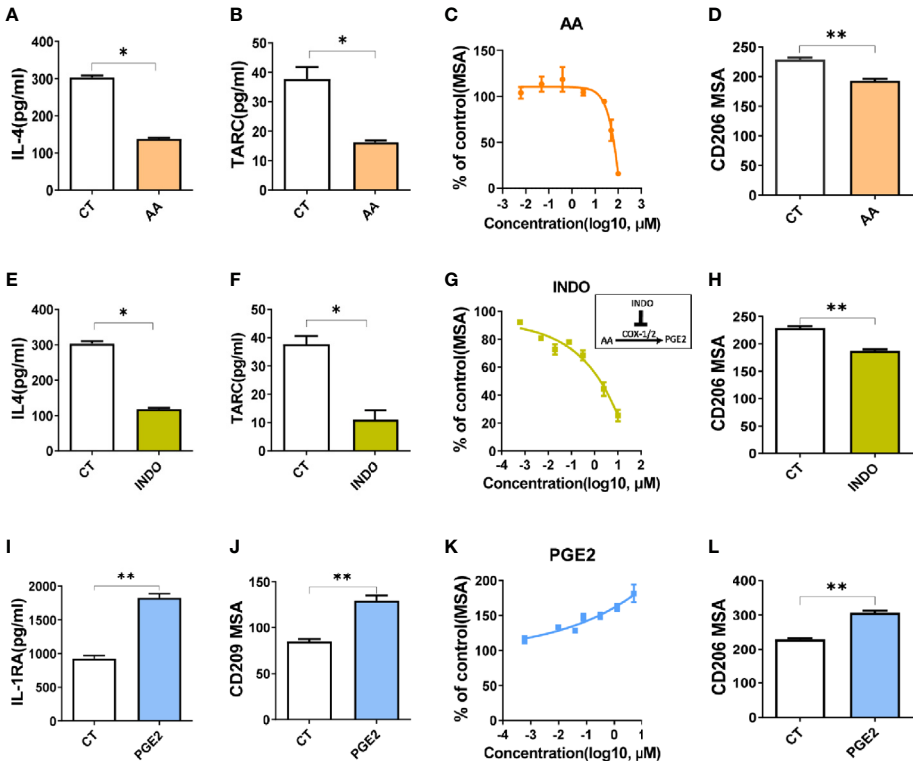


FIGURE 3 | Arachidonic acid and PGE2 inversely regulate M2 polarization. **(A, B, E, F, I)** Cytokines of THP-1 derived M2 macrophages treated with compounds for 48hours during polarization as indicated. **(C, G, K)** CD209 expression curves of THP-1 derived M2 macrophages with treatment as indicated *via* HCl **(D, H, L)** Analysis of CD206 expression of BMDM derived M2 macrophages *via* HCl with treatments as indicated. **(J)** Analysis of CD209 for THP-1 derived M2 macrophages treated as indicated during polarization *via* HCl. CT represents corresponding solvent control. Arachidonic acid (AA, 50 μM), indomethacin (INDO, 10 μM), prostaglandin E2 (PGE2, 10 μM). Error bars represent the mean ± SEM. Data presented are from three biological replicates. *P < 0.05; **P < 0.01.

macrophage M2 polarization in BMDM model as well (**Figure 4C**). These data suggested that PPARγ de-activation was critical for M2 polarization.

Since the inhibition effect of arachidonic acid on M2 polarization (**Figures 3A–D**) was similar to R, we presumed that arachidonic acid inhibited macrophage M2 polarization through activating PPARγ. To test this, we examined the polarization effect of arachidonic acid in the presence of T. When PPARγ de-activated by T, arachidonic acid could not inhibit M2 polarization while PPARγ activated by R could enhance the inhibition of arachidonic acid on CD209 expression, suggesting that PPARγ was involved in the effect of arachidonic acid and might be activated by arachidonic acid (**Figure 4D**). At the same time, we questioned whether PGE2 promoted M2 polarization by suppressing PPARγ activation. We found that PPARγ activation totally reversed M2 polarization mediated by PGE2 while PPARγ de-activation further enhanced M2 polarization mediated by PGE2 (**Figure 4E**). In addition, INDO shared a similar response with arachidonic acid when co-treated with R or T, suggesting that PPARγ was involved in the effect of INDO and might be activated by INDO (**Figure 4F**). To formally address the possibility of PPARγ in bridging arachidonic acid and/PGE2 mediated macrophage M2 polarization, we construct BMDM polarization model from wild type (WT, $Pparg^{+/+\Delta Mono}$) or monocyte specific PPARγ knockout (KO, $Pparg^{-/-\Delta Mono}$) mice. In WT model, M2 marker CD206 was significantly suppressed by arachidonic acid while increased by PGE2 (**Figures 4G, H**). However, in KO mice, when compared with WT, monocyte specific PPARγ knockout significantly abolished or dampened these effects on M2 polarization (**Figures 4G, H**), indicating that arachidonic acid and/PGE2 regulated M2 polarization in a PPARγ-dependent manner. Intriguingly, the suppression of CD206 by INDO was not abolished but was enhanced by monocyte specific PPARγ knockout, suggesting that INDO might have additional mechanisms besides PPARγ activation in regulating M2 polarization of macrophages (**Figure 4I**). Together, these data supported the proposal of a role for PPARγ in bridging arachidonic acid or PGE2 mediated macrophage M2 polarization.

PGE2 Enhances OXPHOS Through Suppressing PPARγ in Promotion of Macrophage M2 Polarization

On the basis of OXPHOS was enhanced in M2 macrophages (**Figure 1B**) and FAO fuels OXPHOS with acetyl-CoA (18), we

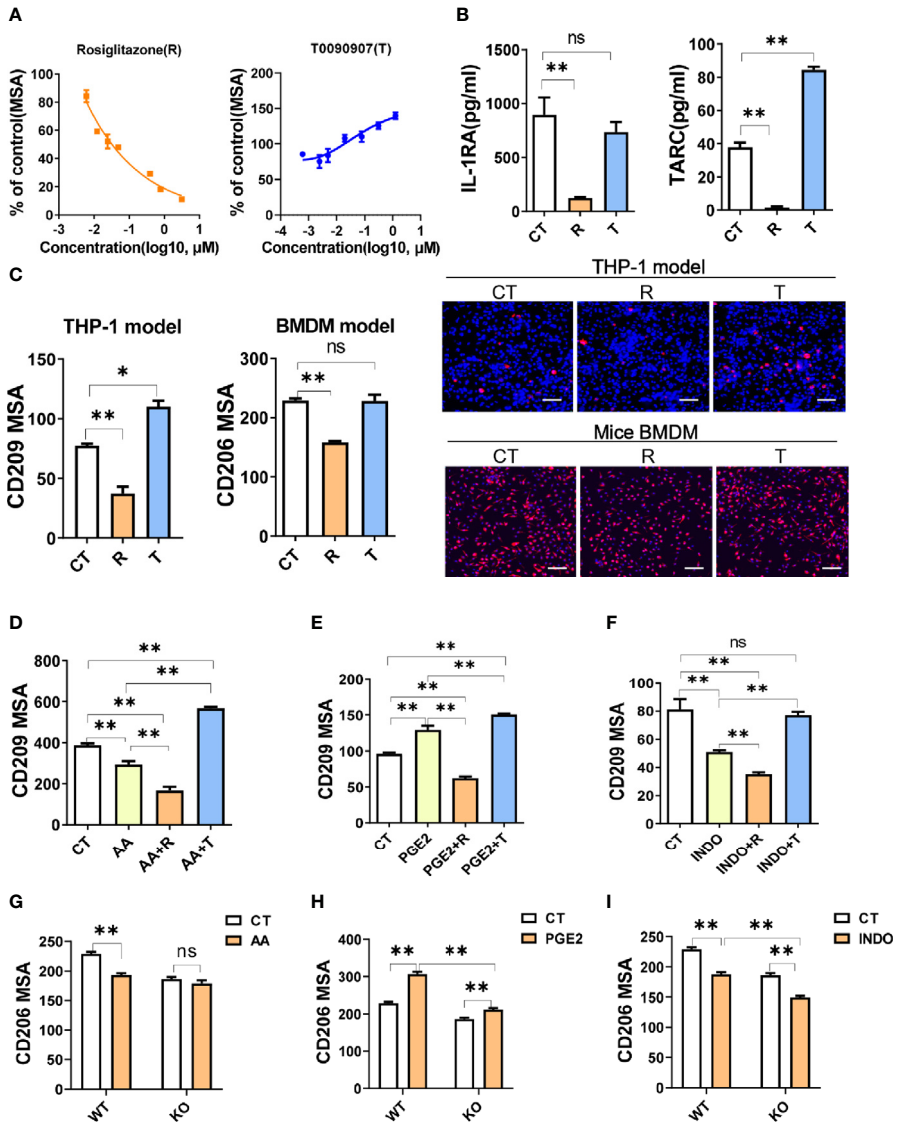


FIGURE 4 | PGE2 facilitates macrophage M2 polarization through PPARγ suppression. **(A)** CD209 expression curves of THP-1 derived M2 macrophages with treatments as indicated. **(B)** Cytokines of THP-1 derived M2 macrophages with treatment as indicated. **(C)** Protein expression of M2 markers (CD209 or CD206) from THP-1 or BMDM derived M2 macrophages with treatment as indicated. Right panel: representative images. Blue staining: nuclei; Red staining: CD209 or CD206. Scale bar: 100 μm. **(D–F)** Protein expression of CD209 in THP-1 derived M2 macrophages with treatment as indicated. **(G–I)** Protein expression of CD206 for BMDM derived M2 macrophages from wide type (WT) or monocyte specific PPARγ knockout mice (KO) with treatment as indicated. CT represents corresponding solvent control. Rosiglitazone (R, 10 μM), T0070907 (T, 1 μM), AA (50 μM), INDO (10 μM), PGE2 (2 μM). Error bars represent the mean ± SEM from three biological replicates. *P < 0.05; **P < 0.01; ns, no significance.

proposed that the polarization effects of PGE2 or AA might be attributed to these two processes. To test this, we conducted transcriptomic analysis of PGE2 treated macrophages in the induction of M2 polarization. Firstly, we explored the top 20 enriched pathways (ranked by normalized enrichment score, NES) by GSEA (**Figure 5A**). When considering false discovery rate q-value (FDR q value, usually no more than 0.25 was acceptable), the 11th pathway, *oxidative phosphorylation*, was the most significantly enriched pathway with highest NES (**Figure 5A** and **Supplementary Table S3**). The enrichment plot of OXPHOS suggests that PGE2 up-regulated OXPHOS remarkably in the induction of M2 polarization (**Figure 5B**). Further comparison on the OXPHOS associated DEGs demonstrated that genes related to mitochondria respiratory complex I (NDUFA8 et al.), II (SDHA), III (UQCRH et al.), IV(COX7A2 et al.), V (ATP5MG et al.) were exclusively increased in the induction M2 but not M1 polarization (**Figure 5C**). This was consistent with the enhancement of OXPHOS in M2 macrophages (**Figure 1C**). To explore whether PPARγ was involved in the enhancement of OXPHOS by PGE2, we tried to

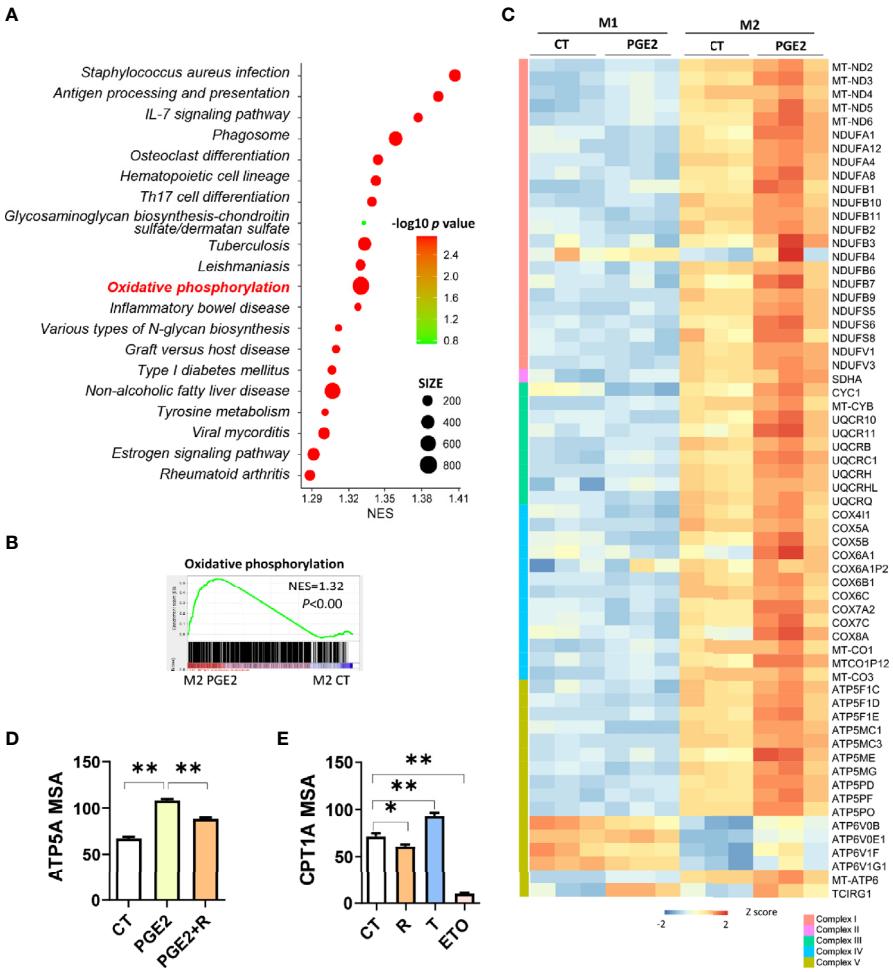


FIGURE 5 | PGE2 enhances OXPHOS through suppressing PPARγ in promotion of macrophage M2 polarization. **(A)** Enrichment pathways [TOP 20, ranked with normalized enrichment score (NES)] in THP-1 derived M2 macrophages with PGE2 treated during polarization. **(B)** Enrichment plot for oxidative phosphorylation in THP-1 derived M2 macrophages with PGE2 treated during polarization from GSEA analysis. **(C)** Heatmap for representative DEGs matching "oxidative phosphorylation" in PGE2 treated M1 or M2 macrophages. **(D)** Protein expression of ATP5A with treatment during polarization as indicated and detected *via* HCl. **(E)** Protein expression of CPT1A with treatment during polarization as indicated. PGE2 (2 μM), R (10 μM), T (1 μM), ETO (Etomoxir, 100 μM, serve as positive control for CPT1A inhibition). *P < 0.05; **P < 0.01.

block this effect by PPARγ activation. As expected, PGE2 dramatically increased the expression of ATP5A, one subunit of ATP synthase (complex V), while PPARγ activation weakened this effect significantly, suggesting that PPARγ was involved in the effect of PGE2 on OXPHOS (**Figure 5D**). In addition, given that FAO can serve as a replenishment pathway for OXPHOS and PPARγ is a key regulator for FAO, we next determine the involvement of PPARγ on FAO. CPT1A expression, a key enzyme for FAO, was significantly inhibited by PPARγ activation while was promoted by PPARγ de-activation (**Figure 5E**). This indicated that PPARγ activation inhibited FAO, thus might weaken OXPHOS. This finding was consist with a previous study that demonstrated the inhibition of OXPHOS by PPARγ activation (19). Collectively, these data revealed that PGE2 enhanced OXPHOS during M2 polarization and PPARγ de-activation was involved in this process.

Arachidonic Acid Metabolism Is Correlated With M2 Polarization in Tumor Microenvironment

The metabolic crosstalk between cancer cells and macrophages suggested that nutrients availability may play a role in immunosuppressive tumor microenvironment (20, 21). M2 type tumor associated macrophages (M2-TAMs) formation have been seen as results of tumor cell "re-education" (22). This suggests that tumor cells derived metabolites may have a role for M2-TAMs formation. In our previous study, we have validated M2-TAMs infiltration in esophageal carcinogenesis (17), thus we questioned whether arachidonic acid metabolism facilitated M2-TAMs polarization in esophageal cancer. Unsurprisingly, in mice ESCC, comparing with non-tumor tissue, several key metabolic genes in arachidonic acid metabolism *(Ptgs2, Cyp4a10, Cyp2b10, Hpgds* and *Alox8)* were

up-regulated in tumor tissues and M2 macrophages marker Arg1 was also increased in tumor tissues (**Figure 6A**), indicating a correlation between arachidonic acid metabolism and M2-TAMs formation. Simultaneously, using transcriptomics data from human ESCC, we also observed that many markers for M2 macrophages such as MRC1, CD209, CD163 and TREM2 were positively correlated to PGE2 biosynthesis (suggested by PTGES and PTGS1) of arachidonic acid metabolism (**Figure 6B**). Thus we next check the correlation between FAO/OXPHOS and M2-TAMs. Several FAO or OXPHOS associated genes (PPARGC1A, COX7A1, SDHA) positively correlated to markers of M2-TAMs (CD200R1, MRC1, CD209, CD163) (**Figure 6C** and **Supplementary Figure S6)**. This suggested the existence of OXPHOS related M2-TAMs formation. Consistently, PGE2 biosynthesis (suggested by PTGES3) and OXPHOS (suggested by UQCRH, COX7A2) were also positively correlated (**Figure 6D**). This was consistent with our *in vitro* observation and support our hypothesis (arachidonic acid metabolism facilitates M2-TAMs polarization in esophageal cancer) well. In addition, by calculating the correlation between PTGS1/PTGS2 and PPARG, we found that PGE2 biosynthesis was negatively correlated to PPARG (**Figure 6E**), which indirectly supported that PGE2 suppressed PPARG. Together, these findings suggest that arachidonic acid metabolism might make contribution to M2-TAMs formation *via* PPARγ-OXPHOS modulation, thus promote tumor progression.

DISCUSSION

Distinct metabolic characteristics help macrophages with particular function during phenotype polarization (13). Lipid mediators are key fatty acid metabolites involved in this process (13), serving as important signals. In the present study, we demonstrate that arachidonic acid metabolism is up-regulated in the induction of macrophage M2 polarization. Arachidonic acid inhibits IL-4/IL-13 stimulated M2 polarization of macrophages. PGE2, an essential metabolite generated from arachidonic acid metabolism, promotes macrophage M2 polarization through inhibiting PPARγ. Contrary to PGE2, inhibition of arachidonic acid metabolism suppresses M2 macrophage polarization. Our data elucidates a previously unappreciated mechanism of Arachidonic acid metabolic PGE2 to regulate macrophage alternative activation through inhibiting PPARγ. This inhibition effect facilitates OXPHOS by enhancing FAO pathway. The newly uncovered connection between

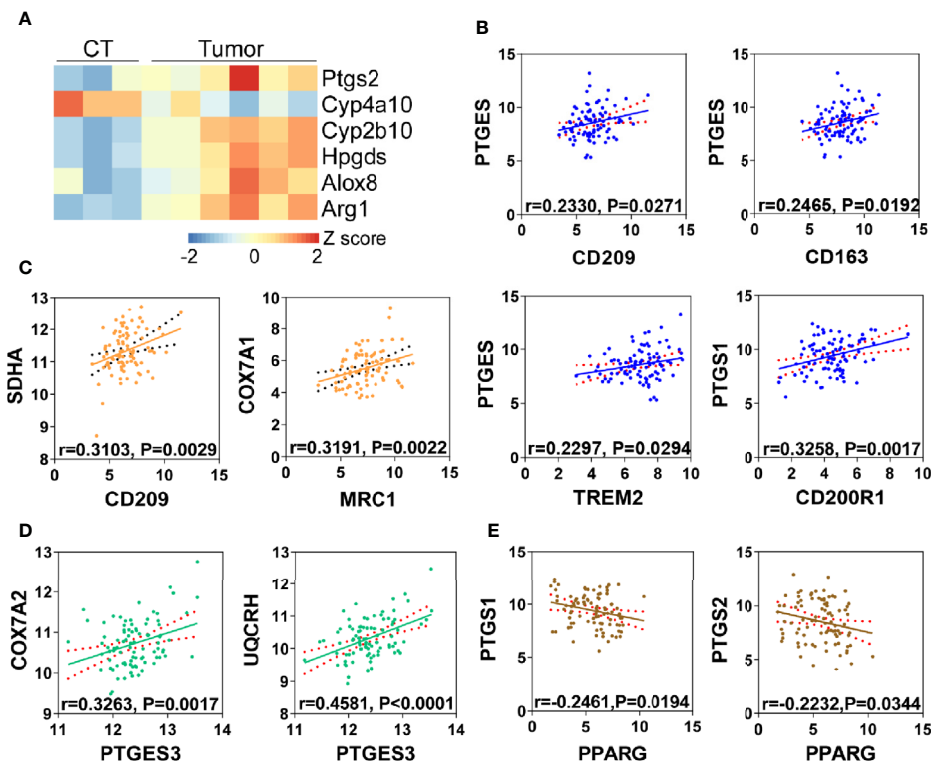


FIGURE 6 | Arachidonic acid metabolism is correlated with M2 polarization in tumor microenvironment. **(A)** Heatmap of differentially expressed genes matching "arachidonic acid metabolism" from mice ESCC or corresponding control tissues (CT). Arg1 was used as M2 macrophages marker. **(B)** Correlation analysis of PGE2 biosynthesis (PTGES, PTGS1) and M2 macrophages (CD209, CD163, CD200R1, TREM2) in human ESCC from TCGA database. **(C)** Correlation analysis of OXPHOS (COX7A1, SDHA) and M2 macrophages (CD209, MRC1) in human ESCC from TCGA database. **(D)** Correlation analysis of oxidative phosphorylation (COX7A2, UQCRH) and PGE2 biosynthesis (PTGES3) in human ESCC from TCGA database. **(E)** Correlation analysis of PPARG and PGE2 biosynthesis (PTGS1, PTGS2) in human ESCC from TCGA database.

arachidonic acid metabolism and macrophage alternative activation is briefly outlined in **Figure 7**. This metabolic regulation of macrophage polarization will affect many physiological and pathological processes eventually.

Metabolism intricately links to immune homeostasis, which largely reflects by polarization of immune cells including macrophages towards differential phenotypes. Like any physiological process, polarization of immune cells requires energy as well as the availability of nutrients, metabolites and oxygen (23). Macrophages in a nutrient deprivation or hypoxia microenvironment acquire distinct phenotypes with those in perivascular areas (24), suggesting an essential role of oxidative metabolism of nutrients. Mitochondrial OXPHOS has been widely accepted as characteristic and necessity for M2 macrophages (25, 26). This biological process is essential in M2 macrophages for ATP and biosynthetic output. Roisin et al. has reviewed how oxidative metabolism controls immune cell function (27). Inhibition of OXPHOS by reducing substrates or inhibiting mitochondrial complex had been found to suppress M2-related genes (Arg1, Mrc1) and surface marker (CD206) (26, 28). In line with these observations, we found that OXPHOS directly control macrophage alternative activation (M2 polarization).

Mitochondria utilizes pyruvate from glucose metabolism, α-KG from glutamine metabolism as well as acetyl-CoA from FAO to feed Krebs cycle and drive OXPHOS. It has been known that in M2 macrophages, FAO fuels OXPHOS thus provides a crucial energy source for M2 polarization (25, 26). Pharmaceutically blockade of FAO diminishes immune function of M2 macrophages or dampens M2 polarization (25) and favors M2-to-M1 repolarization (29), suggesting an essential role of FAO in macrophage M2 polarization. Our data also verify its contribution on M2 polarization by FAO inhibitor, which significantly decreased CD209 dose-dependently. Due to PPARs, especially PPARγ, have been extensively investigated as essential nuclear receptors for macrophage M2 polarization and in lL-4 stimulated M2 polarization, PPARγ and PGC1β are well known regulators of FAO (26, 30), we speculated that PPARγ-regulated FAO might have a role in M2 polarization. Interestingly, our data revealed that PPARγ negatively regulated CPT1A (an important enzyme for FAO) during M2 polarization, suggesting an inhibition of FAO by PPARγ. Collectively, these data demonstrate that FAO regulated by PPARγ are involved in M2 polarization.

In our study, PGE2 was found to dramatically promote M2 polarization and enhance OXPHOS during this process, revealing a possibility that PGE2 promotes macrophage M2 polarization through enhancing OXPHOS. On the basis of previous studies, PPARγ regulated FAO may be involved in this process (31). Besides, our results show that PGE2 directly suppress PPARγ expression and transcription activity (**Supplementary Figure S7**). Therefore, it's conceivable that inhibition of PPARγ to favor FAO can enhance OXPHOS and lead to the promotion effect of PGE2 in M2 polarization. Consistently, PPARγ activation might be a potential mechanism of arachidonic acid in inhibiting M2 polarization. In addition, PGE2-EP4 signaling has been reviewed as a possible mechanism of M2 polarization (32). However, we found that PGE2 promoted M2 polarization even though EP4 was blocked (**Supplementary Figure S8**). This suggests the existence of EP4-independent mechanisms under PGE2 mediated M2 polarization. Therefore, we believe that this PPARγ dependent mechanism is the main contributor in PGE2 mediated M2 polarization.

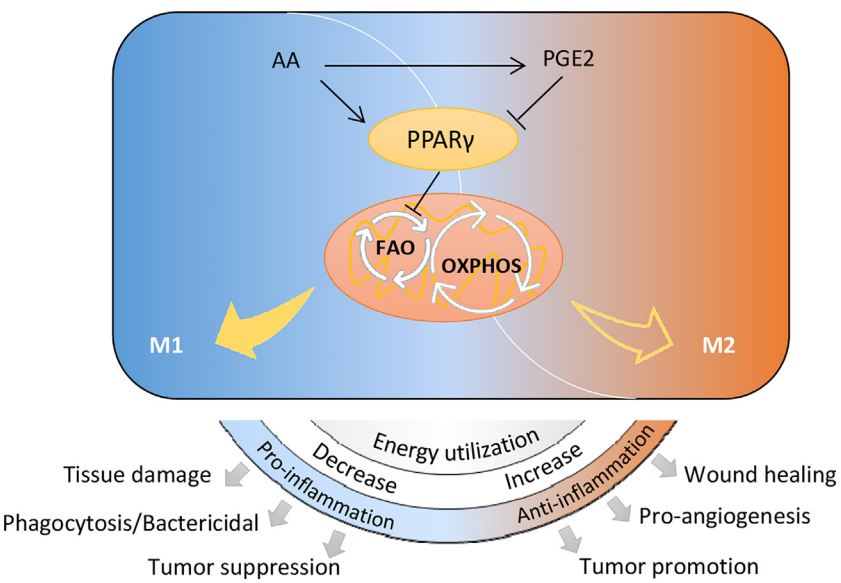


FIGURE 7 | Schematic diagram of arachidonic acid metabolism in controlling macrophage polarization and biological processes.

As for the role of lipolysis on macrophage M2 polarization, previously studies reported that lipolysis inhibition suppressed M2 polarization due to reduced FAO (25) or blocked PGE2 biosynthesis (33). However, the target of JZL184, monoacylglycerol lipase (MAGL), can metabolize 2-arachidonoylglycerol into arachidonic acid (34), thus JZL184 can decrease arachidonic acid intracellular biosynthesis. Based on our data that arachidonic acid inhibited M2 polarization through activating PPARγ, the promotion effect of JZL184 on M2 polarization might be attributed to arachidonic acid reduction. In addition, it should be note that large amount of fatty acids and lipid mediators are pan-agonist for PPARs, thus PPARα and PPARδ may be also involved in arachidonic acid metabolism regulated M2 polarization. We have found that PPARα was not influenced by arachidonic acid or PGE2 (**Supplementary Figure S9A**) while PPAR response element (PPRE) and CD36 could be activated by PGE2 (**Supplementary Figures S9B, C**), suggesting the involvement of PPARs other than PPARα. The role of PPARδ might be also involved in PGE2 mediated macrophage polarization (35, 36). There is a need to clearly compare polarization effects between PPARs and their contribution to PGE2 mediated macrophage polarization. Besides, many other metabolites could also be produced by arachidonic acid metabolism, the polarization effect of this pathway may be more complex beyond our data suggest. Further investigations are needed to clearly clarify our findings.

The identification of PGE2 as a key player for macrophage M2 polarization adds a metabolic explanation for how tumor polarize infiltrated macrophages towards an immunosuppressive M2 type. Previous studies have demonstrated that PGE2 promotes TAMs formation in glioblastoma (33), colorectal cancer (35), ovarian cancer (37), neuroblastoma (38) and prostate cancer (39). Based on the accumulation of macrophages (40) and differential expression of COX-2 in ESCC (41), it could be inferred that above correlation may also exist in the TME of ESCC. Our data supported this opinion and also suggested that besides tumor cell-derived PGE2, macrophage-derived PGE2 may also make contribution to M2-TAMs polarization through PPARγ-OXPHOS pathway. In addition, this correlation may generally exist in many other physiological/pathological conditions. Some *in vivo* experiments have revealed that PGE2 administration promoted M2 macrophage polarization. In a xenograft mouse model of colorectal cancer, PGE2 (17.6 μg/kg/d) treatment increased $CD206^+$ M2 macrophages in TME (42). However, in a mice asthma model, although PGE2 (0.4 mg/kg) administration decreased Ym-1 (M2 marker), it did not affect CD206 expression (43). This uncertain polarization effect is possibly due to the sophisticated responses of various cell types that can be affected by free PGE2 in the lung. These findings indicate that metabolic production of PGE2 may serve as a potential target for the prevention of many macrophages associated diseases including cancers. Reduction of PGE2 by pharmacological inhibition of COX-1/COX-2 may benefit cancer prevention. INDO, a non-specific COX-1/COX-2 inhibitor, has shown chemo-preventive and chemotherapeutic efficacy on colorectal cancer (44).

In summary, we identified that arachidonic acid metabolism notably impact macrophage M2 polarization induced by IL-4/IL-13 through regulating PPARγ and OXPHOS. As one of the key metabolite of arachidonic acid, PGE2 plays a crucial role in promoting macrophage polarization by the inhibition of PPARγ and enhancement of OXPHOS. Our finding renews the current understanding about the functions of arachidonic acid and metabolic PGE2 as immune regulators.

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 Institutional Animal Care and Use Committee of China National Center for Food Safety Risk Assessment.

AUTHOR CONTRIBUTIONS

MX: Writing-Original Draft preparation, Methodology, formal analysis, investigation. XW: Validation, investigation. YL & XG: investigation. XJ: Supervision, Funding acquisition LZ: Supervision, Project administration. HY: Conceptualization, Data Curation, Resources, Writing - Review & Editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.618501/full#supplementary-material

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Significance of PD1 Alternative Splicing in Celiac Disease as a Novel Source for Diagnostic and Therapeutic Target

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Background: We have focused on the alteration of the PD-1/PD-L1 pathway in celiac disease and discussed the roles of the PD1 pathway in regulating the immune response. We explored the idea that the altered mRNA splicing process in key regulatory proteins could represent a novel source to identify diagnostic, prognostic, and therapeutic targets in celiac disease.

Methods: We characterized the PD1 mRNA variants' profile in CD patients and in response to gluten peptides' incubation after *in vitro* experiments. Total RNA from whole blood was isolated, and the coding region of the human *PD-1* mRNA was amplified by cDNA PCR.

Results: PCR amplification of the human PD-1 coding sequence revealed an association between the over-expression of the sPD-1 protein and the PD-1Δex3 transcript in celiac disease. Thus, we have found three novel alternative spliced isoforms, two of which result in a truncated protein and the other isoform with a loss of 14 aa of exon 2 and complete exon 3 (Δ3) which could encode a new soluble form of PD1 (sPD-1).

Conclusions: Our study provides evidence that dietary gluten can modulate processes required for cell homeostasis through the splicing of pre-mRNAs encoding key regulatory proteins, which represents an adaptive mechanism in response to different nutritional conditions.

Keywords: PD1/PDL, celiac disease, alternative splicing, gluten peptides, immune checkpoint

INTRODUCTION

Alternative splicing is the process whereby the cell obtains different proteins from a single gene. Deregulations of the alternative splicing process are associated with the appearance of various aberrant splicing isoforms that may have pathological potential (1). Changes in the splicing pattern can lead to obtaining or losing a domain that could be critical in protein formation, resulting in an alteration in protein stability and/or subcellular localization (2). The isoforms generated from

splicing can have similar, different, and even antagonistic functions, depending on the extent of the changes in the open reading frame, which involves the regulation of gene function. Alternative splicing events play critical roles in immune responses and have been documented in the immune system (3).

Celiac disease (CD) can be classified as an autoimmune disease, influenced by genetic, environmental, and immunological factors (4, 5). It is characterized by an abnormal immune response to prolamins from wheat and other cereals, such as rye and barley, which affects genetically susceptible people. The classic form of CD is manifested by an intestinal injury with villous atrophy, crypt hyperplasia, and inflammatory cell infiltrate (6, 7). Gluten peptides with immunogenic characteristics can trigger both an innate and an adaptive immune response, leading to the clinical and histological manifestations of CD (8). In the pathogenesis of CD, the CD4+ T cells of the lamina propria are the central element, which recognize gliadin peptides modified by the enzyme transglutaminase 2 (TG2), with HLA-DQ2/DQ8 restriction, releasing cytokines involved in inflammation and the development of histological disorders (9, 10).

Gluten is the main compound of wheat proteins and is rich in proline and glutamine residues, which makes it resistant to digestion at the gastrointestinal level. The partial digestion of gluten produces small peptides that cause autoimmune disease in celiac patients (11). Gliadins constitute the most immunogenic fraction of gluten, containing the main stimulating epitopes of T cells DQ2.5-glia-1 (PF/YPQPQLPY), DQ2.5-glia-2 (PQPQLPYPQ), and DQ2.5-glia- 3 (FRPQQPYPQ) (12, 13). The main epitopes are present on the 33-mer peptide, which is the main contributor to gluten immunogenicity (14). Gluten proteins' immunogenicity is the result of canonical epitopes and their variants, so natural substitutions of canonical epitopes may contribute to wheat toxicity, with some being more abundant than the canonical epitopes (14). Gluten proteins have different immunogenic potential and are found in variable proportions among cereal species. For these reasons, it should be considered that amino acid substitutions in the variants of these epitopes can increase, reduce, or suppress CD response (14).

In previous studies, our research group used RNA interference technology (RNAi) to reduce the expression of specific gliadin fractions and immunogenicity with down-regulation of immunodominant peptides (15). Silencing of specific prolamin fractions by RNAi resulted in significant differences in the peptide composition and the number of peptides per protein identified in the PT-digested flour (16).

Programmed cell death-1 (PD-1) is a molecule member of the CD28 family that is expressed in T, B, and myeloid cells and, when activated, is released into blood plasma as soluble PD1 (sPD1) form (17). PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), which are expressed in a variety of cells (18, 19). Interaction between PD-1 and its ligands results in a diverse range of pathological effects in T-cell activation, T-cell tolerance, and immune-mediated tissue damage. The axis PD-1 and its ligands have already been observed with an important role in autoimmune disease regulation (20).

Nielsen et al. (21) reported the presence of five splicing isoforms, including an exon 3-skipped PD-1(PD-1Δ3). Exon 1 encodes a short signal sequence; exon 2 encodes an Ig domain; exon 3 is made up of the stalk and transmembrane domains; exon 4 encodes a sequence of 12 aa that marks the beginning of the cytoplasmic domain; and exon 5 contains the C-terminal intracellular residues and an untranslated region (21). The PD-1Δ3 isoform, with the loss of the exon 3 that encodes the membrane domain, is secreted as a soluble form; whose function will be different, or even antagonistic, to membrane form (21). The presence of soluble forms increases the diversity and complexity of the PD-1/PDL pathway, because soluble forms can represent contributory factors to immune homeostasis and can mediate immunological diseases (22). The modulation of inhibitory and stimulatory pathways may represent the best strategy for treating autoimmune diseases (20).

Only few studies report the role of PD-1/PD-L1 in intestinal inflammation (23, 24). PD-1 is highly expressed on T cells in inflamed colon from IBD patients, and blockade of PD-L1 suppressed experimental colitis (14). The role of PD-L1 in gut mucosa tolerance is known, engagement of PD-1 on T cells by PD-L1 inhibits the activation and proliferation of effector T cells, inducing production of IFN-gamma and IL17A cytokines. PD-L1 expression is normally upregulated during inflammation to prevent overt tissue damage (25).

Previously, we focused on the alteration of the PD-1/PD-L1 pathway in celiac disease and discussed the role of the PD1 pathway in regulating immune response in celiac disease autoimmunity. Increased levels of soluble PD1 are found in patients with celiac disease, when compared to healthy controls, while the sPD1 level is significantly higher than the expression of the PD1 membrane isoform in CD patients (26).

The *PDCD1* gene has been a candidate gene in polymorphism studies (27, 28). Several single-nucleotide polymorphisms (SNPs) have been identified: such as, PD1.1 (rs36084323), PD1.3 (rs11568821), PD1.5 (rs2227982), and PD1.9 (rs2227981), which affect the inhibitory functions of the PD-1 receptor (29). An important function of PD-1 in maintaining the peripheral self-tolerance and prevention of autoimmunity is further supported by the reported association between single nucleotide polymorphisms (SNPs) in the human PD-1 gene with susceptibility to systemic lupus erythematosus (SLE) (27, 28) and rheumatoid arthritis (27, 29). It is not yet clear if these SNPs in *PDCD1*gene are a cause of or simply additional to PD-1 function.

One of the objectives of this study was to investigate the role that PD1 and its ligands have in regulating autoimmunity in celiac disease. Up to now, PD1 variants expression in human leucocyte populations has not been explored in celiac disease; it remains possible that expression pattern of alternative PD1 molecules in persons with CD contributes to its dysregulation as this protein is key in inflammation and autoinmune response. To explore this issue in this study, we characterized the PD1 mRNA variants' profile in CD in response to gluten peptide incubation for *in vitro* experiments. We hypothesized that the presence of a particular SNP genotype may destabilize the PD1 pre-mRNA as it occurs in other human diseases. The genetic

association study was conducted to investigate the possible associations between PD1 single nucleotide polymorphisms (SNPs) and celiac disease in a Spanish population. In summary, we have analyzed the relationships between dietary factor (gluten), PD-1/PD-L axis, genetic polymorphism of PD1 gene and the functions in celiac disease, finding differences in reactivity of individuals to gluten peptides.

EXPERIMENTAL SECTION

Ethics

The study protocol was approved by the local Ethics Committee of the Virgen de las Nieves Hospital (Granada, Spain) and carried out in accordance with the Helsinki Declaration. Written consent was obtained from the parents or legal guardians of the children involved for the publication of any potentially identifiable images or data included in this article.

Patients

This study included patients with active celiac disease with gluten intake (n = 25) (**Table 1**) and healthy controls (n = 5, 60% female, 40% male, age range 7–12 years). The diagnosis of CD was determined by serological testing using anti-endomysial antibodies (AAEMs) and anti-tissue transglutaminase antibodies (AATGs), by typing of the CD-specific human leukocyte antigen (HLA), with confirmation of the disease by small intestine biopsy according to the Marsh classification (30).

Peripheral Blood Mononuclear Cell Isolation and Stimulation

PBMCs were isolated from 6 ml of heparinized blood by Histopaque gradient centrifugation (Sigma Aldrich, Saint Louis, MO, USA) and cultured at a density of 1×10^6 cells/ml in RPMI-1640 culture medium (GIBCO, Grand Island, NY, USA Gibco, Thermo Scientific, Madrid, Spain) supplemented with 10% fetal bovine serum (GIBCO), 1% penicillin–streptomycin, and 0.1% gentamicin (Sigma Aldrich).

Gluten peptides were obtained by using combinations of different RNAi fragments designed to target d γ-gliadin fraction and glutenins. The RNAi lines and transformation vectors used were previously reported (31). Lines were obtained by using combinations of different RNAi fragments designed to target different gliadin fractions—the ω-, α- and γ-gliadin. After silencing by RNAi, there is a variation in the number of peptides per protein and its composition. In our study, we used the 33-mer peptide, as it is the main immunodominant toxic peptide for celiac patients, the wild-type line BW208 (BW), and the D623 line, as this has a higher number of peptides per protein than the wild-type line BW208, and shows an increase in α-gliadin peptides. After 48 h, the PBMCs were incubated with 10 μg/ml PHA (GIBCO) (positive T cell stimulation) for 24 h, and peptides (50 μg/ml) of cell medium

TABLE 1 | Clinical data of celiac patients.

Patient	Weight (kg)	High (Cm)	Atrophy grade	AAG	AEMA	ATGA	HLADQB1	HLADR
Age Range: Babies 0–2 years (56%)								
Celiac 1	9.70	80	4	30.8	1/160	28	0303,0601	7,15(2)
Celiac 2	9.67	79.5	4	68.4	1/160	38	0201,0202	3,7
Celiac 3	7.8	75	3	12	1/160	38	0302,0301	4,4
Celiac 4	9.7	74	4	199	1/160	18	0201,0202	3,7
Celiac 5	7.40	68.5	4	200	1/320	120	0201,0202	3,7
Celiac 6	13	89	4	30	1/160	28	0201/0101	3,7
Celiac 7	11.5	80.5	1	7.55	1/160	20	0201/0202	3,11
Celiac 8	8	76.5	4	200	1/320	90	0201/0202	3,7
Celiac 9	11.3	90	4	111	1/320	118	0201/0202	3,7
Celiac 10	14	92	4	49	1/5	70	0301/0202	11(5),7
Celiac 11	6.6	65	4	2.23	1/5	20	0502/0604	13,15(2)
Celiac 12	12.8	89.5	4	36	1/360	95	0201/0301	7,11
Celiac 13	11.5	83	1	55	1/320	90	0301/0501	11,10
Celiac 14	7.50	66	4	60	1/320	27.70	0201,0501	1,3
Children 3–12 years (44%)								
Celiac 15	13	95	3	65	1/80	25	0302/0301	4,4
Celiac 16	12.30	88	4	200	1/320	90	0201,0501	1,3
Celiac 17	21.5	118	4	19.2	1/160	28	0201,0301	3,11(5)
Celiac 18	26.5	128	4	102	1/320	97	0202,0301	7,11(5)
Celiac 19	14.3	100.4	3	31.5	1/160	28	0301,0302	4,11
Celiac 20	26	130	4	23.5	1/320	165	0202,0301	7,11(5)
Celiac 21	27	125	3	100	1/320	111	0201/0202	3,7
Celiac 22	11.5	89.5	4	144	1/5	10	0502/0602	15,16
Celiac 23	10.6	79	4	20.1	1/5	50	0201/0503	3,14(6)
Celiac 24	23	126	4	19.3	1/20	26	0201/0303	4,13(6)
Celiac 25	12.3	88	4	200	1/320	90	0201,0501	1,3

AAG, anti-gliadin antibody expressed as mg/L; AAEM, anti-endomysial antibody; AATG, anti-transglutaminase antibody expressed U/ml.

were added in each well to stimulate lymphocytes. Supernatants were collected and stored at −80°C for later analysis.

IFN-γ Production

The PT-digested protein extracts were used to study immunogenic potential by assaying IFN-γ release for *in vitro* assays. The supernatants from PBMC culture were collected after 24 h of peptide incubation and stored at −80°C for IFN-γ determination using a commercial ELISA kit in accordance with the manufacturer's instructions (Thermo Fischer Scientific, Waltham, MA, USA Thermo Scientific, Madrid, Spain). The sensitivity of the assay was <2 pg/ml.

Cell Proliferation Analysis

The BrdU Cell Proliferation Assay Kit (Millipore Chemicon, Temecula, CA, USA) for the *in vitro* quantitative detection of newly synthesized DNA from actively proliferating cells was used in PBMC cultures after 48 h of incubation with the different types of peptides. The stimulation index (SI) was calculated by dividing the mean absorbance by 10 at a wavelength of 450 nm after stimulation by the mean absorbance of T cells exposed to the culture medium alone (negative control), then divided by 10. The proliferation of PBMCs was expressed as the mean fluorescence intensity.

RNA Isolation and cDNA Synthesis

Total RNA from whole blood was isolated using QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of isolated RNA was measured using a NanoDrop Lite spectrophotometer (Thermo Scientific, Madison, WI, USA). Total RNA was converted into single-strand cDNA using a Maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific). The quality of the cDNA was confirmed by PCR using human *β-actin* gene specific primers: Actin-F (5′-ATCTGGCAC CACACCTTCTAC AATGAGCTGCG) and Actin-R (5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC).

PD-1 PCR Amplification, Fragment Cloning, and Sequencing

Amplification of the coding region of the human *PD-1* mRNA was performed by cDNA PCR using the primers PD-1-F (5′-GCGGCCAGGATGGTTCTTA) and PD-1-R (5′-TACTCCGTCTGCTCAGGGA) (**Supplementary Material**). The PCR program consisted of an initial denaturalization step at 94°C for 5 min and 40 cycles with 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, with a final extension of 5 min at 72°C. The amplified fragments were analyzed by electrophoresis in 2% agarose gels, eluted, and cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA). Recombinant plasmids were sequenced on both strands by the dideoxy sequencing method.

Genotyping the PD-1.3 and PD-1.5 Polymorphisms of the PD1 Gene

Two single nucleotide polymorphisms (SNPs) were analyzed in CD patients (n = 76) and healthy controls (n = 59) by the PCR-based RFLP restriction fragment length polymorphism method (**Table 2**). The first, (rs11568821), called PD-1.3, is located within intron 4 of the gene. The second, (rs2227981), called PD-1.5, is located within exon 5 of the gene.

Genotyping of the PD-1.3 was analyzed following Hoffmann et al. (32). A 331 bp containing the SNP was amplified by PCR using the primers PD1.3-F and PD1.3-R (**Figure 1A**). The PCR reactions were performed using the DreamTaq Green PCR Master Mix 2× (Thermo Fischer Scientific) using 50 ng of genomic DNA and 10 pmol of each primer in a 25 µl mixture. The PCR cycling profile was as follows: initial denaturation at 95°C (4 min), 35 cycles at 95°C (30 s), 60°C (1 min), 72°C (30 s), with a final elongation step of 72°C for 5 min. Aliquots of 5 µl of the PCR reaction mixture were analyzed by electrophoresis in 2% agarose gels to test the DNA amplification. Aliquots of 10 µl of the PCR reaction mixture were digested by adding five units of the restriction endonuclease *Pst*I (Sigma Aldrich) in a 40 µl reaction mixture that also contained 50 ng of the pUC19 plasmid. This plasmid was used as an internal control in the PCR product digestion. Only digestions with totally linearized plasmid were considered (**Figure 1B**).

For genotyping the PD-1.5 polymorphism, a 333 bp fragment containing the SNP was amplified by PCR using the primers PD1.5-F and PD1.5-R (**Figure 1C**). The PCR reactions were performed using the same conditions as for the PD-1.3 polymorphism. Aliquots of 10 µl of the PCR reaction mixture were digested by adding five units of *Alu*I (Sigma Aldrich, Saint Louis, MO) in a 40 µl reaction mixture. Then 50 ng fragment of the 16S rRNA gene of the ant *Messor structor* was added as an internal control for the digestion (34). When digested, this fragment produced two bands of 403 and 135 bp (**Figure 1D**). Only digestions where both bands were visible were considered.

PD1/PDL1 and PDL2 Immunohistochemistry

Formalin-fixed paraffin-embedded biopsies from patients with active CD and control subjects were analyzed. About 4 µm thick sections were cut, deparaffinized, and rehydrated. Sections were microwaved in 10 mM of citrate buffer (pH 6.0) for antigen retrieval

TABLE 2 | Analysis of the PD1 SNP polymorphisms by PCR-RFLP.

SNP	PCR primers[1]	Restriction endonuclease	Generated fragments
PD1.3 G/A (rs11568821)	PD1.3-F CCAGGCAGCAACCTCAAT	*Pst*I	G, 331 bp
	PD1.3-R GTCCCCCTCTGAAATGTCC		A, 276 bp, 55 bp
PD1.5 C/T (rs2227981)	PD1.5-F AGACGGAGTATGCCACCATT	*Alu*I	C, 249 bp, 84 bp
	PD1.5-R CACTGTGGGCATTGAGACAT		T, 180 bp, 69 bp, 84 bp

[1]*Primers for PD1.3 were taken from Hoffmann et al. (32) and for PD1.5 from Ferreiros-Vidal et al. (33).*

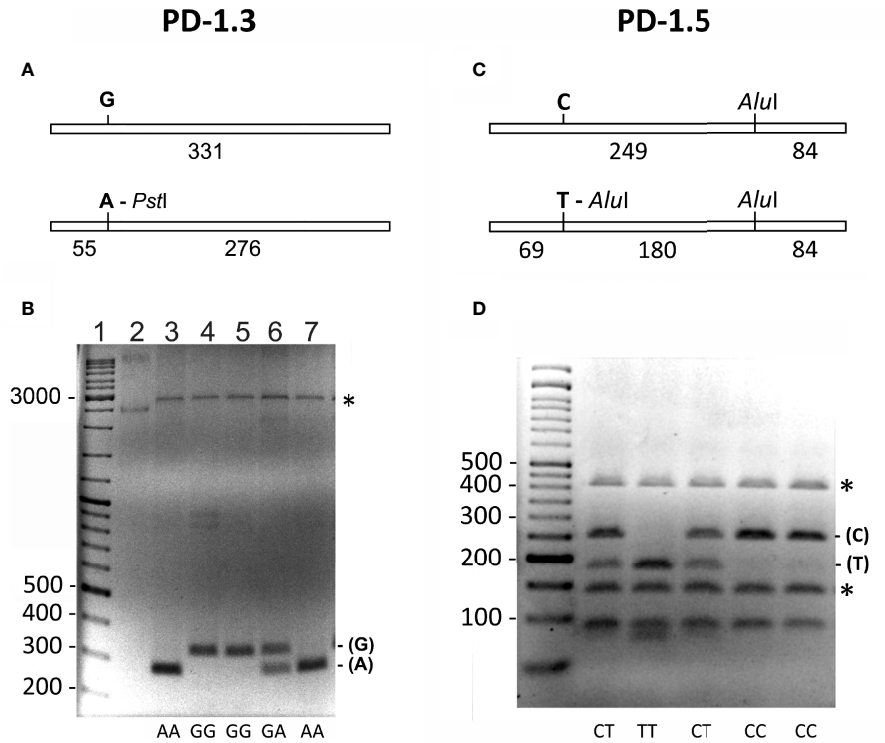

FIGURE 1 | **(A, B)** Genotyping of the (G/A) PD-1.3 polymorphism of the PD1 gene. **(A)** Schematic representation of the 331 bp PCR amplified fragment containing the SNP. The presence of A in the SNP generates a target for the restriction endonuclease *Pst*I that generates two fragments of 55 and 276 bp. **(B)** Electrophoresis of the *Pst*I digested PCR products on a 2% agarose gel, showing the different banding patterns obtained for each genotype (AA, GG and GA). Lane 1 = DNA size marker. Lane 2 = undigested plasmid used as an internal control of the *Pst*I digestion. The numbers on the left indicate the size of DNA fragments in bp. Asterisk marks linearized form of the plasmid used as internal control for the *Pst*I digestion. **(C, D)** Genotyping of the (C/T) PD-1.5 polymorphism of the PD1 gene. **(C)** Schematic representation of the 333 bp PCR amplified fragment containing the SNP. The presence of T in the SNP generates an additional target for the restriction endonuclease *Alu*I that generates three fragments of 69, 180 and 84 bp. **(D)** Electrophoresis of the *Alu*I digested PCR products on a 3% agarose gel, showing the different banding patterns obtained for each genotype (CC, CT and TT). First lane is the DNA size marker. The numbers on the left indicate the size of DNA fragments in bp. Asterisk marks the two fragments generated by the DNA used as an internal control for the *Alu*I digestion.

and cooled in phosphate-buffered saline. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were then treated with the protein-blocking agent, incubated with the primary antibody, followed by the biotinylated secondary antibody and the Streptavidin–Biotin Universal detection system (Immunotech, Marseilles, France). We used anti-human PD1, anti-human PDL1, and anti-human PDL2 antibodies (Abcam, Cambridge, UK). Negative control experiments were performed by incubating sections with isotype-matched IgG1.

ELISA Quantification of sPD1/sPDL1 and sPDL2

We determined the levels of soluble PD1, PDL1, and PDL2 in serum of active celiac and non-celiac patients using a commercial ELISA kit in accordance with the manufacturer's instructions (Sigma-Aldrich) (Invitrogen, Carlsbad, CA, USA) (Thermofisher Scientific). For each assay, the standards as well as the samples were tested in duplicate. To estimate the cytokine concentration (pg ml^{-1}). The sensitivity of the assays was <2 pg/ml. The concentrations of sPD1, sPDL1, and sPDL2 were determined from the optical density according to standard curves.

Statistical Analysis

Statistical analysis of the data was done using Statgraphics software (StatPoint Technologies, Warrenton, VA, USA). The chi-square test was used for comparison of qualitative data. Quantitative data were expressed as a median, and the Mann–Whitney U test was used as a test of significance for comparing two groups. The SNPs were tested for Hardy–Weinberg equilibrium, then their genotypic and allelic disease association analysis was performed. For all tests, the results were considered statistically significant for $p < 0.05$.

RESULTS

Diagnosis: Serological, Genetic, and Histological Analysis

The endomysial antibody (AEMA) was positive in all CD patients included in this study. The anti-transglutaminase antibody (ATGA) was elevated (range 16 ≥ 200) in 95% of CD patients. There were no patients with IgA deficiency. All patients were active for celiac disease (**Table 1**).

Patients with CD had classic signs of the disease in intestinal biopsies with the presence of villi, with total or partial atrophy and increased lymphocyte infiltration. These histological alterations were classified according to the Marsh criteria (types I–IV). Among the celiac patients included in this study, 16% had Marsh III lesions (partial or complete atrophy of the villi and crypt hypertrophy), 76% had Marsh IV hypoplasia (totally atrophied villi, that is, completely flattened), and 8% had Marsh I lesions (increased intraepithelial lymphocytes, but without villus atrophy) (**Table 1**).

HLA genotype frequencies among these patients with CD were as follows: HLA-DQ2 (DQA1*0501, DQB1*0201) was the most frequent allele in the patients (76%). Three patients (24%) were carriers of DQ8 (DQA1*03:01,*03:03–DQB1*03:02).

Stimulatory Response of PBMCs From Patients With CD

The specific cell proliferation capacity and the amount of IFN-γ released against gluten peptides were studied to determine the ability to activate the stimulatory response of PBMC in patients with CD. The immunogenic potential of gluten peptides was quantified by the stimulation index in cultures of patients with CD subjected to a diet containing gluten. In addition, a control group was included without the addition of gluten peptides to the cell cultures with the same culture conditions; these were the reference values to compare with the effect of the peptides in cells of patients with CD. As a positive control assay, we stimulated T-cell by adding phytohemagglutinin (10 µg/ml PHA), and the production of IFN-γ was measured to reflect the proliferation function of T-cells. We chose to study the 33-mer peptide, BW208 and D623 wheat lines, due to their higher cell proliferation and IFN-γ values. The highest values of IFN-γ released were found in the supernatant of T-cells incubated with 33-mer (26.5 ± 1.6 pg/ml), BW208 (22.5 ± 2.6 pg/ml) and D623 wheat line (21.5 ± 1.3 pg/ml), respectively (**Figure 2**). The value of IFN-γ stimulated T-cell by PHA was 16.5 ± 1.8 pg/ml. In healthy controls, the levels of IFN-γ were 0.3 ± 0.4 pg/ml and 8.3 ± 0.7 pg/ml in unstimulated and PHA stimulated T cells, respectively (**Figure 2**).

The results of cell proliferation from celiac PBMCs clearly showed that the peptides 33-mer (SI = 34.7 ± 1.4), BW208 (SI = 29.7 ± 1.5), and D623 (SI = 24.6 ± 1.2) wheat lines induced a weak proliferative response in comparison with the negative control (SI = 4.7 ± 0.4).

We also analyzed the PHA-associated expression of PD1 mRNAs of PBMCs by reverse transcription and subsequent polymerase chain reaction (RT-PCR) in celiac patients and by flow cytometry in healthy controls. In PBMCs without PHA stimulation, we found expression of the full PD1 variant (687 bp) representing the membrane-bound isoform (**Figure 3**). In PHA stimulated cells, an additional band was obtained. The sequencing of that fragment revealed it was an alternatively spliced transcript of the PD1, lacking exon 3. This PD-1 Dex3 variant encodes the soluble form of PD1 (sPD-1); see **Figure 3**.

By flow cytometry were analyzed both $CD4^+$ and $CD8^+$ populations for detection of PD1 in healthy controls, either (A) untreated or (B) treated with PHA. As indicated in **Figure 4**, we found an increase in PD1 expression correlated with T-cell stimulation when PHA was added under the conditions of culture employed in this study. Generally, $CD4^+$ populations demonstrated predictable increases in PD1 expression, while $CD8^+$ populations were slightly less likely to follow this pattern, indicating that the cells were successfully stimulated (**Figure 4**).

PD1/PDL1/PDL2 Expression in Celiac Disease by Immunohistochemistry

In this study, we tested two antibodies that recognized PD1 protein which different immunogens. One of them was a recombinant anti-PD1 antibody in which immunogen is a synthetic peptide within Human PD1 aa 1–100 (N terminal) with membrane cellular localization and contains the one Ig-like V-type (immunoglobulin-like) domain. The other was a monoclonal antibody in which immunogen is an NK-like

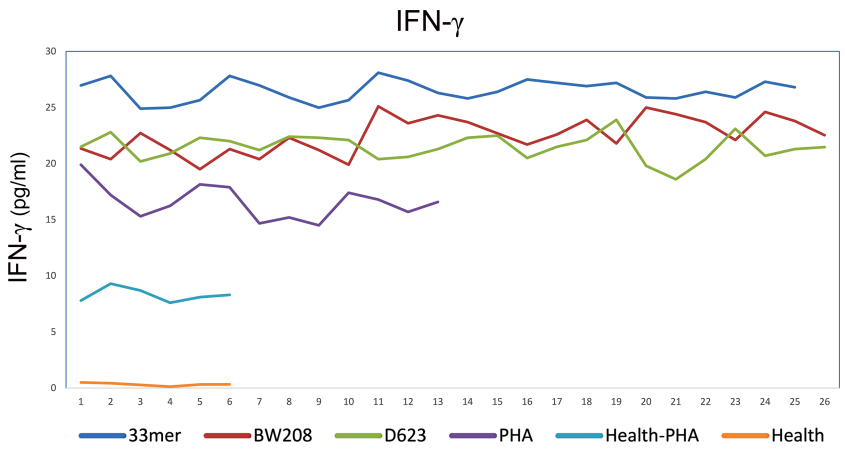

FIGURE 2 | IFN-gamma release by PBMCs of CD patients stimulated with different peptides and PHA as positive control and of healthy controls stimulated and unstimulated with PHA. In axis X the patients and in axis Y the levels of IFN-gamma expressed in pg/ml.

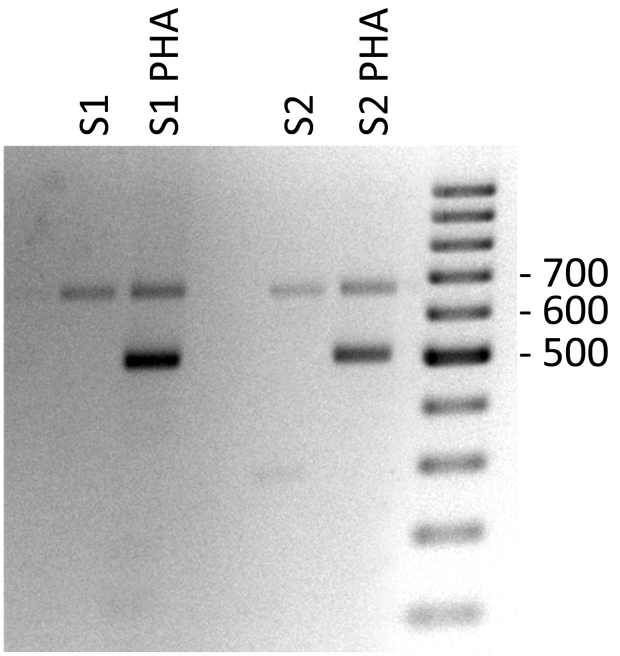

FIGURE 3 | Gel electrophoresis of PD-1 cDNA amplification products from PHA stimulated PBMCs of celiac patients as control positive of T cell stimulation.

leukemia cell line that expresses PD1, with membrane cellular localization, and also contains the one Ig-like V-type (immunoglobulin-like) domain. The epitope is in the range 24–170 aa protein. We found the same immunohistochemistry results with the two antibodies tested, giving negative PD1 expression in biopsies of patients with celiac disease (**Figure 5A**).

Indeed, all patients tested showed a positive expression of PDL1 and PDL2 with a different degree of immunoreactivity. PD-L1 is expressed on lamina propria cells of active CD patients (**Figure 5B**), and PDL2 is expressed on intestinal epithelial cells of active CD patients (**Figure 5C**).

Detection of sPD1/PDL1 and sPDL2 in Serum From Patients With CD

ELISA was used to examine the serum level of sPD-L1 and sPD-L2 in patients with CD to test its utility as a candidate biomarker. We showed that levels of sPD-1 were considerably higher in the serum of patients with celiac disease (n = 25) compared with healthy controls (n = 5) (9,123 ± 120 pg/ml *vs* 288 ± 36 pg/ml); see **Figure 6**. The mean levels of PDL1 and PDL2 were 1,723 ± 150 pg ml^{-1} *vs* 320 ± 65 pg ml^{-1} and 997.10 ± 126 *vs* 320 ± 78 in sera from patients with CD and healthy control, respectively.

Identification of Alternatively Spliced Variants of PD-1 mRNA in Peripheral Blood Mononuclear Cells Stimulated With Gliadin Peptides

In this study, we describe for the first time the expression of different splice variants for the PD-1 gene in CD patients. In an attempt to evaluate whether the regulation of splicing could be modified during the course of CD, we studied the pattern of PD1 isoforms in peripheral leucocytes from patients with CD, in comparison with healthy controls.

The effect of gluten peptide intervention on the expression pattern of splicing machinery components was evaluated in PBMCs from patients with celiac disease. PBMCs cultured from patients with CD and healthy controls were stimulated with different types of peptides (33-mer, BW, and D623). PCR amplification of the human PD-1 coding sequence revealed the expression by human PBMCs of four splice variants: flPD-1, PD-1Dex3, PD-1Dex2, PD-1Dex2,3 with sizes about 687, 531, 327, and 171 bp, respectively (**Figure 7**). We also found two new alternative spliced isoforms that retained part of different introns, with sizes of 781 and 715 bp, as well as another isoform with a loss of 42 bp from exon 2 and a complete exon 3 (Δ3) with a size of 489 bp (**Figure 7**). All amplified products were cloned and sequenced. The largest band (687 bp) represents the membrane-bound form, showing complete homology with the published membrane PD-1 sequence (GenBank Accession No. NM_005018). The 531, 327, and 171 bp correspond to previously described variants (PD-1Dex2, PD-1Dex3, and PD-1Dex2,3) that lack the exon 2, exon 3, or both (**Figure 7** and **Supplementary Figure S1**).

The PMBC stimulation with BW wild-type peptide showed the expression of the PD1 form (687 bp) representing the membrane-bound form while also producing an alternatively spliced transcript of the PD1 with the sequence encoded by exon 3 being skipped, encoding the soluble form of PD1 (**Figure 8**). The stimulation with the D623 line that presented a higher number of peptides per protein than the BW wild-type line showed expression by human PBMCs of four splice variants: flPD-1, PD-1Dex3, PD-1Dex2, PD-1Dex2,3, presenting variability of expression in each patient with celiac, as shown in **Figure 8**. In patient 2, the stimulation with line D623 presented a new PD1 isoform (Δ3*, band 489 bp) with a loss of 42 bp at the beginning of exon 2 and without exon 3 (**Figure 7** and **Supplementary Figure S1**). This deletion does not change the reading frame (**Supplementary Material S4**) and could produce a protein which lacks the first 14 aa codified by exon 2. This isoform could encode another soluble and new isoform of PD1 (sPD-1).

Stimulating the PMBCs with 33-mer showed the expression of two PD1 alternative spliced isoforms with sizes of 781 and 715 bp, respectively (**Figures 7**, **8B**, **Supplementary Figure S1**). In patient 2, a large 33m-2 band of 781 bp retains all exons and a 95 bp fragment of the end of the intron that separates exons 3 and 4 (Splicing 3′ site that establishes the end of one intron and the beginning of the next exon). The 95 bp sequence introduces a premature stop codon (TAA) that could produce a truncated protein without the aa encoded by exons 4 and 5 (**Supplementary Figure S2**). In patient 3, a large 33m-3 band of 715 bp retains all exons and a 25 bp fragment at the beginning of the intron between exons 2 and 3, with the last three bp of this intron (Splicing 5′ site that establishes the end of one exon and the beginning of the next intron); see **Figures 7**, **8B** and **Supplementary Figure S1**. These nucleotides generate a

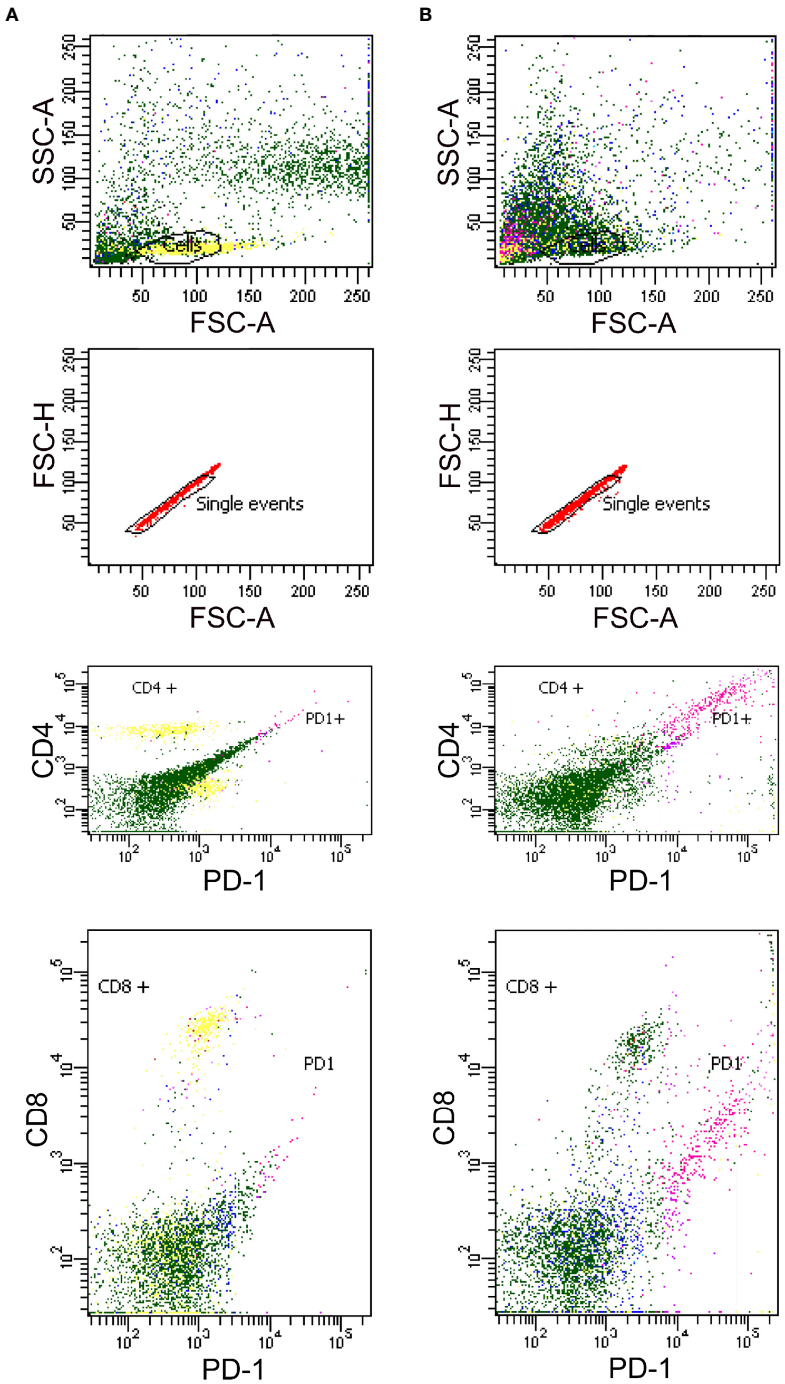

FIGURE 4 | Detection of PD1 in Human PBMCs from healthy controls by flow cytometry either unstimulated **(A)** and stimulated with PHA **(B)**.

change in the reading frame and therefore a change in the aa sequence from this point, so the protein will surely be non-functional (**Supplementary Figure S2**). It was not possible to establish a correlation between the degree of inflammatory activity of celiac disease according to the Marsh classification and the expression of the different isoforms of PD1 described in this study. The control group (C), corresponding to PMBCs of patients with CD without posterior peptide stimulation *in vitro*, revealed the expression of the PD1 form (687 bp), representing the membrane-bound form, while other CD patients present expression of the soluble form of PD1 (sPD-1); see **Figure 8B**.

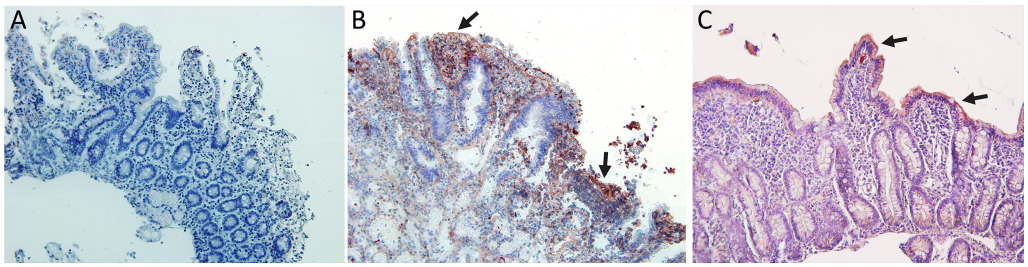

FIGURE 5 | Immunohistochemistry analysis: **(A)** Negative immunostaining for PD1 in celiac disease patients. **(B)** Immunostaining for PDL1 in celiac disease patients showing expression in lamina propria cells and in Lieberkhün crypts. **(C)** Immunostaining for PDL2 in CD patients showing expression in epithelial cells. Magnification 200×. Arrows: cells with immunoreaction.

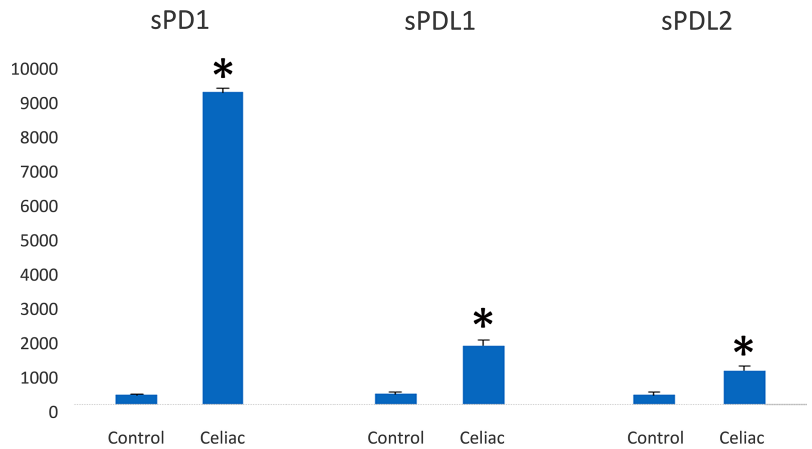

FIGURE 6 | PD-1/PD-L1 expression in the serum of celiac disease patients and healthy controls (pg/ml). PD-1 and PD-L1 are highly expressed in serum from celiac disease patients (n = 25) in relation to healthy controls (n = 5). Significant difference at $*p < 0.05$ is shown.

Finally, gel electrophoresis of PD-1 cDNA amplification products from PBMCs stimulated with gluten peptides in healthy controls showed the expression of the PD1 form (687 bp), representing the membrane-bound form (**Figure 8A**).

PD-1 Genotype and Allele Frequencies

The allele and genotype frequencies of PD-1.3 and PD-1.5 SNPs are shown in **Table 3**. In this study, the observed genotype frequencies in the control and CD groups were consistent with the Hardy–Weinberg equilibrium.

For the PD-1.3 SNPs, the frequency of the GG genotype was higher in patients with CD as well as in healthy controls, while the AA genotype was less abundant in both groups. Analysis by Pearson's x2 and Fisher's exact test showed there was no significant association between CD and the genotypes and alleles (p>0.05). Moreover, there was no meaningful association between the gender of patients with CD and specific PD-1.3 genotypes. In the CD group, the odds of celiac for AA were 1.08 times as great as the odds of celiac for GG, and the odds for AG were 0.89 times as great as the odds for GG. Meanwhile, the healthy control group odds for AG were 1.13 times as great as the healthy control group odds for GG, and for AA this was 0.92 times as great as the odds for GG. However, this difference in CD susceptibility between the AA and GG genotypes was not statistically significant.

For the PD-1.5 SNP, the frequency of the CT genotype was higher in patients with CD and in healthy controls, while the TT genotype was less abundant in both healthy control groups. Analysis by Pearson's x2 and Fisher's exact test showed there was no significant association between CD and the genotypes and alleles (p>0.05). Moreover, there was no meaningful association between the gender of patients with CD, while CC and TT genotypes were the most and least frequent genotype in the CD and healthy control.

Susceptibility to CD among subjects with the TT genotype seemed much higher than that among the subjects with the CC genotype; the odds ratios of CD patients for TT was 0.83 times as great as the odds of celiac for CC. In contrast, the odds ratios of healthy control for TT was 1.20 times as great as the odds ratios of the healthy control for CC. However, this difference in CD

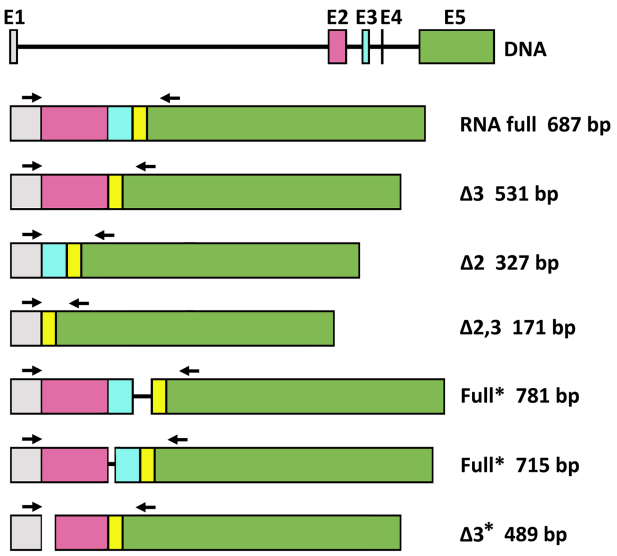

FIGURE 7 | Schematic diagram for PD1 splicing variants found in this study. flPD-1, PD-1Dex3, PD-1Dex2, PD-1Dex2,3 with sizes about 687, 531, 327, and 171 bp, respectively. New alternative spliced isoforms that retained part of different introns, with sizes of 781 and 715 bp, and isoform with a loss of 42 bp from exon 2 and a complete exon 3 (Δ3) with a size of 489 bp. Asterisks indicate the new isoforms found in this study.

susceptibility between the TT and CC genotypes was not statistically significant.

Nevertheless, a small difference between the female and male patients could not be excluded completely due to the small number of male patients with CD in this study.

DISCUSSION

Celiac disease is a chronic and systemic autoimmune pathology characterized by a reaction to gluten in genetically predisposed individuals. It causes severe damage to the mucosa of the small intestine, with atrophy of the intestinal villi causing poor absorption of nutrients (35). The disease is polygenic involving genes from the major histocompatibility antigen (MHC) complex such as HLA DQ2 and DQ8 and, less frequently, other non-MHC genes (35). Studies of genome-wide association scans have demonstrated gene alterations at the level of regulators of the immune response in celiac disease (36, 37).

PD-1/PD-L1 binding prevents an excessive immune response and protects tissues from damage through the induction of immune tolerance in normal tissues (38). The inflammatory signaling and epigenetic alterations regulates the PD1/PD-L1 expression (39). In this study, we tested two antibodies that recognized PD1 protein with different immunogens:

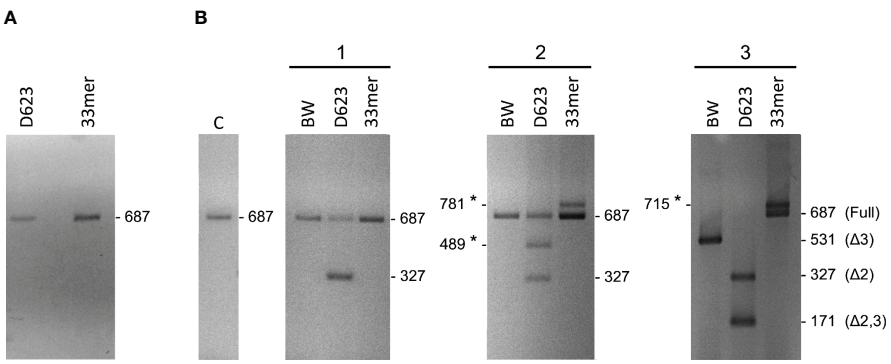

FIGURE 8 | Gel electrophoresis of PD-1 cDNA amplification products from PBMCs stimulated with gluten peptides in healthy controls **(A)** and in celiac patients **(B)**. With the used primers in the present study, the sizes of the respective PCR products were 687 bp for the full-length PD-1 transcript, 531 bp for PD-1Dex3, 327 bp for PD-1Dex2, 171 bp for PD-1Dex2,3, and three new isoforms with 781, 715, and 489 bp. Asterisks indicate the new isoforms found in this study.

TABLE 3 | Allele and genotype frequencies of polymorphic sites of the PD1 gene in celiac Spanish population.

PD-1.5 SNP frequencies			PD-1.3 SNP frequencies		
Genotypes	Control	Celiac	Genotypes	Control	Celiac
CC	42% (24)	43.5% (33)	GG	65% (37)	67% (51)
CT	49% (28)	46% (35)	GA	31.5% (18)	29% (22)
TT	12% (7)	10.5% (8)	AA	3.5% (2)	4% (3)
Alleles	Control	Celiac	Alleles	Control	Celiac
C	64.4% (76)	66% (101)	G	80.7% (92)	81.6% (124)
T	35.6% (42)	33.5% (51)	A	19.3% (22)	18.4% (28)

(i) a synthetic peptide within Human PD1 aa 1–100 (N terminal), and (ii) with the epitope in the range 24–170 aa protein. Both antibodies with membrane cellular localization contain one Ig-like V-type (immunoglobulin-like) domain. We demonstrated a negative expression of PD1 in the intestinal biopsy of active CD patients, with the same results for the two antibodies tested suggesting an important role for the PD-1/PDLs pathway in regulating mucosal tolerance *in situ*.

In a previous study, we assessed cell surface expression of PD1 on freshly isolated PBMCs by flow cytometry analysis. In CD patients, CD4+ and CD8+ T cells presented significantly decreased PD-1 protein expression (26). Thus, PD-1 function would be compromised in CD4+ and CD8+ T cells, indicating the establishment of an inappropriate activation state (40). A dysregulation of immunosuppression mechanisms that can lead to abnormal and persistent T-cell activation and cytokine production occurs in celiac disease. PD1 plays a crucial role in the tolerance mechanisms of central and peripheral T cells, helping to protect tissues from autoimmune responses. Without PD1 expression, excessive tissue damage mediated by the immune response can have devastating consequences in the protection of self-tissues from autoimmune responses.

The expression of sPD-1 could increase the maturation of dendritic cells (DCs), which could be accompanied by upregulation of major histocompatibility complex II (MHC II) (40). sPD-1-regulated DC maturation is mediated by activated T lymphocytes and may be influenced by increased T cell responses (40). Soluble PD-1 can play an adjuvant role in enhancing antigen-specific T-cell immunity responses. A deficit in PD1 gene expression has been shown to result in inadequate suppression of autoreactive lymphocytes, an aberrant presence of activated T cells and autoantibody production (40).

In this study, we found expression of PD-L1 and PD-L2 in serum and in intestinal biopsies of patients with active CD. These findings are consistent with other studies that show a higher expression of PD-L1 in other human autoimmune diseases. PD-L1 is known to be upregulated in inflamed intestinal tissue (41, 42). PD1 expressed on the surface of activated T cells, by binding to its PD-L1 ligand, sends inhibitory signals to T cells; this being a significant molecular mechanism for the control of antigen-specific T cells, thus avoiding excessive tissue damage induced by immune responses (43). In relation to cytokine expression, interferon (IFN)-γ plays an important role in the positive regulation of PD-L1 expression in tumor tissues (44).

In addition to the inhibitory effect of PD-L1 on inflammation, previous studies also showed that epithelial expression of PD-L1 induced by bacterial pathogens could inhibit T-cell functions (45). Thus, some species of commensal bacteria may contribute to chronic inflammation by upregulating the epithelial expression of PD-L1 in an inflammatory environment to inhibit the function of T cells in the mucosa.

PD-L2, as a receptor of PD1, is involved in the costimulatory signal essential for T-cell proliferation and IFN-γ production (46). PD-L2 generally expressed at a lower level may favor PD-L1 as the primary binding ligand of PD-1, except during Th2 responses when PD-L2 is upregulated (47, 48). Several research groups have shown that PD-L2 expression can be induced on a wide variety of other immune cells and non-immune cells depending on microenvironmental stimuli (49–51). PD-L1 and PD-L2 expression depends on distinct stimuli, and their expression patterns suggest both overlapping and differential roles in immune regulation (11).

Few studies have assessed PD-L2 expression in autoimmune diseases, especially in SLE (33, 52). We found PD-L2 expression on the intestinal biopsy from patients with CD. An interesting speculation would be that, in CD, the soluble PD-L2 could act as a decoy ligand to increase PD-1 activation of immune cells to further amplify the immunopathological damage. Additional studies would be required, however, to test this theory.

Wan and colleagues demonstrated that the soluble PD-1 in serum of RA patients was the translational product of the PD-1Δex3 mRNA transcript (53). We identified the over-expression of the sPD-1 protein and the PD-1Δex3 transcript in CD patients. These results provide new evidence that PD-1 is associated with CD and that its soluble form might play a key role during the phase of T cell exhaustion and the primary activation of T cells. Soluble PD-1 can be used as an adjuvant to increase T cell immunity. This study suggests that it is likely that the soluble PD-1Δ3 isoform still retains the ability to bind to PD-L1/PD-L2 and that, by interfering with its signaling pathway, it has antagonistic effects on PD-1. In patients with CD, the excessive sPD-1 could serve as an "antibody" to block the PD-1/PD-Ls pathway and lead to aberrant T-cell proliferation.

Soluble forms of many immune regulatory molecules, both co-stimulatory and co-inhibitory molecules, are detected in plasma of CD patients including sCTLA-4, sHLA-G, sCD27, and sIL-33 (54–56). These soluble forms are produced through alternative splice variants. sCTLA-4 is present in serum as a functional protein and correlates with mucosal injury (54)

We have identified four alternative spliced PD-1 mRNA transcripts of PD-1 (PD-1Dex2, PD-1Dex3, PD-1Dex2,3, and PD-1Dex2,3,4) in addition to the full length isoform encoded by exon 1 (leader peptide), exon 2 (extracellular IgV-like domain), exon 3 (transmembrane domain), exons 4 and 5 (intracellular domain). These transcripts were verified by cloning and sequencing, and the different splice variants did not represent amplified artifacts during PCR reactions.

The PD-1Dex2 mRNA transcript is generated from an alternative junction of the PD-1 gene where the sequence encoded by exon 2 is deleted. If this PD-1Dex2 mRNA transcript were translated, the protein product would be expressed as a molecule membrane lacking its binding properties to PD-L1/2 receptors. This splicing does not affect the reading frame and the biological significance of this mRNA transcription could be the result of a splicing error occurring in parallel with increased mRNA expression during cell proliferation development.

Similarly, we found expression of the PD-1Dex2,3 mRNA transcript in PBMC from patients with celiac disease subsequently stimulated with gluten-derived peptides. We have no evidence of any biological function of the putative protein encoded by this transcript, since this truncated protein would lack PD-L1/2 receptor binding properties. However, the PD-1Dex2,3 transcript does not affect the reading pattern and, if it

were translated, it would introduce glycine at position 26 compared to aspartic acid in the complete form fl-PD1

Two new splice alternatives of PD1 variants that retain part of different introns with sizes of 781 and 715 bp were found. The 781 bp band has all the exons and a 95 bp sequence that introduces a premature stop codon (TAA) that leads to a loss in the region encoded by exons 4 and 5 as a result of a truncated protein. This protein with premature termination will be more or less serious depending on the area in which it occurs. The band at 715 bp has all the exons and a 28 bp fragment that corresponds to the beginning of the intron between exons 2 and 3. The reading pattern changes from the zone of the intron, and a totally different amino acid sequence is generated, so the protein will surely be non-functional.

Finally, a new isoform of 489 bp that has a deletion at the beginning of exon 2, being a multiple of three nucleotides, was found. This isoform does not change the reading pattern. The biological importance of this new mRNA transcript is unknown, although the level of this transcript was lower compared to the other transcripts we found in patients with celiac disease. This mRNA transcript would correspond to a new soluble isoform that would probably have antagonistic effects on the membrane shape by interfering with its signaling pathway and could also preserve the ability to bind to the PD-L1/PD-L2 ligands.

The PD-1 gene is located on chromosome 2q37.3 near another region 2q33.3 related to autoimmunity (57). To determine the associations between these PD-1 genetic variations and CD in the population of southern Spain, PD-1 SNPs were evaluated in genomic DNAs extracted from control groups and from patients with CD.

One of the selected polymorphisms was the PD-1.3 SNP, since it alters the inhibitory effect of PD-1 and increases the activity of lymphocytes. Substitution of guanine (G) for adenine (A) at nucleotide + 7,146 in intron 4 alters transcriptional regulation and PD-1 expression at this polymorphic site during disruption of runt-related transcription factor 1 binding (RUNX1) (28, 58). PD1.5 polymorphism does not exert any change in the final amino acid structure of the protein, and the link imbalance between the PD1.5 variation and other PDCD1 gene polymorphisms can lead to modify expression at the mRNA and protein level.

To our knowledge, this study primarily showed that PD1.3 and PD1.5 polymorphisms were not associated with risk of CD, possibly indicating the absence of any interaction between them. We have observed a tendency for an increase in PD1.3 G and PD1.5 C alleles in patients with CD in comparison with healthy controls, although this difference was not statistically significant. More such studies with a larger sample size would be needed in the Spanish population to confirm these observations. In addition, it must be considered that there may be other SNPs that we have not been able to identify in the current study.

Based on the results shown, we can suggest that the splicing machinery would act as a biological sensor to adapt gene expression to the pathophysiological conditions that arise (59). Dysregulation of the gene could lead to an imbalance in the splice variants present in cells at any given time and in response to external factors. Among these factors, the existence of gliadin-derived peptides in the serum of patients with CD could modulate the expression of relevant splice components and the function of the splicing machinery. Our study provides primary evidence that a dietary intervention of gluten peptides could alter the expression pattern of the splicing machinery at risk for CD. In this study, although we observed splicing complex formation in the presence of active peptides, it is not possible to use these assays to determine whether the active peptides inhibited splicing by interfering with conformational rearrangements in the splice.

The alternative splicing process may represent a physiological mechanism to maintain cell homeostasis. This is suggested in different studies that show that nutrients can modulate gene expression and, in particular, the splicing of pre-mRNA that encodes regulatory proteins (60). Minimal alterations in the alternative splicing process could lead to the production of deficient proteins that contribute to the development of some human diseases (61).

CONCLUSIONS

In view of our results, we propose that gluten peptides may modulate the processes necessary for cellular homeostasis in a specific way through the alteration of gene expression; in particular in the splicing of pre-mRNA that encodes regulatory proteins' key, as PD1. Our results indicate that alternative PD1 splicing could explain the complex pathophysiology that occurs in each patient with CD, and it can be hypothesized that there is differential regulation of PD1 splice variants in CD. These alterations in the production of different isoforms of PD1 could contribute to the development of CD.

Since PD1.3 and PD1.5 polymorphisms were present in both patients with CD and in healthy controls and no significant evidence was observed between both groups, it could not be concluded that this polymorphism was associated with CD.

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 local Ethics Committee of the Hospital "Virgen de las Nieves" (Granada, Spain) approved the study protocol. Written consent was obtained from parents or legal guardians of children involved.

AUTHOR CONTRIBUTIONS

Conceptualization: PL, ML-C, TP, and MT. Methodology: CP, PL, ML-C, FB, TP, and MT. Formal analysis: CP, PL, ML-C, FB, TP, and MT. Resources: MT, and PL. Writing—original draft preparation: MT and PL. Writing—review and editing: CP, PL, ML-C., FB, TP, and MT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.678400/full#supplementary-material

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Vitamin D and Exercise Are Major Determinants of Natural Killer Cell Activity, Which Is Age- and Gender-Specific

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Background: The coronavirus-19 disease (COVID-19) pandemic reminds us of the importance of immune function, even in immunologically normal individuals. Multiple lifestyle factors are known to influence the immune function.

Objective: The aim was to investigate the association between NK cell activity (NKA) and multiple factors including vitamin D, physical exercise, age, and gender.

Methods: This was a cross-sectional association study using health check-up and NKA data of 2,095 subjects collected from 2016 to 2018 in a health check-up center in the Republic of Korea. NKA was measured using the interferon-γ (IFN-γ) stimulation method. The association of NKA with 25-(OH)-vitamin D (25(OH)D) and other factors was investigated by multiple logistic regression analysis.

Results: The average age of subjects was 48.8 ± 11.6 years (52.9% of subjects were female). Among 2,095 subjects, 1,427 had normal NKA (NKA ≥ 500 pg IFN-γ/mL), while 506 had low NKA (100 ≤ NKA < 500 pg/mL), and 162 subjects had very low NKA (NKA < 100 pg/mL). Compared to men with low 25(OH)D serum level (< 20 ng/mL), vitamin D replete men (30–39.9 ng/mL) had significantly lower risk of very low NKA (OR: 0.358; 95% CI: 0.138, 0.929; $P = 0.035$). In women, both low exercise (OR: 0.529; 95% CI: 0.299, 0.939; $P = 0.030$) and medium to high exercise (OR: 0.522; 95% CI: 0.277, 0.981; $P = 0.043$) decreased the risk compared to lack of physical exercise. Interestingly, in men and women older than 60 years, physical exercise significantly decreased the risk. Older-age was associated with increased risk of very low NKA in men, but not in women.

Conclusion: Physical exercise and vitamin D were associated with NKA in a gender- and age-dependent manner. Age was a major risk factor of very low NKA in men but not in women.

Keywords: vitamin D, NK cell activity, exercise, immunosenescence, immunity

INTRODUCTION

The coronavirus-19 disease (COVID-19) pandemic caused by SARS-CoV-2 prompted us to keep our immune function healthy to protect the body from the pathogen. Physical exercise, nutrition and emotional status were frequently named as the main factors that can influence the immune function. Thus, lifestyle that includes regular physical exercise, healthy dietary and emotional habits were emphasized besides wearing masks and social distancing (1–5).

Immune markers that can represent immune function in immuno-competent individuals is still under search. In this regard, earlier research tried to elucidate immune characters that can predict healthy longevity. Decreased immune responses represented by CD4+:CD8+ ratio (<1), poor T-cell proliferation response to mitogens *in vitro*, increased number of CD8+ T lymphocytes and so on were associated with increased mortality in the elderly (6–9). However, those findings could not be reproduced (10, 11). Most of the immune function tests used in clinical practice examine critical functional defects that are present in immunodeficiency diseases. Yet, disease development in immuno-competent individuals is also closely associated with impaired immune function. Overwork, fatigue, and emotional stress are well acknowledged to negatively affect the immune function. However, there are not many tests that can evaluate the immune function in immunologically normal individuals, that is varied by stressors in everyday life. Commercialized natural killer (NK) cell activity (NKA) assay is an immune function test that may fill the need. The NKA test measures the amount of interferon-gamma (IFN-γ) released by activated NK cells contained in 1ml of peripheral blood (12). Therefore, it represents the secretory function of NK cells. Also, it may indirectly indicate the amount of NK cells contained in 1 mL of the peripheral blood approximately. Studies on NKA have demonstrated that decreased NKA is significantly associated with increased cancer incidence in the stomach (13), colorectum (14), and prostate (15, 16).

NK cells, as members of the innate immune system, are early responders of immune reactions. Functional defects of NK cells results in immunodeficiency syndrome which pose the individuals at the risk of critical infections or cancer development (17). Decreased NK cell function in immunologically normal individuals were associated with increased risk for cancer development (13–16, 18, 19). Decreased NK cell function in the immunologically normal elderly was associated with increased risk for severe infection and mortality (20, 21). Further, studies on COVID-19 suggested that NK cell may play a critical role in the early response which can determine overall outcome of the disease. For example, severe COVID-19 cases were characterized by depleted peripheral NK cell counts compared to mild cases or healthy controls (22–26). $CD56^{dim}$ NK cells, which mediate cytotoxicity, were depleted in ventilator-dependent patients, and $CD56^{bright}$ NK cells, that is immune-regulatory producing IFN-γ, were significantly depleted in all COVID-19 patients (23).

The traits of NK cells and the NKA test suggest that the NKA may serve as a useful surrogate marker to evaluate immune function of the immunologically normal individuals. We postulated that there exist lifestyle characteristics which define better immune function. Immune function is affected by multiple factors such as age, gender, nutrition, exercise, and underlying diseases. We investigated how each factor was associated with the NKA.

METHODS

Study Population

Between January 2016 and June 2018, individuals aged ≥ 18 years (n = 3,066) who had undergone a comprehensive health check-up and NKA test at Chaum Life Center (Seoul, Republic of Korea) were screened (**Figure 1**). For the enrolled individuals, health check-up data were retrieved from electronic medical records. A certain proportion of individuals had the health check-up annually or biannually at the same institution, which resulted in multiple enrollments. In such case, only the first check-up data were included, and the check-up data of the following years were excluded (n=486). Exclusion criteria included suspicious findings in health check-up results requiring immediate biopsy confirmation for cancer (n = 64), active allergic disease under treatment (n = 57), recent use of antibiotics (n = 8), immunosuppressants (n = 2), or herbal medicines (n = 8), and history of malignant disease (n = 163), autoimmune diseases such as inflammatory bowel disease (n = 61), and other inflammatory or immune-related diseases [chronic hepatitis B and C infections (n = 78); chronic liver disease identified in liver ultrasonography, except for fatty liver (n = 3); bronchiectasis (n = 1); chronic pancreatitis (n = 1); thrombocytopenia (n = 1); and multiple of the above (n=38)]. Individuals with missing variables were automatically excluded during statistical analyses. This study was approved by the Institutional Review Board of CHA Bundang Medical Center (IRB number 2018-06-033-004) and was conducted according to the Declaration of Helsinki. As a retrospective study, the requirement of informed consent was waived.

Lifestyle Factors

Every individual undergoing health check-up was asked to fill out a questionnaire sent by mail 2 weeks before and to submit it on arrival. The questionnaire asked medical and social history and lifestyle habits including alcohol intake habits, smoking habits and physical exercise habits. Regarding physical exercise habits, it asked about exercise frequency (no exercise, 2–4 times per month, 2-4 times per week, or everyday) and exercise duration per workout session (less than 1-h, 1–2-h, or more than 2-h).

For alcohol intake habits, individuals who did not drink at all or those who drank once a month or less frequently with a limit of 1–2 standard servings per session were defined as non-drinkers. Individuals who drank up to 3 times per week with a limit of 1–2 standard servings per session were defined as light drinkers. Individuals who drank more than light drinkers either in frequency or quantity were defined as heavy drinkers (27–29).

For exercise habits, medium exercise was defined as exercise 2–4 times per week with 1–2-h duration per workout session or

everyday exercise with less than 1-h duration per session (30–32). Low and high exercises were defined as less or more exercise than medium exercise in terms of workout frequency or time per session, respectively.

During the check-up, height and body weight were measured. Body mass index (BMI) was calculated by dividing body weight in kilograms by height in square meters. BMI was categorized with cut-offs of < 18.5 kg/m^2 (underweight), 18.5–27.5 kg/m^2 (normal to overweight), and ≥ 27.5 kg/m^2 (obese), as suggested for Asian populations by the World Health Organization (33).

Laboratory Measurement

NKA was measured using the NK Vue® kit (ATGen, Seongnam, Republic of Korea) containing a recombinant cytokine that specifically activates NK cells to release interferon-γ (IFN-γ) (12). According to the manufacturer's instructions, 1 mL of peripheral blood was directly added to the test kit and incubated for 20–24 h at 37°C. Upon completion of incubation, the supernatant was collected and centrifuged at 3,000 × *g* for 3 min. The final supernatant was measured for IFN-γ content in pg/mL using enzyme-linked immunosorbent assay. This study used reference ranges provided by the test-kit manufacturer (13–16) and defined NKA < 100 pg IFN-γ/mL as very low, 100 pg/mL ≤ NKA < 500 pg/mL as low, and NKA ≥ 500 pg/mL as normal. Electrochemiluminescence-binding assay based on competition principles (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure serum level of 25-(OH)-vitamin D (25(OH)D). Health check-up factors, including NKA, 25(OH)D, and Hb A1c, were measured at the Department of Laboratory Medicine, CHA Gangnam Medical Center (Seoul, Republic of Korea).

Statistical Analysis

For univariable analyses, continuous variables were assessed using Student's *t*-test or analysis of variance (ANOVA), and categorical variables were examined by Pearson's Chi squared test. To identify the risk factors for or protective factors against low NKA, we compared the very low NKA group with the normal NKA group using multiple logistic regression analyses. Variables were eligible for entry into a multiple logistic regression model if they were significantly associated with $P < 0.1$ in univariable analyses or clinically important. Multiple logistic regression analyses were performed in gender, age, and exercise groups to find differential influences of relevant factors in each subgroup. Correlation was tested with Pearson's correlation test. IBM SPSS statistics version 21 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Box plots were created using the R software (version 3.5.1).

RESULTS

Population Characteristics

Among the initially screened 3,066 electronic medical records, 486 records were excluded due to multiple check-ups. Thereafter, 485 subjects were excluded according to the predefined exclusion criteria as already described above. Finally, 2,095 subjects were included for analyses (**Figure 1**).

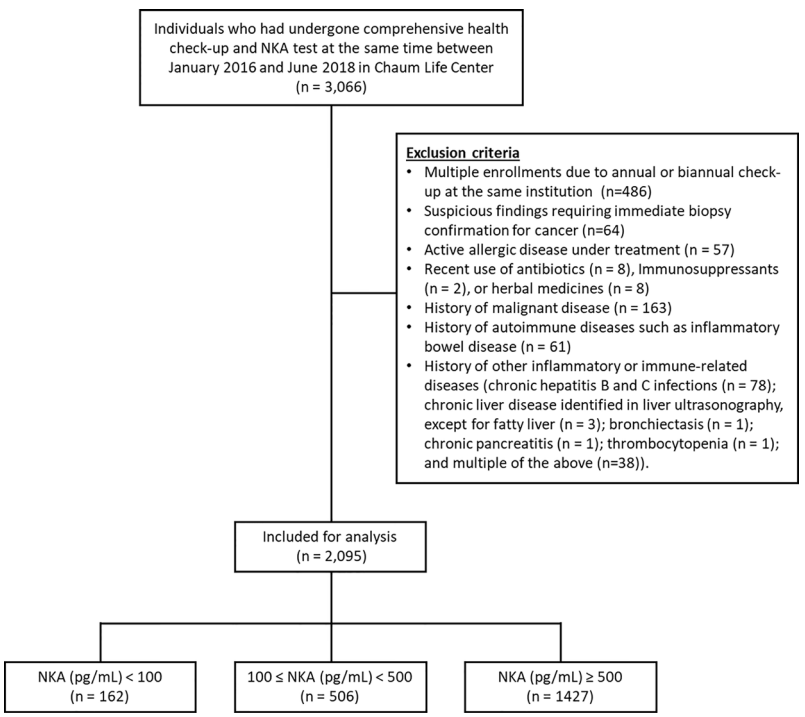

FIGURE 1 | Participant flow chart. NKA, natural killer cell activity.

The mean (± SD) age of the study population was 48.8 ± 11.6 years, 52.9% were female, and the mean (± SD) NKA value was 1,243 ± 1,044 pg INF-γ/mL. Population characteristics according to NKA values are provided in **Table 1**. There were 162 subjects with very low NKA value (NKA < 100 pg/mL), 506 with low NKA (100 ≤ NKA < 500 pg/mL), and 1,427 with normal NKA (NKA ≥ 500 pg/mL). The mean ages of groups with very low NKA, low NKA and normal NKA were 50.72 ± 13.01, 49.19 ± 11.76 and 48.47 ± 11.35 years, respectively ($P = 0.047$). In the very low NKA and low NKA group, the proportions of uncontrolled blood glucose level represented by Hb A1c ≥ 7.5 were higher than the normal NKA group (4.4%, 3.5% *vs.* 1.5%, $P = 0.034$). In the very low NKA and low NKA group, the proportions of vitamin D deficiency represented by serum level of 25(OH)D < 20 were higher than the normal NKA group though it could not reach statistical significance (52%, 48.9% *vs.* 44.4%, $P = 0.092$). The distributions of NKA value according to age and 25(OH)D are depicted in **Figures 2**, **3** respectively. Gender, smoking habit, alcohol use, BMI, hypertension, hyperlipidemia and diabetes mellitus (DM) were not associated with NKA.

TABLE 1 | Population characteristics according to NKA.

Characteristics	Very low NKA (NKA < 100 pg/mL, n = 162)		Low NKA (100 pg/mL ≤ NKA< 500 pg/mL, n = 506)		Normal NKA (NKA ≥ 500 pg/mL, n = 1427)		*P* value[1]	*P* value[2]
Age (years, mean)	50.72	± 13.01	49.19	± 11.76	48.47	± 11.35	**0.047**	**0.036**
Age (years)								
18-30	10	(6.2%)	25	(5%)	86	(6.0%)	0.119	**0.018**
31-40	25	(15.4%)	97	(19.2%)	267	(18.9%)		
41-50	45	(27.8%)	146	(28.9%)	453	(31.7%)		
51-60	46	(28.4%)	154	(30.5%)	414	(293%)		
61-70	25	(15.4%)	66	(13.1%)	168	(11.8%)		
71-80	9	(5.6%)	16	(3.2%)	35	(2.5%)		
81-90	2	(1.2%)	1	(0.2%)	2	(0.1%)		
Gender								
Women	86	(53.1%)	272	(53.8%)	750	(52.6%)	0.897	0.898
Men	76	(46.9%)	234	(46.2%)	677	(47.4%)		
Smoking								
Non-smoker	85	(54.8%)	272	(55.5%)	743	(53.3%)	0.904	0.839
Ex-smoker	41	(26.5%)	120	(24.5%)	362	(26.0%)		
Current smoker	29	(18.7%)	98	(20.0%)	289	(20.7%)		
Alcohol								
Non-drinker	65	(41.9%)	201	(40.8%)	499	(35.8%)	0.236	0.299
Light-drinker	31	(20.0%)	92	(18.7%)	290	(20.8%)		
Heavy-drinker	59	(38.1%)	200	(40.6%)	605	(43.4%)		
Physical exercise								
Lack of exercise	61	(39.9%)	163	(33.7%)	391	(28.5%)	0.058	**0.026**
Low exercise	53	(34.6%)	170	(35.2%)	533	(38.9%)		
Medium exercise	30	(19.6%)	107	(22.2%)	317	(23.1%)		
High exercise	9	(5.9%)	43	(8.9%)	129	(9.4%)		
Body mass index (kg/m^2)								
<18.5	16	(10.0%)	31	(6.2%)	80	(5.7%)	0.301	0.094
≥18.5, <27.4	126	(78.8%)	409	(81.2%)	1161	(82.2%)		
≥27.5	18	(11.3%)	64	(12.7%)	172	(12.2%)		
Hb A1c (%)								
<5.7	102	(63.8%)	321	(63.8%)	901	(63.5%)	**0.034**	0.063
≥5.7, <6.5	45	(28.1%)	141	(28.0%)	445	(31.4%)		
≥6.5, <7.5	6	(3.8%)	23	(4.6%)	51	(3.6%)		
≥7.5	7	(4.4%)	18	(3.6%)	21	(1.5%)		
25-(OH)-Vitamin D (ng/ml)								
<20	77	(52.0%)	223	(48.9%)	553	(44.4%)	0.092	0.196
≥20, <30	47	(31.8%)	145	(31.8%)	413	(33.1%)		
≥30, <40	17	(11.5%)	59	(12.9%)	219	(17.6%)		
≥40	7	(4.7%)	29	(6.4%)	61	(4.9%)		
Hypertension	23	(14.5%)	76	(15.1%)	192	(13.6%)	0.710	0.768
Hyperlipidemia	20	(12.6%)	56	(11.1%)	184	(13.0%)	0.529	0.867
Diabetes	10	(6.3%)	33	(6.5%)	64	(4.5%)	0.173	0.324

[1]P values were calculated using AVONA or Pearson's Chi squared test with 3 group variables, NKA (pg/mL) < 100, 100 ≤ NKA < 500 and NKA ≥ 500.

[2]P values were calculated using Student's t-test or Pearson's Chi squared test with 2 group variables, NKA (pg/mL) < 100 and NKA ≥ 500.

NKA, natural killer cell activity.

P values in bold denote statistical significance at the P < 0.05 level.

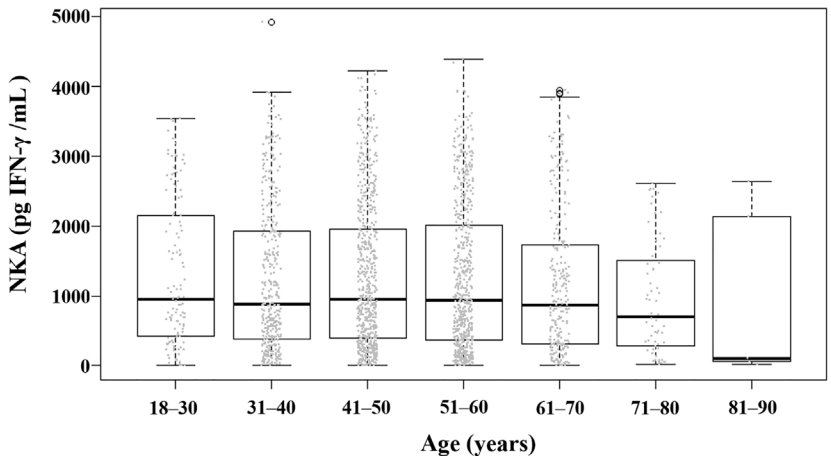

FIGURE 2 | Distribution of NKA value according to age. Each dot represents the individual value of NKA. Box plots show group medians, interquartile range (IQR), and spread of data with outliers for each group. NKA, natural killer cell activity.

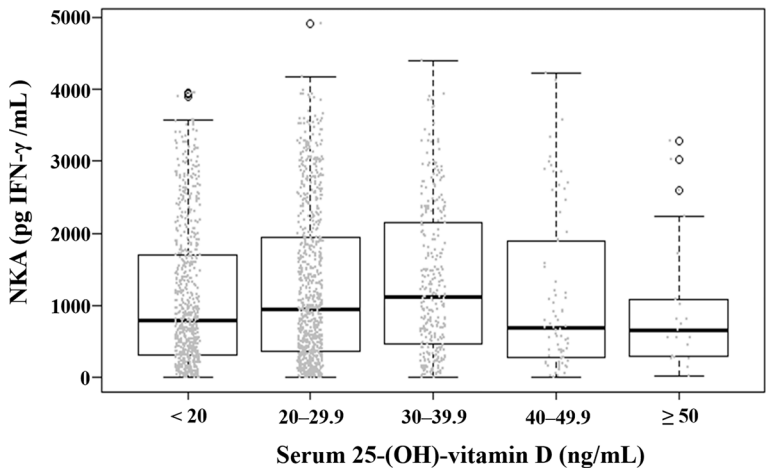

FIGURE 3 | Distribution of natural killer cell activity value according to serum level of 25-(OH)-vitamin D. Each dot represents the individual value of natural killer cell activity. Box plots show group medians, interquartile range (IQR), and spread of data with outliers for each group. NKA, natural killer cell activity.

Vitamin D, Exercise, and NKA

Compared to 25(OH)D < 20 ng/mL, the 25(OH)D 30–39.9 ng/mL significantly decreased the risk of very low NKA in men (OR: 0.358; 95% CI: 0.138, 0.929; $P = 0.035$). But this association was not observed in women (**Table 2**). The risks of very low NKA in the total population are depicted according to 25(OH)D level in **Figure 4**.

Compared to lack of physical exercise, low exercise (OR: 0.529; 95% CI: 0.299, 0.939; $P = 0.030$) and medium to high exercise (OR: 0.522; 95% CI: 0.277, 0.981; $P = 0.043$) decreased the risk of very low NKA in women (**Table 2**). In men, physical exercise was not associated with the risk (**Table 2**).

We checked the correlation between 25(OH)D (continuous variable) and exercise (categorical variable). 25(OH)D had a significant correlation with exercise ($P < 0.001$, r = 0.173). The mean value of 25(OH)D level increased gradually from the no-exercise group (19.2 ± 9.92 ng/mL) to low exercise (22.4 ± 10.8 ng/mL), medium exercise (23.3 ± 10.0 ng/mL), and high exercise group (24.9 ± 10.5 ng/mL) (**Figure 5**).

To investigate the independent association of vitamin D with NKA, we performed multiple logistic regression analyses in subgroups divided by exercise (**Table 3**). In the subjects who did not exercise, 25(OH)D 20–29.9 ng/mL decreased the risk of very low NKA (OR: 0.449; 95% CI: 0.202, 0.998; $P = 0.049$).

NKA and Age

In men, older-age was associated with increased risk of very low NKA (**Table 2**). Compared to age 18-40 years, the age-associated

TABLE 2 | Factors associated with very low NKA < 100 pg/mL compared to normal NKA ≥ 500 pg/mL.

	Total population		Women		Men	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Gender						
Women	1 (ref)		–		–	
Men	0.928 (0.649, 1.33)	0.680	–		–	
Age (years)						
18-40	1 (ref)		1 (ref)		1 (ref)	
41-50	1.06 (0.649, 1.74)	0.812	0.882 (0.485, 1.60)	0.680	1.80 (0.701, 4.61)	0.222
51-60	1.25 (0.756, 2.06)	0.387	0.577 (0.289, 1.15)	0.119	3.74 (1.53, 9.12)	**0.004**
61-70	1.75 (0.945, 3.24)	0.075	0.774 (0.322, 1.86)	0.567	5.59 (2.00, 15.6)	**0.001**
≥71	3.26 (1.38, 7.70)	**0.007**	0.940 (0.196, 4.51)	0.938	12.4 (3.62, 42.7)	**<0.001**
Physical exercise						
Lack of exercise	1 (ref)		1 (ref)		1 (ref)	
Low exercise	0.691 (0.454, 1.05)	0.086	0.529 (0.299, 0.939)	**0.030**	0.914 (0.470, 1.78)	0.790
Medium to high exercise	0.551 (0.345, 0.879)	**0.012**	0.522 (0.277, 0.981)	**0.043**	0.612 (0.295, 1.27)	0.188
HbA1c (%)						
<6.5	1 (ref)		1 (ref)		1 (ref)	
≥6.5	1.18 (0.573, 2.41)	0.659	2.312 (0.728, 7.35)	0.155	0.745 (0.291, 1.91)	0.538
25-(OH) Vitamin D (ng/ml)						
<20	1 (ref)		1 (ref)		1 (ref)	
≥20, <30	0.765 (0.508, 1.15)	0.200	0.751 (0.417, 1.35)	0.338	0.772 (0.425, 1.40)	0.396
≥30, <40	0.557 (0.315, 0.984)	**0.044**	0.754 (0.368, 1.55)	0.440	0.358 (0.138, 0.93)	**0.035**
≥40	0.720 (0.293, 1.77)	0.474	0.754 (0.214, 2.65)	0.659	0.867 (0.232, 3.23)	0.831

NKA, natural killer cell activity.

P values in bold denote statistical significance at the P < 0.05 level.

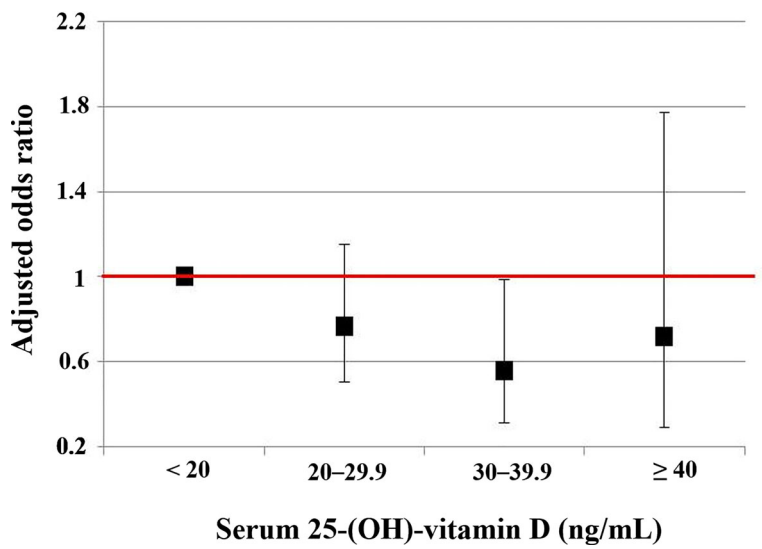

FIGURE 4 | The risks of the very low NKA < 100 pg/mL according to serum level of 25-(OH)-vitamin D compared to normal NKA ≥ 500 pg/mL. This is a result from a multiple regression analysis, and numeric data are shown in the first column of **Table 2**. Vertical lines show 95% confidence intervals.

risk in men was stepwise increase from age 51–60 years (OR: 3.74; 95% CI: 1.53, 9.12; $P = 0.004$) to age 61–70 years (OR: 5.59; 95% CI: 2.00, 15.6; $P = 0.001$) and age ≥ 71 years (OR: 12.4; 95% CI: 3.62, 42.7; $P < 0.001$) (**Table 2**). On the other hand, women did not show the age-associated changes in risk (**Table 2**).

In men with age ≤ 60 years (age 18–60 years), 25(OH)D level 30–39.9 ng/mL significantly decreased the risk of very low NKA (OR: 0.112; 95% CI: 0.0147, 0.855; $P = 0.035$) (**Table 4**). In women with age ≤ 60 years, there was no significantly associated factor.

In subjects with age > 60 years, men had a higher risk of very low NKA than women (OR: 2.48; 95% CI: 1.01, 6.08; $P = 0.048$) (**Table 5**). There was no significant difference between age 61–70 years and age > 70 years in the risk of very low NKA (**Table 5**). Compared to lack of exercise, low exercise (OR: 0.154; 95% CI:

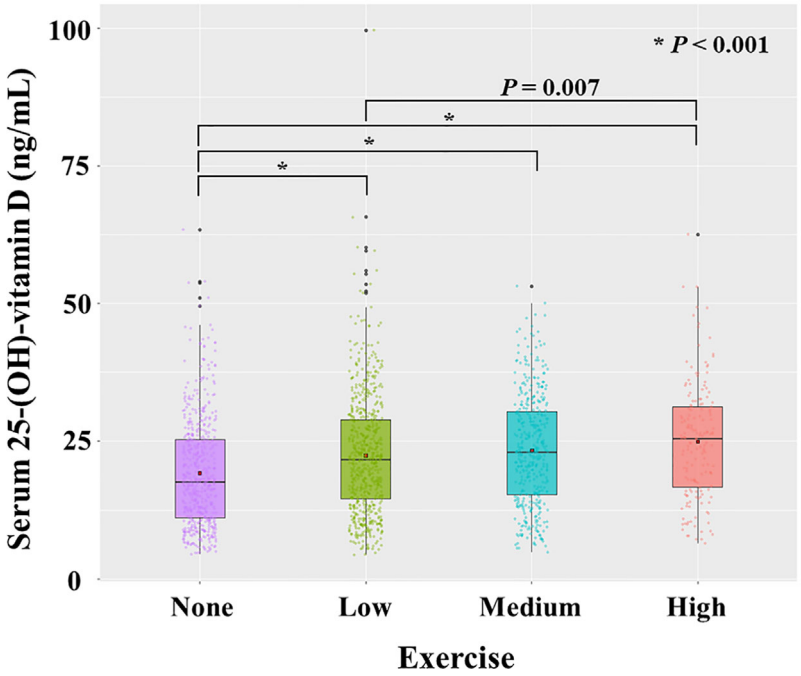

FIGURE 5 | Distribution of serum level of 25-(OH)-vitamin D according to exercise. Each dot represents the individual value of 25-(OH)-vitamin D. Box plots show group medians, interquartile range (IQR), and spread of data with outliers for each group. A dot in each box plot shows the mean value. *P* values were calculated with student *t* test.

TABLE 3 | Factors associated with very low NKA < 100 pg/mL compared to normal NKA ≥ 500 pg/mL in subjects who do or do not exercise.

	Subjects who do not exercise		Subjects who exercise	
	OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**
Gender				
Women	1 (ref)		1 (ref)	
Men	0.579 (0.306, 1.10)	0.094	1.19 (0.757, 1.86)	0.457
Age (years)				
18-40	1 (ref)		1 (ref)	
41-50	1.01 (0.476, 2.12)	0.990	1.05 (0.540, 2.03)	0.892
51-60	1.25 (0.553, 2.84)	0.589	1.18 (0.615, 2.24)	0.625
61-70	3.34 (1.19, 9.40)	**0.022**	1.23 (0.557, 2.70)	0.612
≥ 71	21.0 (1.63, 271)	**0.020**	2.38 (0.879, 6.47)	0.088
Physical exercise				
Low exercise	–		1 (ref)	
Medium to high exercise	–		0.801 (0.509, 1.26)	0.337
HbA1c (5)				
<6.5	1 (ref)		1 (ref)	
≥6.5	1.26 (0.328, 4.87)	0.733	1.18 (0.495, 2.79)	0.714
25-(OH) Vitamin D (ng/ml)				
<20	1 (ref)		1 (ref)	
≥20, <30	0.449 (0.202, 0.998)	**0.049**	0.953 (0.579, 1.57)	0.851
≥30, <40	0.783 (0.297, 2.06)	0.620	0.513 (0.252, 1.04)	0.066
≥40	1.59 (0.364, 6.90)	0.540	0.530 (0.155, 1.81)	0.311

NKA, natural killer cell activity; OR, odds ratio; CI, confidence interval; ref, reference.
P values in bold denote statistical significance at the P < 0.05 level.

0.050, 0.473; $P = 0.001$) and medium to high exercise (OR: 0.180; 95% CI: 0.060, 0.537; $P = 0.002$) decreased the risk significantly (**Table 5**). These associations were observed both in men and women with age > 60 years in separate subgroup analyses (**Table 5**).

DISCUSSION

We investigated how age, gender, vitamin D, and physical exercise were associated with immune function determined by

TABLE 4 | Factors associated with very low NKA < 100 pg/mL compared to normal NKA ≥ 500 pg/mL in subjects with age ≤ 60.

	Total population (age ≤ 60)		Women (age ≤ 60)		Men (age ≤ 60)	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Gender						
Women	1 (ref)		–		–	
Men	0.697 (0.460, 1.06)	0.089	–		–	
Age (years)						
18-40	1 (ref)		1 (ref)		1 (ref)	
41-50	1.08 (0.656, 1.76)	0.775	0.859 (0.473, 1.56)	0.619	1.86 (0.725, 4.80)	0.196
51-60	1.17 (0.694, 1.97)	0.558	0.547 (0.268, 1.12)	0.097	3.50 (1.40, 8.74)	**0.007**
Physical exercise						
Lack of exercise	1 (ref)		1 (ref)		1 (ref)	
Low exercise	0.862 (0.540, 1.38)	0.535	0.654 (0.357, 1.20)	0.169	1.31 (0.600, 2.88)	0.495
Medium to high exercise	0.676 (0.396, 1.15)	0.150	0.616 (0.309, 1.23)	0.169	0.920 (0.379, 2.23)	0.853
HbA1c (%)						
<6.5	1 (ref)		1 (ref)		1 (ref)	
≥6.5	0.984 (0.338, 2.87)	0.977	2.93 (0.748, 11.5)	0.123	0.289 (0.0375, 2.23)	0.233
25-(OH) Vitamin D (ng/ml)						
<20	1 (ref)		1 (ref)		1 (ref)	
≥20, <30	0.673 (0.417, 1.09)	0.105	0.747 (0.393, 1.42)	0.374	0.603 (0.288, 1.26)	0.180
≥30, <40	0.496 (0.246, 1.00)	**0.050**	0.803 (0.367, 1.76)	0.583	0.112 (0.0147, 0.855)	**0.035**
≥40	1.127 (0.419, 3.03)	0.813	1.23 (0.340, 4.41)	0.756	1.28 (0.258, 6.31)	0.765

NKA, natural killer cell activity; OR, odds ratio; CI, confidence interval; ref, reference.
P values in bold denote statistical significance at the P < 0.05 level.

TABLE 5 | Factors associated with very low NKA < 100 pg/mL compared to normal NKA ≥ 500 pg/mL in subjects with age > 60.

	Total population (age >60)		Women (age >60)		Men (age >60)	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Gender						
Women	1 (ref)		–		–	
Men	2.48 (1.01, 6.08)	0.048	–		–	
Age (years)						
61-70	1 (ref)		1 (ref)		1 (ref)	
≥ 71	2.04 (0.797, 5.24)	0.137	1.62 (0.267, 9.88)	0.599	2.43 (0.788, 7.50)	0.122
Physical exercise						
Lack of exercise	1 (ref)		1 (ref)		1 (ref)	
Low exercise	0.154 (0.0501, 0.473)	**0.001**	0.108 (0.0144, 0.810)	**0.030**	0.188 (0.0423, 0.836)	**0.028**
Medium to high exercise	0.180 (0.0603, 0.537)	**0.002**	0.180 (0.0323, 1.00)	**0.050**	0.191 (0.0441, 0.828)	**0.027**
HbA1c (%)						
<6.5	1 (ref)		1 (ref)		1 (ref)	
≥6.5	1.06 (0.340, 3.33)	0.915	2.45 (0.219, 27.4)	0.468	0.861 (0.240, 3.09)	0.819
25-(OH)-Vitamin D (ng/ml)						
<20	1 (ref)		1 (ref)		1 (ref)	
≥20, <30	1.02 (0.372, 2.82)	0.964	0.662 (0.127, 3.46)	0.625	1.48 (0.379, 5.80)	0.572
≥30, <40	0.834 (0.266, 2.62)	0.755	0.573 (0.0894, 3.67)	0.557	1.22 (0.264, 5.59)	0.802
≥40	0.312 (0.0342, 2.84)	0.301	–		0.922 (0.0750, 11.3)	0.949

NKA, natural killer cell activity; OR, odds ratio; CI, confidence interval; ref, reference.
P values in bold denote statistical significance at the P < 0.05 level.

NKA. The association of each factor with NKA varied widely according to age and gender. Age was the determining factor for the very low NKA in men but not in women. Sufficient level of vitamin D reduced the risk for very low NK in men, and physical exercise reduced the risk in women. In men with age < 60 years, sufficient level of vitamin D reduced the risk for the very low NKA. In the subjects with age ≥ 60, physical exercise reduced the risk both in men and women. DM is well-known to diminish immune function, but neither DM nor hyperglycemia represented by Hb A1c showed significant association with NKA when adjusted for other factors. To the best of our knowledge, this is the first observation demonstrating age- and gender-specific association of vitamin D and physical exercise with NKA.

Vitamin D plays an important role in immune health by affecting the maturation and differentiation of various immune cells, inducing production of antiviral peptide cathelicidins and defensins, and reducing production of pro-inflammatory cytokines (34, 35). Thus, vitamin D insufficiency [i.e., serum 25 (OH)D level 20–29.9 ng/mL] or deficiency [serum 25(OH)D

level < 20 ng/mL] are associated with increased infection susceptibility, increased cancer incidence, poor survival of cancer patients, and development of autoimmune diseases (34, 36, 37). For example, recent studies suggested that vitamin D insufficiency and deficiency were associated with increased susceptibility to COVID-19 and severe presentation and mortality from it (38–40). Vitamin D, having an immune modulatory function, is considered to reduce the production of pro-inflammatory cytokines, thus, reducing the severity and mortality of COVID-19 (35). Therefore, in case of vitamin D deficiency, vitamin D supplementation or rapid correction of 25 (OH)D level with high-dose regimens were recommended by experts (35, 41, 42). For normal calcium homeostasis and bone health, many studies showed that serum 25(OH)D around 20 ng/mL is required (43). Yet, the optimal concentration of vitamin D for proper immune reactions has not been determined. For normal immune function, it is considered that much higher level than 25(OH)D 20 ng/mL is required (36). *In vitro*, 65 ng/mL 25(OH)D has been found to be more effective than 30 ng/mL or 90 ng/mL in inducing human NK cell-mediated antibody-dependent cell cytotoxicity (44). In adults, 25(OH)D ≥ 38 ng/mL, compared to lower levels, was associated with lower incidence of acute viral respiratory infection (45). In women, 25(OH)D ≥ 40 ng/mL was associated with lower risk of invasive cancer (46). On the other hand, serum 25(OH)D level revealed a U-shaped association with all-cause mortality. The all-cause mortality was the lowest with serum 25(OH)D levels of 30–40 ng/mL in a general population comprising of 26,916 European individuals (47) and a hospitalized population comprising of 24,094 adult patients (48). Our results indicated that the serum 25(OH)D level of 30–40 ng/ml might be optimal for the NKA. The correction target 40–60 ng/mL against COVID-19 suggested by some experts (41) may have directed in terms of immune modulatory function. Our results demonstrated that vitamin D supplementation might be a good strategy to take care of immune health. Albeit, caution should be taken in vitamin D supplementation because excessive vitamin D may cause disturbance of calcium metabolism.

Physical exercise is a well-known factor that enhances immune function. Many studies reported that physical exercise improved health outcome of cancer patients and ameliorated immunosenescence in the elderly population (49–53). A meta-analysis demonstrated that higher level of habitual physical activity is associated with reduced risk of community-acquired infectious disease and mortality by it (54). Also, physical activity interventions resulted in improved state of immune markers: increased CD4 cell counts, increased salivary immunoglobulin IgA concentration, decreased neutrophil counts, and higher antibody concentration after vaccination (54). One more important factor between the physical exercise and immune function is the intensity or duration of the exercise. In an animal study, mice infected with influenza showed significantly higher survival with moderate exercise, but prolonged exercise had a worse outcome than that of the control, although it was not statistically significant (55). Similar findings were observed in humans. Early observations suggested that athletes who were subjected to strenuous exercise were more prone to getting infectious diseases during outbreaks of the infectious diseases (56). Following studies demonstrated that immune function increased immediately after maximal exercise but depressed afterwards, and it took some time for the immune function to fully recover (57–60). Based on these findings, it was hypothesized that there exists an "open window" for opportunistic infection lasting up to 72 hours after a prolonged endurance exercise, so repeated strenuous exercise without adequate recovery may prolong the "open window" and lead to impaired immune function (57, 61). Therefore, moderate physical exercise in intensity and duration followed by adequate recovery time should be emphasized in lifestyle management. The current study demonstrated that the lack of physical exercise was associated with the risk of very low NKA. This was consistent with a previous study which showed that physical inactivity was associated with decreased NKA (62). Furthermore, our study suggested that physical exercise may be immunologically more beneficial to women.

As life expectancy increases, strategies to enhance healthy life expectancy are of attention (63, 64). As human body ages, immune function also ages, so called immunosenescence. Immunosenescence results in persistent low-grade inflammation, autoimmune diseases, allergic diseases, poor vaccine responses, increased susceptibility to severe infections, increased cancer incidence, and high mortality in the elderly (65–70). Research effort began as early as 1980's to find immune characters that define healthy longevity. A Swedish longitudinal cohort study with octogenarians and nonagenarians revealed that a combination of immune characters (inverted $CD4^+$:$CD8^+$ ratio (< 1), poor T-cell proliferation response to mitogens *in vitro*, and increased number of $CD8^+$ T lymphocytes representing effector-memory T cells) was associated with mortality (6–8). However, this trend was not reproduced in more recent studies conducted in other countries (10, 11). In our study, we presented age-associated changes in NKA: older-age increased the risk of the very low NKA in men. Compared to adaptive or other innate immune cells, NK cell function is better preserved along the immunosenescence process owing to the reciprocal increase in NK cell counts to compensate per-cell functional decrease (71, 72). Thus, NKA or NK cell counts may serve as a good parameter of immunosenescence or healthy immune aging. Further, the present study showed that physical exercise reduced the risk for very low NKA in individuals with age ≥ 60 years. This is consistent with previous studies that investigated the role of physical exercise in ameliorating immunosenescence. For example, moderate cardiovascular exercise in healthy older adults resulted in increased seroprotection after influenza vaccination while balance and flexibility intervention did not (73), and low-dose combined resistance and endurance training for 6 weeks in the elderly resulted in improvements in immune markers: an increase of the CD4+/CD8+ T cell ratio and decrease in systemic levels of interleukin (IL-) 6, IL-8, IL-10 and vascular endothelial growth factor (VEGF) (74). Recent knowledge indicates that there may be a cause and an effect relationship between physical exercise and health outcomes mediated by enhanced immune function (53).

It is discussed that the key role in that relationship may be played by skeletal muscle that the muscle takes an immune regulatory function by producing myokines, that is cytokines released by muscle cells (53). Thus, avoiding sarcopenia by physical exercise may serve as a potential strategy to delay immunosenescence in the elderly (53).

Generally, women live longer and are healthier than men (63). This gender-dependent life expectancy can be closely linked to a gender-specific difference in immunity that is attributed to lifestyle and biological factors (75). Women carry two X chromosomes containing many genes related to immunocompetence, including Toll-like receptors, cytokine receptors, immune-response-related proteins, and transcriptional and translational control effectors (76). In contrast, the Y chromosome contains male-specific inflammatory genes (77). Also, sex hormones affect immune cells differently. Estradiol is immune-enhancing, whereas testosterone is immune-suppressive (75). With advancing age, decline in adaptive immune parameters is greater in men (75), and reciprocal increase in NK cells is greater in women (78). In our study, NKA decreased with age in men but not in women. This supports that NK cells compensate immunosenescence better in women than in men.

This study has some limitations. It was a retrospective study conducted at a single health check-up center that it may have selection bias. The questionnaire used in the health check-up did not ask the intensity of the physical exercise but the frequency and duration only. The population size was not large enough to allow significant results in all meaningful factors including hyperglycemia. Nevertheless, we analyzed the data as accurately as possible and obtained several novel insights on gender, age, exercise, vitamin D, and immune function.

The present study demonstrated that vitamin D reduced the risk of very low NKA in men, and physical exercise did so in women and in individuals with age ≥ 60 years. Age was a major risk factor of very low NKA in men but not in women. Thus, there were gender- and age-dependent differences in factors that was associated with NK cell immune function. We hope these findings help to delineate an individulized strategy to enhance immune function in the immunologically normal population.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data pertains to our institute, and further analyses are now being undertaken. Requests to access the datasets should be directed to SO, ohsooyoun@hotmail.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CHA Bundang Medical Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SO, SC, and S-WC contributed to the study design, data collection, study analysis, manuscript writing, critical review of the manuscript, and final approval of the manuscript submission. SH, JK, YC, JL, and KK assisted in analyzing and interpreting the data, critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Enhancing Checkpoint Inhibitor Therapy in Solid Tissue Cancers: The Role of Diet, the Microbiome & Microbiome-Derived Metabolites

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Host immunity plays a central role in the regulation of anti-tumour responses during checkpoint inhibitor therapy (CIT). The mechanisms involved in long lasting remission remain unclear. Animal studies have revealed that the microbiome influences the host immune response. This is supported by human studies linking a higher microbial richness and diversity with enhanced responses to CIT. This review focuses on the role of diet, the microbiome and the microbiome-derived metabolome in enhancing responses to current CIT in solid tissue cancers. The Western diet has been associated with dysbiosis, inflammation and numerous metabolic disorders. There is preliminary evidence that lifestyle factors including a high fibre diet are associated with improved responses to CIT via a potential effect on the microbiota. The mechanisms through which the microbiota may regulate long-term immunotherapy responses have yet to be determined, although bacterial-metabolites including short chain fatty acids (SCFAs) are recognized to have an impact on T cell differentiation, and may affect T effector/regulatory T cell balance. SCFAs were also shown to enhance the memory potential of activated CD8 T cells. Many therapeutic approaches including dietary manipulation and fecal transplantation are currently being explored in order to enhance immunotherapy responses. The microbiome-derived metabolome may be one means through which bacterial metabolic products can be monitored from the start of treatment and could be used to identify patients at risk of poor immunotherapy responses. The current review will discuss recent advances and bring together literature from related fields in nutrition, oncology and immunology to discuss possible means of modulating immunity to improve responses to current CIT.

Keywords: cancer immunotherapy, microbiome, metabolome, checkpoint inhibitor therapy, short chain fatty acids

INTRODUCTION

Checkpoint inhibitor therapy (CIT) has revolutionized cancer treatment paradigms to date, but much progress remains to be made. In fact, 60-70% of patients do not respond to single agent immunotherapy (1–3). Clinicians are in need of predictive biomarkers in order to successfully identify patients who are most likely to have a long-lasting treatment response (4). Novel therapeutic targets designed to boost responses to existing CIT would enhance and expand therapeutic efficacy and application. Animal studies have confirmed that both spontaneous tumor-specific T cell responses as well as subsequent responses to CIT are microbiota dependent (5, 6). Clinical studies have corroborated these findings with compelling evidence that microbial richness and diversity is associated with a durable response to immunotherapy (7).

Diet remains the major determinant of microbial composition and a high quality diet that is rich in fibre has been associated with improved immunotherapy responses (8). The following review will focus on new developments relating to diet, the microbiome and the microbiome-metabolome with respect to augmenting immunotherapy responses. We will discuss dietary manipulation, use of pre and pro-biotics and fecal transplantation and their potential impact on the outcomes of checkpoint inhibitor therapy. The microbiome-derived metabolome is a new area under investigation and warrants discussion as both a potential novel predictive biomarker and a target for enhancing responses to treatment (9, 10).

DIET AND RESPONSES TO CHECKPOINT INHIBITOR THERAPY

The microbiome is defined as the trillions of bacteria, viruses and fungi colonizing most surfaces of the human body (11). Diet remains the major determinant of the composition of the gut microbiome and a variation in nutrients can induce significant changes within a 24-hour period (12, 13). The standard western diet (WD) which is typically characterized as a high fat, high carbohydrate and low fibre diet. influences the microbiota in many ways including increased bile acid secretion into the gastrointestinal tract, generation of bile-tolerant organisms, dysbiosis and decreased downstream production of short chain fatty acids (SCFA) (14–17) (**Figure 1**).

The intestinal microbiota is a key regulator of immune response during both health and disease (18). For example, the composition of the gut microbiota is known to differ significantly between agrarian and western societies and may provide an explanation for the dramatic rise of allergic and autoimmune disease states in western countries (15, 17, 19–21).

De Filippo et al. (2010) showed that children residing in the remote African community of Burkina Faso (BF) had significantly altered gut microbial composition, compared to those residing in European cities (EU) (19). Key changes for children from BF included enrichment in Bacteroidetes and depletion of Firmicutes ($p < 0.001$) together with significantly higher SCFA production ($p < 0.001$) compared to EU children. *Shigella* and *Escherichiae* were also significantly under-represented for BF children. These findings have been attributed to the predominantly plant-based, high-fibre diet that is consumed in rural Africa (19). The health benefits of a high fibre diet have been apparent over many years of research and have been typically associated with increased SCFA production driving regulation of immunological tolerance and promoting gut homeostasis. The impact of diet on checkpoint inhibitor therapy responses is presently under investigation in view of research supporting the importance of the microbiome as a regulator of immune response (8).

Initial data from a study of 113 melanoma patients undergoing CIT confirmed that a high fibre diet was associated with increased microbial richness and highest odds of response (8). Whole grains, fruits and vegetables were associated with a 'responder' microbial signature, whilst sugars and processed meat had a negative association (8). Patients who were following a high fibre diet were five times more likely to respond to CIT, compared to patients on a low fibre-diet (OR = 5.3, 95% CI: 1.02 – 26.3) (8).

At present, there are no specific dietary guidelines for patients undergoing immunotherapy and evidence for dietary approaches remains preliminary. Multiple publications over the past 10 years have suggested that dietary fibre and specifically SCFA are key regulators of T cell homeostasis (22–30) (**Table 1**). These early-stage findings from Spencer et al. appear to support the concept that fibre is immunomodulatory (8).

IMPACT OF SHORT CHAIN FATTY ACIDS ON IMMUNITY AND T CELL FUNCTION

SCFA including acetate, butyrate and propionate are the bacterial metabolites of fermented fibre and are known regulators of T cell differentiation and function (22–31) (**Figure 1**). Exactly how a high fibre diet enhances checkpoint inhibitor therapy, which relies on CD8+ T cell infiltration into the tumor requires further investigation. Pre-clinical studies suggest that the effects of SCFA on T cell differentiation may depend on the immunological context and the concentration of the relevant SCFA (27, 29). Park et al. (27) showed that T cell differentiation is affected by the local cytokine milieu and that SCFA could promote either effector or regulatory T (Treg) cell development. Kesphol et al. (29) showed that the effects of butyrate on T cell differentiation were in fact dose dependent. Physiological concentrations of butyrate could promote Treg production, whereas higher butyrate concentrations could enhance development of IFNγ producing Tregs or conventional T cells. SCFA have also been shown to enhance generation of macrophage and dendritic cell precursors *via* systemic effects on bone marrow haemataopoiesis (30). Most recently, Bachem et al. (31) showed that the microbiota could affect CD8+ T cell function. In particular, butyrate was shown to enhance the memory potential of activated CD8+ T cells (31).

Enhancing immunotherapy responses through use of dietary fibre and its impact on SCFA production requires further study,

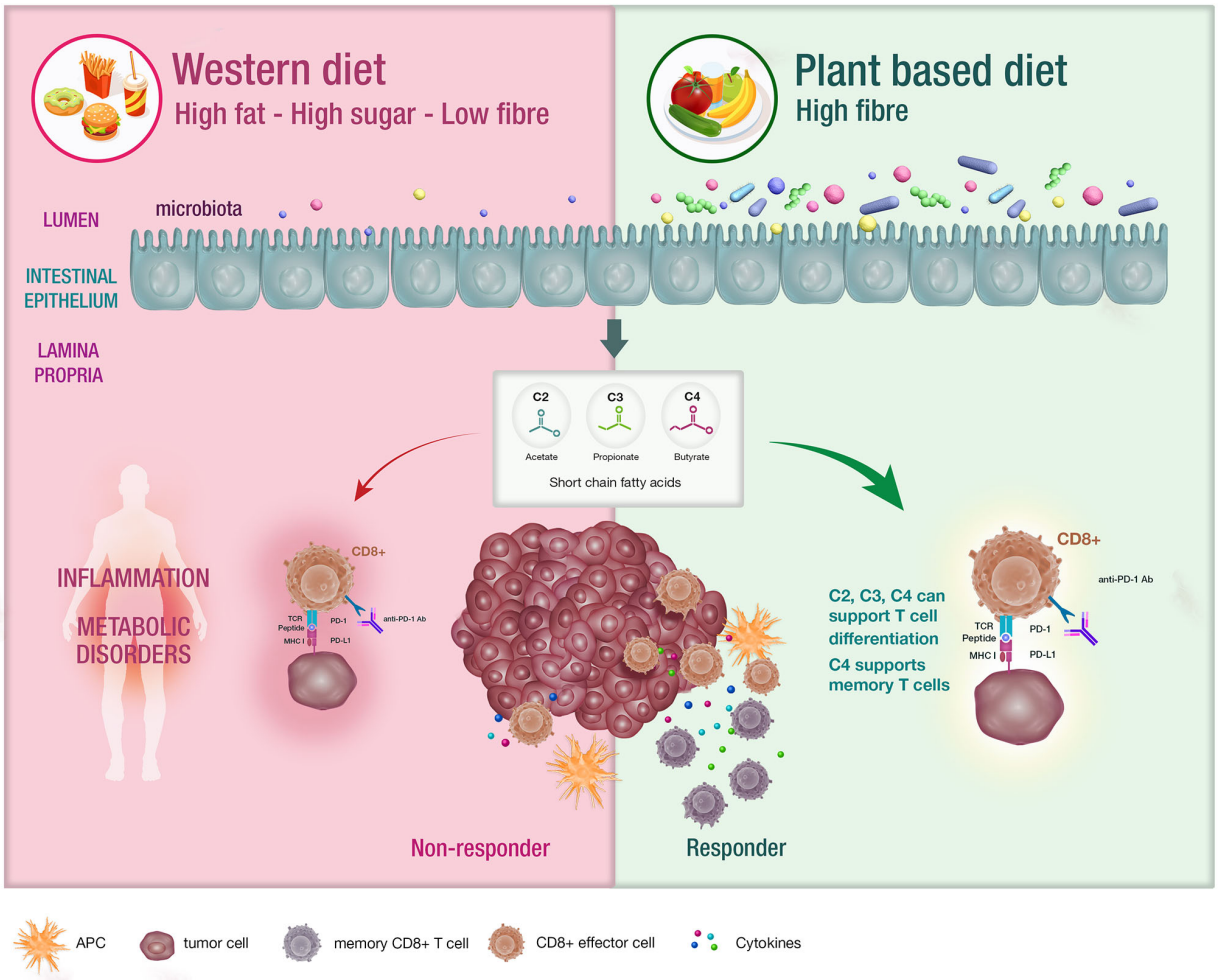

FIGURE 1 | The impact of diet on the microbiota and systemic immunity. Diet has an impact on the host microbiota with the western diet being linked with dysbiosis. Conversely, diets that are high in fibre have been associated with bacterial diversity in the microbiota , which is postulated to support appropriate T cell differentiation. These patients are likely to experience enhanced responses to CIT.

although this would be a straight forward and risk averse intervention. Elucidating the mechanisms through which fibre may enhance treatment responses is likely to be a more complex question that may be addressed further through study of the microbiome-derived metabolome in humans (9, 10).

ANTIBIOTICS IMPAIR IMMUNOTHERAPY OUTCOMES

Dysbiosis and perturbation of gut flora is a known consequence of antibiotic use (32). Infection and antibiotic use are common occurrences during the course of cancer treatment. There is now a well-documented association between antibiotic use and poor therapeutic outcome. Patients with antibiotic exposure have impaired treatment responses, including decreased response rates, shorter progression free and diminished overall survival (33–38). There is a wide variation in the cited overall survival times for solid tissue cancers treated with CIT for groups with and without antibiotic exposure (34). Nonetheless, the impact of antibiotics appears to be clinically significant and detrimental. In a large, retrospective study of 568 stage III and stage IV melanoma patients, the authors confirmed that the antibiotic exposed group had a significantly worse OS of 27.4 months, compared to 43.7 months for the antibiotic-unexposed group (hazard ratio 1.81, 95% confidence interval 1.27-2.57, $p < 0.001$) (38). At this stage, mechanistic studies are lacking and most of the available data is retrospective (33). There is also a possibility that patients requiring antibiotic therapy are potentially a more unwell group in terms of susceptibility to infection and compromised immunity leading to suboptimal treatment outcomes (33). Further clinical trials are awaited to determine which interventions could be implemented to improve clinical outcomes for patients who find themselves in this common scenario.

TABLE 1 | Summary of studies implicating short chain fatty acids as modulators of T cell differentiation and function.

Study	Summary of study findings
Arpiai et al. (23)	- Butyrate facilitated extrathymic generation of Treg cells - *De novo* generation of Tregs was potentiated by propionate
Furusawa et al. (24)	Butyrate induced the differentiation of Treg cells *in vitro* and *in vivo*
Smith et al. (25)	Short chain fatty acids regulate the size and function of the colonic Treg pool
Park et al. (27)	SCFAs could promote T-cell differentiation into effector or regulatory T cells to promote either immunity or immune tolerance depending on immunological milieu.
Kesphol et al. (29)	- Lower butyrate concentrations facilitated differentiation of Tregs *in vitro* and *in vivo* - Higher concentrations of butyrate promoted IFN-γ-producing Tregs or conventional T cells
Bachem et al. (31)	- SCFA enhance the memory potential of antigen-activated $CD8^+$ T cells - Butyrate promoted memory potential of activated $CD8^+$ T cells, enhanced metabolism and promoted long-term survival as memory cells.

THE MICROBIOME AND REGULATION OF RESPONSES TO CHECKPOINT INHIBITOR THERAPY

In 2015, landmark pre-clinical mouse studies confirmed that the anti-tumor effects of CTLA-4 and PD-L1 blockade were facilitated by commensal intestinal flora (5, 6). Sivan et al. (2015) studied two groups of genetically similar mice from two different commercial sources (The Jackson Laboratory (JAX) and Taconic Biosciences (TAC)) with distinct microbiota composition (5). At baseline, JAX mice and TAC mice were noted to have significantly different rates of spontaneous melanoma growth. This was attributed to differences in spontaneous immunity between the two groups. High intra-tumoral CD8 T cell infiltration was associated with low melanoma growth (JAX mice), whilst low intra-tumoral CD8 T cell infiltration was associated with accelerated melanoma growth (TAC mice) (5). These differences in T cell immunity were shaped by the composition of the microbiota. Fecal microbiota transplant (FMT) of JAX mice (via oral gavage) to TAC recipients was sufficient to augment CD8+ T cell infiltration into tumor, and slow the melanoma growth rate to the same extent as treatment with an anti-programmed-death-ligand-1 antibody (anti-PD-1 Ab). *Bifodobacterium* were identified as being critical to antitumor immunity and could mediate therapeutic effects by enhancing host antitumor T cell responses including peripheral T cell induction, CD 8+ T cell infiltration into tumor and dendritic cell activation, which led to enhanced CD8+ T cell priming. Interestingly, Vetizou et al. (2015) (6) found that responses to CTLA-4 blockade were dependent on the presence of *Bacteroides*. In this study, therapeutic responses to anti-CTLA-4 mAb were tested in germ free and antibiotic treated mice. The anti-tumor effects of anti-CTLA-4 mAb were significantly compromised in these two groups but could be re-established following colonization with *B. fragilis* (6).

Since 2015, multiple clinical studies have supported the findings that the efficacy of CIT is microbiota dependent (7, 39–42). Whilst a single, consistent microbial 'responder' signature has not been identified, microbial richness with a high alpha diversity has been key findings associated with CIT responsiveness (7). Alpha diversity refers to the ecological richness of a given microbiome sample (43) as opposed to beta diversity, which is the diversity of microbes between two different samples. Bacterial species that have been associated with a treatment response have included taxa within the Ruminococcaceae family of the Firmicutes phylum (44). A lack of response has been associated with bacterial taxa within the Bacteroidales order of the Bacteroidetes phylum (44). Investigators have noted that there is an absence of definitive overlap between responder microbial signatures as described in various clinical studies, suggesting that efficacy may not rely entirely on a specific strain of bacteria but more likely on how the microbiome interacts with the immune system *via* the production of metabolites. It is likely that a favorable microbiome can lead to enhanced antigen presentation and effector T cell function leading to improved local anti-tumor responses and systemic immunity (7). Historically, tumor infiltration with CD8+ T cells has been associated with a favorable prognosis, which is in agreement with recent findings in immunotherapy (45–48).

The microbiota is an exciting therapeutic target that could be of enormous value. Mouse studies have demonstrated the role of FMT as a means of altering the microbiota to successfully effect tumor control. Whilst FMT is an established technique for treatment of refractory *C Difficile* infection (CDI), it is not a standard technique for other dysbiotic states (49). Further study is needed to investigate the mechanisms through which FMT is able to reconstitute a functional gut microbiome in the CDI setting. There may be unique ecological factors during CDI that render FMT effective, whereas in other dysbiotic states, such as ulcerative colitis, results have not been as great. Other more practical issues with FMT will include presence or absence of facilities to carry out the procedure, our lack of understanding of what constitutes an ideal donor and the obvious difficulties with standardization of fecal donor specimens (50). Clinical trials (NCT03353402; NCT03341143) are presently underway using fecal donor material from complete responders and a phase I trial has been completed (NCT03353402) confirming safety of this procedure in a small group of patients (51). We suggest that the fecal and serum microbiome-derived metabolome will provide greater insight into the functional metabolic products of specific microbial communities and will be able to quantify these, providing researchers with new therapeutic applications.

Probiotics are defined as live organisms that are taken orally in order to provide health benefits to the host. Conventional probiotics are available over the counter and usually contain limited bacterial strains. Probiotics have been associated with detrimental effects during checkpoint inhibitor therapy including lower microbiome diversity (8). Whilst murine models have

confirmed the proof of principle in that certain commensals appear essential to immunotherapy responses, the complexity of the microbiome in humans is such that it would be unrealistic to re-create a responder phenotype with conventional probiotics. In an elegant study, Suez et al. (2018) randomized healthy human volunteers to treatment with broad spectrum antibiotics followed by either watchful waiting, FMT or treatment with an 11-strain probiotic cocktail (52). FMT resulted in rapid reconstitution of indigenous microbial flora, whereas probiotics resulted in significant delays to reconstitution of normal flora that lasted up to 5 months post probiotic cessation. At present, patients on CIT should be cautioned against the use of probiotics as they appear to be detrimental in this setting.

THE MICROBIOME-DERIVED-METABOLOME

The metabolome is a relatively new concept that describes the metabolites in a biological system. Metabolomics is performed utilizing mass spectrometry based techniques and can look at the end metabolic products of gut bacteria (either fecal or serum samples). These may represent the metabolic end products of the bacteria that are present. As the ideal 'microbial' responder signature has not been identified, there is a possibility that different bacterial communities may ultimately exert similar immunologic outcomes through common metabolic end products, such as SCFA.

Investigators have assessed the role of the microbiome-derived metabolome in patients undergoing anti-PD-1 therapy and have found that those who were classed as good responders had higher levels of SCFA, compared to patients who had early progressive disease (9, 10). Nomura et al. (2020) assessed serum and fecal metabolites in 52 patients with mixed solid tissue cancers undergoing single agent immunotherapy (9). Fecal concentrations of acetic acid, propionic acid, butyric acid, valeric acid (p =0.05 - 0.002) and plasma isovaleric acid ($p < 0.01$) were associated with significantly prolonged progression free survival times (9). Botticelli et al. (2020) showed that non-small cell lung cancer (NSCLC) patients (n = 11) who had early disease progression within 3 months of starting nivolumab had fecal samples that were characterized by low levels of SCFAs (propionic, butyric, acetic, valeric acids), compared to long-term responders (progression free survival > 12 months) (10). In contrast to these studies, a separate group, which looked at (mostly melanoma) patients undergoing anti-CTLA-4 Ab monotherapy (n = 85) found that elevated levels of SCFAs were associated with disease progression (53). In this study, low baseline butyrate and propionate were associated with longer PFS ($p = 0.0015$ and $p = 0.0029$ respectively). Much larger studies with a single-tumor focus looking at both single agent and combination immunotherapy are required to confirm these findings.

We postulate that the microbiome-derived-metabolome is a predictive biomarker of response and may be able to identify patients who are at greater need of early intervention (e.g. dietary) in order to augment immunotherapy responses. Serial monitoring of the microbiome-metabolome may also be possible during a patient's treatment in order to assess levels of SCFA as a guide to immunological response.

A therapeutic application is a theoretical possibility. Metabolites can be more readily quantified and regulated compared to complex bacterial ecosystems and may be easier to manipulate in order to induce an immune response. SCFA administration has been utilized in the setting of dysbiosis with autoimmune bowel disease (54). Whilst results have not been favorable to date, the exploration of this approach has not been complete (54).

DISCUSSION

The advent of CIT has led to a new exploration of the host-tumor relationship and has raised many questions over what drives an effective host immune response. We now know, perhaps unsurprisingly, that both animals and humans with better baseline systemic and anti-tumor immunity go on to have better responses to CIT.

A key question is how to identify immunotherapy responders and more importantly, how to improve clinical outcomes for the non-responders. We have a range of different targets that could be manipulated although we are still awaiting the results of multiple studies that will direct our approach.

The function of the immune system is inextricably linked to the microbiota and we have clear evidence that perturbation of bacterial ecology through antibiotics has functional implications for immunotherapy, whereas potential enhancements could be achieved through nutritional manipulation.

Clinicians and scientists continue to search for a consistent, responder microbiome signature, although it is likely that there is more than one microbial profile that may be associated with good anti-tumor immunity. Microbial communities ultimately exert their effects through metabolic end products such as SCFA and different congregates of micro-organisms may produce the same beneficial metabolites.

Preliminary findings suggest that fibre is emerging as a modulator of immune response and this may not be surprising given the plethora of health benefits that have been associated with a plant-based, high-fibre diet. Further mechanistic studies are needed to define the immunomodulatory role of fibre, given the fact that it has traditionally been associated with Treg induction and immunological tolerance. Recent mouse studies have confirmed that SCFA including butyrate have activity in enhancing CD8+ T cell memory function and this has helped to shed light on the complex role that SCFA have in immune regulation (31).

FMT is presently under study as a technique for enhancing tumor control by introducing a responder microbiome. It is certainly appealing as a means of replacing an entire microbiota with one from a known immunotherapy-responder. Whilst this technique been successful in the setting of refractory CDI, it is unclear whether this FMT will gain traction in the immunotherapy setting, given its inconsistent results in other dysbiotic states.

The microbiome-derived-metabolome is a new concept that refers to measurement of the metabolic end products of the microbiome in either serum or feces. Metabolites, such as SCFA can be quantified and may have a potential application as a predictive biomarker and as a target for manipulating the host immune response. Furthermore, the adaptive mature of the microbiome-immune cross talk suggests that metabolite-based therapeutics might offer attractive new therapeutic avenues to enhance the immune response to CIT and provide a positive feedback signal to the microbial ecosystem, possibly extending the duration of therapeutic benefit.

The future may hold baseline microbiome and microbiome-metabolome profiling of patients at baseline as well as at several time points throughout their immunotherapy treatment. Correlation of the metagenomic and metabolomic aspects of the microbiome is required in order to have a better functional understanding of the human immune response during CIT.

AUTHOR CONTRIBUTIONS

AM and SN prepared the manuscript. NK and JC provided intellectual and editorial feedback. All authors contributed to the article and approved the submitted version.

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Physical Activity in Adolescents Living in Rural and Urban New Caledonia: The Role of Socioenvironmental Factors and the Association With Weight Status

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Physical activity (PA) is an important factor for the prevention of overweight and obesity, particularly during adolescence. This study focuses on the understudied adolescent population of New Caledonia with the aim to (1) determine the daily PA levels and estimate the sedentary time through out-of-school sitting time; (2) highlight the influence of sociodemographic and environmental factors, and (3) assess the associations of PA and sitting time with overweight and obesity. A sample of 508 school-going adolescents living in New Caledonia was surveyed about their PA habits using the International Physical Activity Questionnaire–Short Form, as well as about the context in which they usually engage in PA. The influences of the place of living and ethnic community were also investigated. Results indicated that about 66% of the adolescents performed an average of at least 60 min of PA daily. Both Melanesian adolescent boys and girls were more active than Caucasian adolescents but only when they lived in rural areas (females: 115 vs. 93 min/day, $p = 0.018$; males: 133 vs. 97 min/day, $p = 0.018$). Indeed, PA was reduced in an urban environment (females: 88 min/day; males: 95 min/day, $p = 0.028$; rural vs. urban in Melanesian adolescents). Melanesian adolescents also spent less time in out-of-school sitting than Caucasian adolescents independently of where they lived (females: 164 vs. 295 min/day, $p < 0.001$; males: 167 vs. 239 min/day, $p = 0.001$). Feeling safe was positively associated with PA levels (females: $OR_{adj} = 2.85$, $p < 0.001$; males: $OR_{adj} = 4.45$, $p < 0.001$). In the adolescent boys, accessibility to a suitable place was also an important factor ($OR_{adj} = 2.94$, $p = 0.002$). Finally, while PA and sitting time were negatively associated with overweight in male adolescents ($OR_{adj} = 0.28$, $p = 0.044$ and $OR_{adj} = 0.39$, $p = 0.004$), they were not in females. Living in a rural area allowed the Melanesian adolescents to maintain a more active lifestyle with more physical activities and less sitting time. Our results also indicated that safety was an important driver for engagement in PA. The urban environment in New Caledonia appears to be a contributor of a less active lifestyle in adolescents.

Keywords: exercise, sedentarity, lifestyle, Melanesian, Polynesian, Pacific, obesity, sitting time

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INTRODUCTION

Many factors are related to obesity, including gender, ethnic background, income level and educational background (1–3). Lifestyles and habits are also related to the development of overweight and obesity, particularly increased fast food and soft drink consumption, frequent dieting attempts, low physical activity (PA), and long hours watching television (1, 4). Sedentary time, which is a major risk factor for non-communicable diseases, has increased worldwide in the past few decades (5, 6). In addition, and despite the health benefits of PA (7), adolescents around the world do not meet the current guidelines (5, 8–11). Yet in children and adolescents, PA confers such benefits as improved physical fitness (including both cardiorespiratory and muscular fitness), cardiometabolic health (improved control of blood pressure, dyslipidemia, glucose, insulin resistance), bone health, cognitive outcomes (academic performance, executive function), and mental health (reduced symptoms of depression), and it is favorably associated with adiposity (12). While doing some PA is better than doing none, these benefits require an average of at least 60 min per day of moderate- to vigorous-intensity PA (MVPA) across the week (13, 14). This means that <60 min/day of MVPA would be considered as insufficient even if an individual was active all day in the course of professional activity. Conversely, someone having high sedentary time because of professional activity (in an office, for instance) could be considered as physically active provided that he/she has practiced at least 60 min/day of MVPA (13). Similarly, a schooled adolescent who is seated all day long in school can have sufficient MVPA time (before or after school times) to be considered physically active. Notably, sedentary behavior is often associated with unhealthy habits such as snacking and high screen times (4).

The social environment, which can influence health behaviors like PA and sedentary time, has received increasing attention in recent years (2, 3, 15–18). Influences from family and friends can occur through social pressure, social modeling and imitation, social comparison, and behavior approximation (8, 15, 19–22). For example, studies have highlighted that parental modeling and parental support may be associated with the child's and adolescent's PA (22–24). Friends may be more influential than other social contacts, however, because of a clearer understanding of the information coming friends, resulting in better internalization (16, 25, 26). Although social influences on PA occur throughout life, they are particularly important during adolescence, especially since behavior acquired at a young age can determine behaviors and lifestyle choices into adulthood (8, 23).

In the Pacific region, islanders have increasingly adopted Western modes of living in the past few decades (27, 28). This has caused fundamental changes in lifestyles and a dramatic increase in non-communicable diseases (29). Globalization, trade liberalization and increasing urbanization have all contributed to shifts in PA and diet, leading to a steadily increasing prevalence of overweight (30–35). The Polynesian and Melanesian populations of the French territories in the Pacific are particularly exposed to lifestyle Westernization (36–39). New Caledonia has the particularity of approaching the economic level of Western countries due to industrial and mining activities. While some Caledonians live in cities and have adopted a Western lifestyle, others still live a more traditional Pacific lifestyle, which is characterized by fishing, agriculture and cultural activities that follow traditional customs (e.g., house construction, weddings, mourning, customary ceremonies) and is generally associated with high daily PA (40). Conversely, Oceanian adolescents living in urban areas may adopt a more sedentary lifestyle (32, 41) that favors obesity and its health consequences. In New Caledonia, Zongo et al. investigated Melanesian adolescents' physical fitness, PA and body composition using questionnaires and anthropometric measures (42). They found that Melanesian adolescents living in rural environments had good physical fitness, were more active, and had a higher percentage of body fat than Melanesian adolescents living in urban environments, especially boys (42). However, from a pilot study using wrist activity trackers, Galy et al. found that rural Melanesian adolescents performed only ∼30 min of moderate to vigorous PA per day on average, which is half the World Health Organization (WHO) recommendations (11, 43). Although specific health education programs are needed in the schools of New Caledonia, as in other Asia-Pacific countries (44), little is known about the patterns of PA in this region and the reasons why certain people adopt or maintain active lifestyles whereas others do not (42). Moreover, studies have suggested that financial limitations, family commitments, time constraints, and road safety issues restrict healthy lifestyle behaviors in the Melanesian population from Vanuatu, especially for women (45–47).

Several studies conducted in a range of countries have identified ethnicity as a factor of PA levels (2, 19, 23, 48, 49), while others have found no consistent relations (50). In any case, it is not yet known whether ethnicity is associated with PA levels in the adolescents from the multicultural population of New Caledonia. We thus hypothesized that living in rural areas with a traditional Melanesian lifestyle would be associated with higher PA levels and less sedentary time.

This study aimed to (i) assess the time that New Caledonian adolescents spend in PA and in a sitting position, (ii) analyze the possible associations between PA and sitting time and the sociodemographic or environmental characteristics, and (iii) identify the associations of PA and sitting time with overweight in these adolescents.

MATERIALS AND METHODS

Data Collection and Participants

Data were collected from surveys completed by school-going adolescents living in New Caledonia from May 2015 to April 2016. Parents gave informed written consent prior to the children's participation in the study, in line with the legal requirements and the Declaration of Helsinki. The protocol was also approved by the Human Research Ethics Committee of the University of New Caledonia.

New Caledonia is a South Pacific archipelago located between 162–169°E longitude and 19–23°S latitude (**Figure 1**). It is divided into three provinces (Northern Province, Southern

FIGURE 1 | Towns and villages (Koumac, Hienghène, Lifou, Païta, Nouméa, and Mont-Dore) that had schools involved in the study on a New Caledonia map.

Province, and Loyalty Islands Province) with marked differences in ethnic composition, socioeconomic status (SES), and urbanization. In 2019, the New Caledonia population numbered 271,407. Among them, 75% lived in Southern Province, which includes Grand Noumea (the only urban area on the archipelago) that accounted 67% of the total population. Another 18% lived in Northern Province and 7% in Loyalty Islands Province. This same year, 24% of New Caledonians declared they were Caucasian (European), 41% Melanesian (Kanak), and 10% Polynesian (Tahitian, Wallisian and Futunian), with 24% belonging to other ethnic communities (Indonesian, Ni-Van, Vietnamese, Asiatic, Métis and others). Around 9% of the population was 11–16 years old (51). Thirteen secondary public schools (40%) were located in rural areas and 20 (60%) in urban areas (52).

Five secondary public schools were selected for this study: one in Loyalty Islands Province (rural area), two in Northern Province (east and west coasts, rural areas) and two in Southern Province (Noumea, the capital and only urban area). The selection criterion was school size ($n > 200$) to ensure that sufficient data could be collected in a single visit. Based on this criterion, only one school was eligible in the Loyalty Islands Province, four schools were eligible in the Northern Province (two on each coast), and eight in the Southern Province. Schools were then randomly selected and contacted to obtain agreement from the principal and the teaching team (**Figure 1**). Two classes were then randomly selected in each of four grades (levels) by a staff member, providing around 180 adolescents per school. In each school, we were able to collect data on 90% of the expected sample. The missing 10% was due to students who either were absent or did not provide parental consent. Adolescents with missing data ($n = 276$) and those from ethnic groups other than Melanesian, Caucasian or Polynesian ($n = 4$) were then excluded because of the small sample size of these groups. Finally, the thresholds of outliers for PA and sedentary time were computed with the following formula:

$$t_x = \bar{x} + 1.5 \times \sigma_x \quad (1)$$

where x is the variable of interest (i.e., PA or sedentary time), t_x the computed threshold, $\bar{x}$ the average of x, and σ_x the standard deviation of x. Adolescents with PA $> t_{PA} = 326$ min/day or sedentary time $> t_{ST} = 720$ min/day were removed from the study ($n = 55$). We note that 18,759 students were enrolled in New Caledonian middle schools in 2015, and this study ultimately included 508 adolescents, representing 2.7% of this population (53).

Anthropometric Parameters

Anthropometric measurements were made by a trained member of the research team. Adolescents were measured in light indoor clothing with emptied pockets and no shoes. Height was measured with the participant looking straight ahead, heels connecting with a wall mounted stadiometer (Leicester Tanita HR 001, Tanita Corporation, Tokyo, Japan) that was accurate to the nearest 0.1 cm. Weight was determined using a scale (Tanita HA 503, Tanita Corporation, Tokyo, Japan) accurate to the nearest 0.1 kg. Body mass index (BMI) was calculated by dividing weight in kilograms by height squared in meters: BMI = weight/height^2. The BMI z-scores and percentiles were calculated using the LMS reference values, with the weight status classification defined according to the criteria of the International Obesity Task Force (IOTF) (54), which classifies BMI values according to age and gender as thin (underweight), normal weight, overweight, or obese. The underweight and normal categories were merged, as were the overweight and obese categories, giving a final weight status with only two categories: not overweight (NO) and overweight (O).

Sociodemographic Characteristics

Demographic data included in the analyses were age, gender, ethnic community and SES. Ethnic community was self-reported by the adolescents using an anonymous questionnaire and categorized as usually processed by the *Institut de la statistique et des études économiques* (ISEE: Institute of Statistics and Economic Studies) census and as recommended by the *Institut national de la santé et de la recherche médicale* (INSERM: the National Institute of Health and Medical Research) report on New Caledonia (55), but they were told they could choose only one ethnic group. The possible ethnic communities were: Kanak (native Melanesian), Caledonian European (European origin and born in New Caledonia), Metropolitan European, Wallisian–Futunian, Tahitian, and other. In the current study, the Caledonian European and Metropolitan European communities were merged into the Caucasian category, and the Wallisian–Futunian and Tahitian communities were merged into the Polynesian category. SES was indexed on the basis of the occupation of the household reference person (defined as the householder with the highest income) using the National Statistics Socio-Economic classification (56). For the present analyses, we generated three categories: managerial and professional occupations (high SES), intermediate occupations (intermediate SES), and routine and manual occupations (low SES).

The 2014 census in New Caledonia (57) used a European standard (58) to assess the degree of urbanization. An urban area was thus defined as a densely populated area comprising at least 50,000 inhabitants in a continuous zone with more than 500 inhabitants per km^2. A semi-urban area was defined as having more than 50,000 inhabitants in a continuous zone of over 100 inhabitants per km^2 adjacent to an urban area. A rural area was any area that did not fulfill the conditions required to qualify as being urban or semi-urban.

Physical Activity and Out-Of-School Sitting Time

Adolescents self-reported their own PA and sitting time *via* the French version of the International Physical Activity Questionnaire–Short Form (IPAQ-SF), which assessed PA over the last 7 days (59–61). The IPAQ-SF classes activity into four categories: sitting, walking, moderate intensity (e.g., leisure cycling), and vigorous intensity (e.g., running or aerobics). Using the IPAQ-SF scoring system, the total number of days and minutes of PA were calculated for each participant as recommended on the IPAQ website (62). The IPAQ is also widely used to provide a proxy of sedentary time (63, 64). We averaged the resulting PA times and out-of-school sitting times in min/day. Then, both the PA and sitting times were dichotomized using the following thresholds: PA time $\geq$ 60 min/day and out-of-school sitting time $\geq$ 120 min/day. We focused on out-of-school sitting time because all participants attended school and followed a similar curriculum. We thus considered that sitting time during school hours was similar.

Socioenvironmental Factors Affecting Physical Activity

The adolescents were asked the following questions about the number of siblings: (1) "How many sisters have you got?" and (2) "How many brothers have you got?" Then the following binary questions (adolescents answered yes or no) were asked in order to assess the impact of the socioenvironmental factors: "When I practice physical activity or sport, it is because…" (3) Peers: "… a friend comes with me" [Yes/No]; (4) Family: "… a family member comes with me" [Yes/No]; (5) Safety: "…I feel safe" [Yes/No]; and (6) Accessibility: "… it is easy to find a place where I can be active" [Yes/No]. Questions were extracted from the study of Jego et al. (65) and adapted to the New Caledonian context.

Statistics

The analysis consisted of assessing differences in PA, sitting time, social factors associated with PA or sport, and anthropometric variables (mass, height, BMI, IOTF z-score, and weight status) according to sex, ethnic community and place of living (rural or urban). All the results are presented for female and male adolescents.

The differences between two groups (e.g., rural and urban) were determined with a means equality test (Student or Welch *t*-test) for continuous variables. When conditions for the application of the *t*-test were not verified, the non-parametric Wilcoxon test was used. The differences according to a factor having more than two categories (ethnic community: Melanesian, Caucasian, and Polynesian) were tested using a one-way ANOVA when conditions of normality (or at least a distribution close to the normal distribution and a sufficient sample size) and homoscedasticity were satisfied, otherwise the Kruskal-Wallis test was implemented. *Post-hoc* tests were implemented when the differences between factor groups were significant after the one-way ANOVA or Kruskal-Wallis test:

TABLE 1 | Associations between PA time and socioenvironmental factors one by one adjusted with the confounding factors (place of living, ethnic community, age) and interactions.

PA time ≥ 60 min/day	Female				Male			
	OR [95% CI]	*p*-value	OR_{adj} [95% CI]	*p*-value	OR [95% CI]	*p*-value	OR_{adj} [95% CI]	*p*-value
Siblings	1.08 [0.97;1.22]	0.159	1.01 [0.90;1.14]	$0.868^{C,A}$	1.17 [1.00;1.38]	0.061	1.27 [1.05;1.59]	$0.025^{L,\#1}$
Peers	1.74 [1.03;2.94]	0.039	1.79 [1.02;3.14]	$0.041^{C,A}$	1.48 [0.83;2.64]	0.186	3.05 [1.09;8.89]	$0.036^{C,\#2}$
Family	1.67 [0.98;2.84]	0.056	2.35 [1.22;4.52]	$0.010^{L,C,A,*}$	1.54 [0.87;2.74]	0.139	1.54 [0.87;2.74]	0.139
Safety	2.87 [1.69;4.91]	<0.001	2.85 [1.63;5.01]	$<0.001^{C,A}$	4.14 [2.28;7.64]	<0.001	4.45 [2.42;8.36]	$<0.001^{L}$
Accessibility	1.21 [0.67;2.12]	0.520	1.53 [0.82;2.82]	$0.179^{C,A}$	2.82 [1.48;5.39]	0.002	2.94 [1.51;5.77]	0.002^{C}

OR, odds-ratio; OR_{adj}, adjusted odds-ratio; CI, confidence interval.
Confounding factors included in the model.
[L] Place of living.
[C] Ethnic community.
[S] SES.
[A] Age.
Interaction factors included in the model.
[*] Family with Place of living ($OR_{adj,Urban} = 0.22$, $p_{Urban} = 0.024$).
[#1] Siblings with Place of living ($OR_{adj,Urban} = 0.60$, $p_{Urban} = 0.018$).
[#2] Peers with Ethnic community ($OR_{adj,Melanesian} = 0.38$, $p_{Melanesian} = 0.147$, $OR_{adj,Polynesian} = 0.16$, $p_{Polynesian} = 0.131$).

the Tukey *post-hoc* test when using a one-way ANOVA and the Steel-Dwass-Critchlow-Fligner multiple comparison test when using the Kruskal-Wallis test (66). For significant differences in categorical variables, we used the χ^2 test when the Cochran rules were not violated, otherwise the Fisher exact test was implemented. When factors had more than two categories, the *post-hoc* test *p*-values were corrected with the Bonferroni adjustment in cases of significance. Indicators for effect size (EFI) were computed and then categorized in effect size magnitudes. EFI for numerical factors were η^2 and Kruskal-Wallis effect size and EFI for categorical factors was Cramer V. The effect size magnitude for numerical factors was determined as follows: small (S: EFI $<$ 0.06), moderate (M: 0.06 $\leq$ EFI $<$ 0.14) and large (L: EFI $\geq$ 0.14). The effect size magnitude for categorical factors was determined as follows: small (S: EFI $<$ 0.21 when df $=$ 2 and EFI $<$ 0.15 when df $=$ 4), moderate (M: 0.21 $\leq$ EFI $<$ 0.35 when df $=$ 2 and 0.15 $\leq$ EFI $<$ 0.25 when df $=$ 4), and large (L: EFI $\geq$ 0.35 when df $=$ 2 and EFI $\geq$ 0.25 when df $=$ 4) (67).

Factors associated with active time (PA $\geq$ 60 min/day) and sitting time (out-of-school sitting time $\geq$ 120 min/day) were first assessed *via* odds ratios (OR) and odds ratios adjusted for socioeconomic confounding factors and interaction factors (OR_{adj}). Finally, multivariate logistic regressions were computed in order to assess the association between the IOTF weight status (with two categories: NO and O) and active vs. sitting time.

We determined the inclusion of the confounding factors and interaction factors (**Tables 1**–**3**) in the adjusted models (for OR_{adj} computing) by a background selection. Only confounding factors and interaction factors whose *p*-values were lower than 0.20 ($p < 0.20$) were included in the models.

The analyses were conducted using R 3.5.1 (68) with a first species risk probability level set at $\alpha = 0.05$.

RESULTS

Physical Activity and Out-of-School Sitting Time

Overall, around 66% of the adolescents, both females and males, declared engaging in PA more than 60 min/day. Moreover, our results showed that Melanesian adolescents living in rural areas were the most physically active and spent the least out-of-school sitting time. This active living was not found in the urban environment.

A greater proportion of Melanesian adolescents engaged in 60 min/day of PA in rural compared to urban areas (75.78% vs. 46.67% in girls, $p = 0.028$; 74.79% vs. 45.83% in boys, $p = 0.010$; **Supplementary Tables 1, 2**). **Figure 2** shows that male Melanesian adolescents who lived in a rural environment spent on average 133 min/day in PA compared to 95 min/day for those living in an urban environment ($p = 0.028$; **Supplementary Table 2**). While a similar trend was observed for female adolescents (115 min/day in rural vs. 88 min/day in urban, $p = 0.118$), this was not statistically significant.

In the rural environment, both female and male Melanesian adolescents were more physically active compared with Caucasians (115 vs. 93 min/day in girls, $p = 0.018$; 133 vs. 97 min/day in boys, $p = 0.018$; **Figure 2**, **Supplementary Tables 3, 4**). In this environment, 75.78% of Melanesian vs. 52.73% of Caucasian female adolescents engaged in PA for at least 60 min/day on average ($p = 0.006$; **Supplementary Table 3**). Both male and female Melanesian adolescents spent less out-of-school sitting time on average than their Caucasian counterparts in the rural environment (166 vs. 271 min/day in girls, $p = 0.008$; 170 vs. 231 min/day in boys, $p = 0.019$; **Figure 2**, **Supplementary Tables 3, 4**), whereas this was true only for females in the urban environment (156 vs. 332 min/day, $p = 0.019$; **Figure 2**, **Supplementary Table 3**). In rural areas, 42.86% of Melanesian vs. 71.79% of Caucasian male

TABLE 2 | Associations between out-of-school sitting time and socioenvironmental factors one by one adjusted with the confounding factors (place of living, ethnic community, age) and interactions.

Out-of-school sitting time ≥ 120 min/day	Female				Male			
	OR [95% CI]	*p*-value	OR_{adj} [95% CI]	*p*-value	OR [95% CI]	*p*-value	OR_{adj} [95% CI]	*p*-value
Siblings	0.87 [0.78;0.96]	0.007	0.93 [0.83;1.03]	$0.179^{C,S}$	0.91 [0.80;1.03]	0.160	0.62 [0.39;0.90]	$0.019^{C,A,\#1}$
Peers	0.90 [0.56;1.46]	0.673	0.20 [0.04;0.79]	$0.031^{C,S,*1}$	0.83 [0.48;1.43]	0.515	0.82 [0.46;1.44]	0.482^{C}
Family	0.90 [0.55;1.46]	0.658	0.95 [0.56;1.60]	$0.834^{C,S}$	0.88 [0.52;1.48]	0.622	0.82 [0.43;1.58]	$0.558^{L,C,A,\#2}$
Safety	1.51 [0.93;2.45]	0.098	0.75 [0.25;2.17]	$0.595^{L,C,S,*2}$	0.67 [0.38;1.18]	0.170	0.00 [0.00;0.52]	$0.030^{C,A,\#3}$
Accessibility	1.61 [0.95;2.75]	0.080	0.01 [0.00;2.81]	$0.111^{C,S,A,*3}$	0.95 [0.51;1.78]	0.882	0.86 [0.45;1.74]	$0.734^{C,A}$

OR, odds-ratio; OR_{adj}, adjusted odds-ratio; CI, confidence interval.
Confounding factors included in the model.
^{L} Place of living.
^{C} Ethnic community.
^{S} SES.
^{A} Age.
Interaction factors included in the model.
^{*1} Peers with Ethnic community ($OR_{adj,Melanesian} = 1.65$, $p_{Melanesian} = 0.424$; $OR_{adj,Polynesian} = 0.15$, $p_{Polynesian} = 0.197$), Peers with SES ($OR_{adj,Intermediate} = 5.12$, $p_{Intermediate} = 0.045$; $OR_{adj,Low} = 4.52$, $p_{Low} = 0.043$).
^{*2} Safety with Place of living ($OR_{adj,Urban} = 0.32$, $p_{Urban} = 0.144$), Safety with Ethnic community ($OR_{adj,Melanesian} = 5.18$, $p_{Melanesian} = 0.009$; $OR_{adj,Polynesian} = 3.91$, $p_{Polynesian} = 0.317$).
^{*3} Accessibility with Age ($OR_{adj} = 1.45$, $p = 0.085$).
$^{\#1}$ Siblings with Ethnic community ($OR_{adj,Melanesian} = 1.65$, $p_{Melanesian} = 0.021$; $OR_{adj,Polynesian} = 2.63$, $p_{Polynesian} = 0.093$).
$^{\#2}$ Family with Place of living ($OR_{adj,Urban} = 2.75$, $p_{Urban} = 0.144$).
$^{\#3}$ Safety with Age ($OR_{adj} = 1.60$, $p = 0.034$).

TABLE 3 | Association between PA and out-of-school sitting time with weight status (being overweight) in female and male adolescents.

IOTF weight status	Female				Male			
	OR [95% CI]	*p*-value	OR_{adj} [95% CI]^{AF}	*p*-value	OR [95% CI]	*p*-value	OR_{adj} [95% CI]^{AM}	*p*-value
Active time ≥ 60 min/day	0.79 [0.47;1.34]	0.376	0.62 [0.34;1.10]	0.103	0.84 [0.47;1.54]	0.061	0.28 [0.08;0.95]	0.044
Out-of-school sitting time ≥ 120 min/day	1.18 [0.73;1.91]	0.501	1.55 [0.92;2.62]	0.101	0.41 [0.23;0.71]	0.002	0.39 [0.20;0.73]	0.004

OR, odds-ratio; OR_{adj}, adjusted odds-ratio; CI, confidence interval.
^{AF} Adjusted with Place of living ($OR_{adj,Urban} = 0.28$, $p_{Urban} = 0.002$) and Ethnic community ($OR_{adj,Melanesian} = 1.99$, $p_{Melanesian} = 0.030$, $OR_{adj,Polynesian} = 7.86$, $p_{Polynesian} = 0.001$).
^{AM} Adjusted with Ethnic community ($OR_{adj,Melanesian} = 0.68$, $p_{Melanesian} = 0.520$, $OR_{adj,Polynesian} = 5.16$, $p_{Polynesian} = 0.092$), SES ($OR_{adj,Intermediate} = 2.49$, $p_{Intermediate} = 0.028$, $OR_{adj,Low} = 2.20$, $p_{Low} = 0.046$) and interactions between Ethnic community and Active time ($OR_{adj,Melanesian} = 3.90$, $p_{Melanesian} = 0.074$, $OR_{adj,Polynesian} = 0.87$, $p_{Polynesian} = 0.911$).

adolescents declared staying seated more than 120 min/day out of school ($p = 0.009$; **Supplementary Tables 2, 4**).

Factors Associated With PA and Out-Of-School Sitting Time

Most adolescents identified specific contexts (peers, family, safety and accessibility) as important (answered yes to the binary questions) when it comes to engaging in PA (**Supplementary Tables 1, 2**, **Figures 3**, **4**). Accessibility was especially important for both Melanesian and Caucasian adolescents (68% yes in Melanesian females, 76% yes in Melanesian males, and 85% yes in Caucasians), while safety was more consistently reported as important by Melanesian adolescents (63% yes in females and 73% yes in males). In urban areas, accessibility to a suitable place was identified as important more consistently by female Caucasian (88.89%) than Melanesian (46.67%) adolescents ($p = 0.008$; **Figure 3D**, **Supplementary Table 3**). An accessible area for PA was also an important factor for both males (76.22% Melanesians, 85.07% Caucasians, and 50.00% Polynesians; **Supplementary Table 2**) and females (67.61% Melanesians, 84.62% Caucasians, and 66.67% Polynesians; **Supplementary Table 1**). For male adolescents, having access to a safe area was also important (72.73% Melanesians, 59.70% Caucasians, and 43.75% Polynesians; **Figure 4C**, **Supplementary Table 2**) and this was particularly true for Melanesians living in the urban area compared with Polynesians (87.50% vs. 45.45%, $p = 0.044$; **Supplementary Table 4**).

Some of these socioenvironmental factors appeared associated with PA and out-of-school sitting time. In female adolescents, practicing with peers ($OR_{adj} = 1.79$, $p = 0.041$), with family ($OR_{adj} = 2.35$, $p = 0.010$) and in a safe environment ($OR_{adj} = 2.85$, $p < 0.001$) were positively associated with PA (**Table 1**). However, interactions between place of living and family should be taken into account. As shown in **Figure 5A**, the effect of engaging with family members was positive on PA in girls living in rural areas, while in urban areas the effect was not significant. In male adolescents, the number of siblings ($OR_{adj} = 1.27$, $p = 0.025$), peers ($OR_{adj} = 3.05$, $p = 0.036$), safety ($OR_{adj} = 4.45$, $p < 0.001$), and accessibility ($OR_{adj} = 2.94$, $p = 0.002$) were positively associated with PA (**Table 1**). There were significant interactions between place of living and siblings ($OR_{adj,Urban} = 0.60$, $p_{Urban} =$

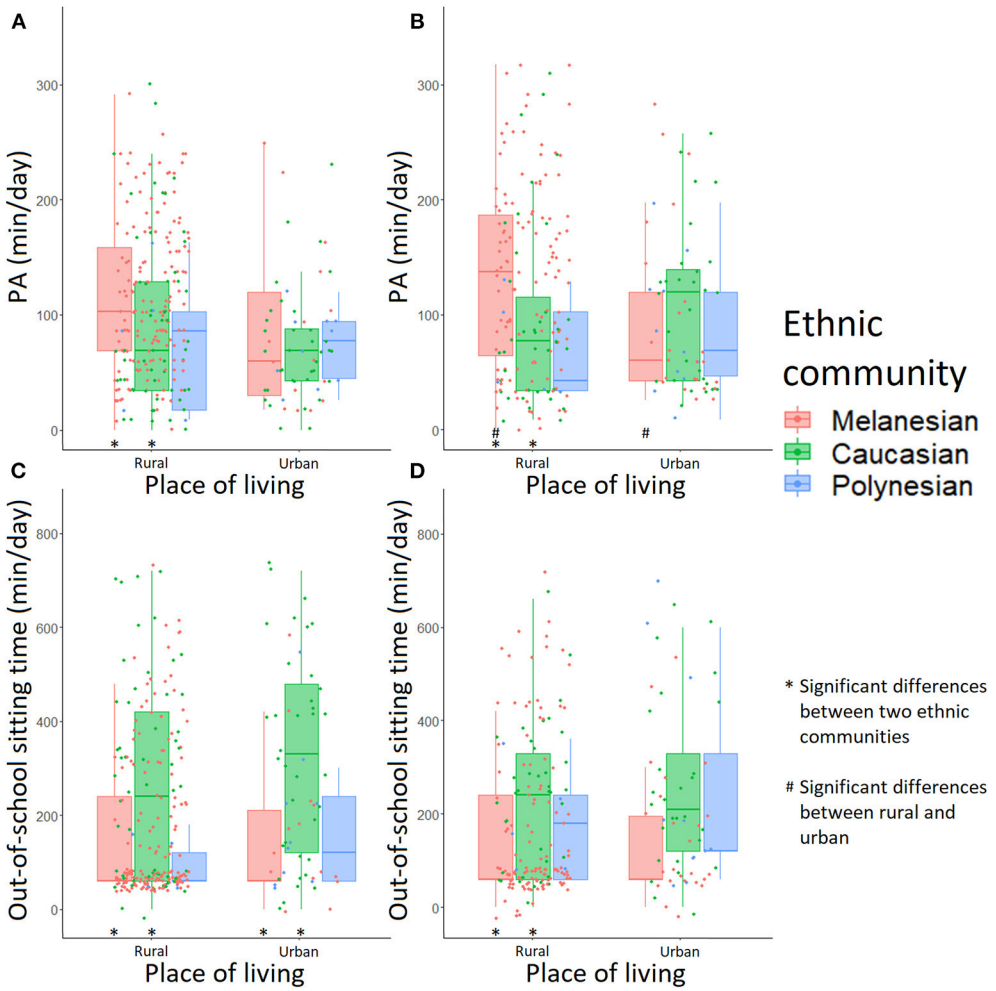

FIGURE 2 | Time spent in physical activity and sitting time in adolescents according to ethnic community and place of living: **(A)** PA in female adolescents, **(B)** PA in male adolescents, **(C)** out-of-school sitting time in female adolescents and **(D)** out-of-school sitting time in male adolescents. A "*" note means a significant difference between two ethnic communities in a single place of living; a "#" note means a significant difference between rural and urban environment in a single ethnic community.

0.018). **Figure 5B** highlights that the impact of siblings seemed positive, especially in rural areas, when male adolescents had more than three brothers and sisters.

Only the factor peers was negatively associated with the out-of-school sitting time of female adolescents ($OR_{adj} = 0.20$, $p = 0.031$, **Table 2**). There was a significant interaction between SES and peers ($OR_{adj,Intermediate} = 5.12$, $p_{Intermediate} = 0.045$; $OR_{adj,Low} = 4.52$, $p_{Low} = 0.043$), and between ethnic community and safety ($OR_{adj,Melanesian} = 5.18$, $p_{Melanesian} = 0.009$). As shown in **Figure 6A**, peers had a negative effect on sitting time especially in high SES. **Figure 6B** shows that female Melanesian adolescents claiming that feeling safe when practicing PA is important (safety) were more likely to declare a sitting time $\geq$ 120 min/day when compared with female Melanesian adolescents for whom safety was not so important. In male adolescents, siblings ($OR_{adj} = 0.62$, $p = 0.019$) and safety ($OR_{adj} = 0.00$, $p = 0.030$) were negatively associated with out-of-school sitting time. We found significant interactions between ethnic community and siblings ($OR_{adj,Melanesian} = 1.65$, $p_{Melanesian} = 0.021$; $OR_{adj,Polynesian} = 2.63$, $p_{Polynesian} = 0.093$) and between age and safety ($OR_{adj} = 1.60$, $p = 0.034$). **Figure 6C** shows that the number of siblings had a negative association with sitting time only in Caucasians, whereas it was positive in Polynesian male adolescents. Moreover, safety seemed negatively associated with sitting time in male adolescents younger than 13 years old (**Figure 6D**).

Anthropometry and Sociodemographic Factors

Around 40% of the Melanesian, <25% of the Caucasian, and 60% of the Polynesian adolescents were O (**Supplementary Tables 1, 2**), but differences were observed according to sex and place of living. The IOTF z-score average in Melanesian female adolescents living rural areas was 1.03, whereas it was 0.24 in those living in urban areas ($p = 0.006$; **Supplementary Table 1**). Caucasian female adolescents had a higher IOTF z-score in rural areas than in urban areas (0.89 in

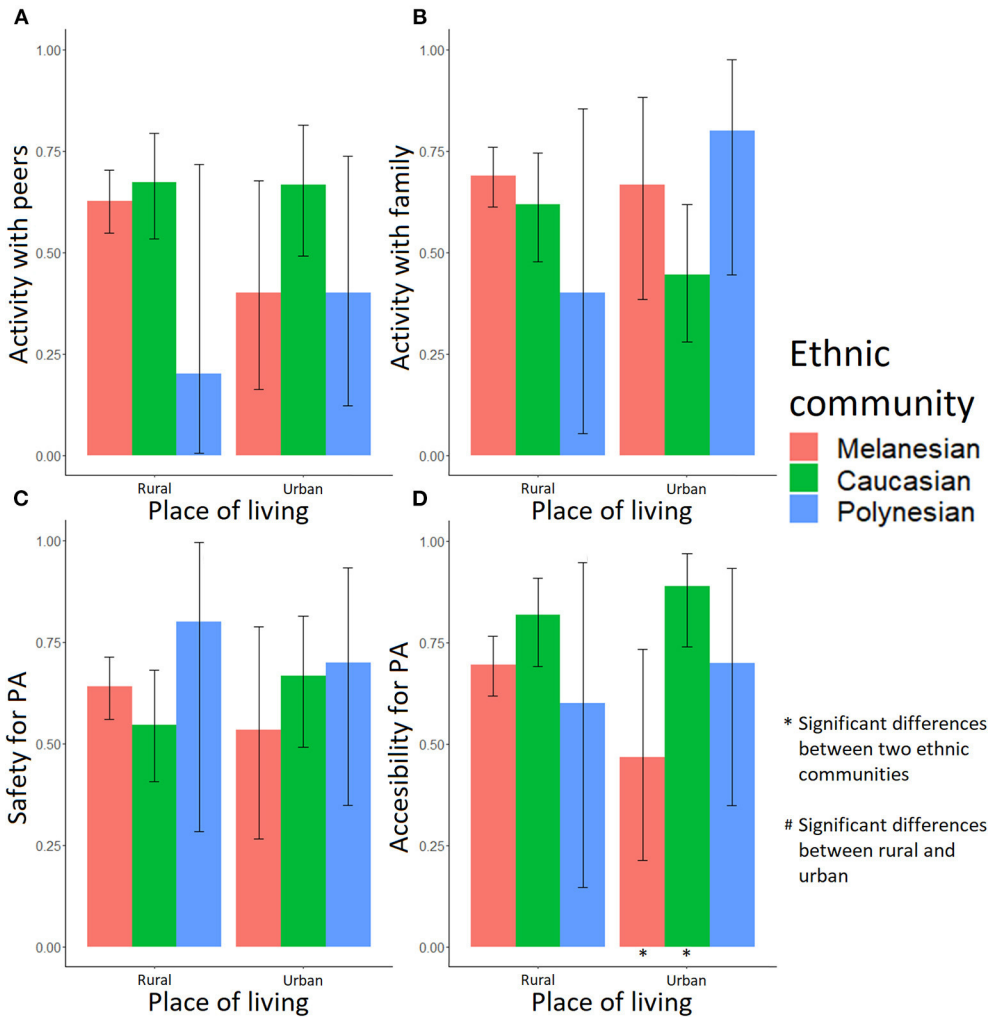

FIGURE 3 | Fractions of female adolescents for whom the socioenvironmental factors are important in PA practice. Activity: **(A)** with peers, **(B)** with family, **(C)** in safety, and **(D)** easy access. A "*" note means a significant difference between two ethnic communities in a single place of living; a "#" note means a significant difference between rural and urban environment in a single ethnic community.

rural vs. 0.02 in urban, $p = 0.001$), and the prevalence of O was higher in rural areas than in urban areas (34.55% O in rural vs. 11.11% O in urban, $p = 0.023$). In Caucasian male adolescents, the IOTF z-score was higher in rural areas than in urban areas (0.64 vs. −0.02, $p = 0.031$; **Supplementary Table 2**).

Supplementary Table 3 shows no significant anthropometric differences in rural female adolescents between ethnic communities. In urban areas, there were significant differences for the IOTF z-score (0.02 vs. 1.11, $p = 0.017$) and IOTF weight status (11.11% O vs. 50.00% O, $p = 0.045$) when Caucasians and Polynesians were compared. **Supplementary Table 4** shows significant differences in male adolescents between ethnic communities, but these differences were not systematically found in comparisons according to the place of living. There were no differences in rural areas according to ethnic community. In urban areas, differences were significant for the IOTF z-score (0.96 in Melanesians vs. −0.02 in Caucasians, $p = 0.023$; −0.02 in Caucasians vs. 1.16 in Polynesians, $p = 0.035$) and IOTF weight status ($p = 0.024$ but there was no significance with the *post-hoc* test).

Association Between PA, Out-of-School Sitting Time and Weight Status

Through adjusted odds ratios, **Table 3** shows that the weight status associated with being active (i.e., PA ≥ 60 min/day) and with out-of-school sitting time ≥ 120 min/day was controlled by socioeconomic factors. In females, place of living and ethnic community were associated with IOTF weight status, whereas PA and sitting time were not. A female adolescent living in the urban context was less likely to be overweight or obese ($OR_{adj} = 0.28$, $p = 0.002$) but Melanesian ($OR_{adj} = 1.99$, $p = 0.030$) and Polynesian ($OR_{adj} = 7.86$, $p = 0.001$) females were more likely to be overweight or obese than the Caucasian females. In male adolescents, SES and both PA and sitting time were associated

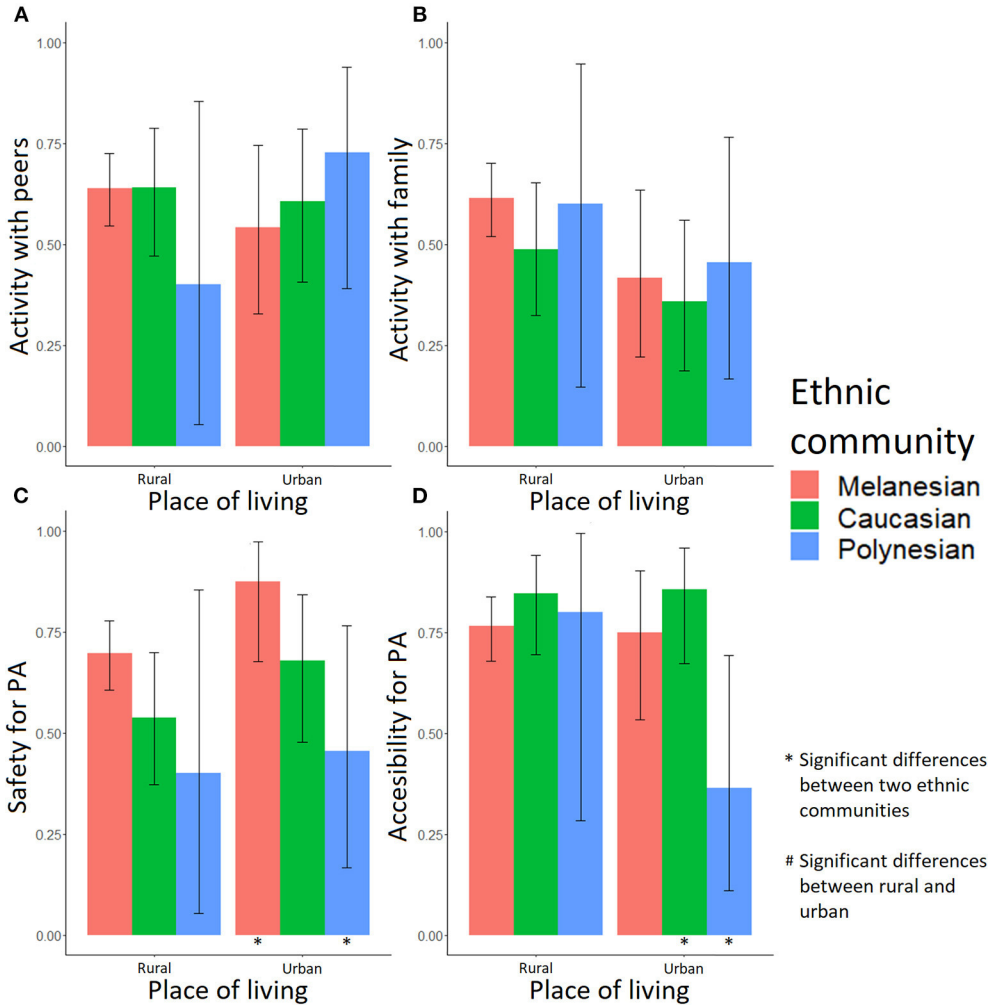

FIGURE 4 | Fractions of male adolescents for whom the socioenvironmental factors are important in PA practice. Activity: **(A)** with peers, **(B)** with family, **(C)** in safety, and **(D)** easy access. A "*" note means a significant difference between two ethnic communities in a single place of living; a "#" note means a significant difference between rural and urban environment in a single ethnic community.

with IOTF weight status. Boys from intermediate ($OR_{adj} = 2.49$, $p = 0.028$) or low ($OR_{adj} = 2.20$, $p = 0.046$) SES were more likely to be overweight or obese than boys from high SES. Active male adolescents (PA $\geq$ 60 min/day) and those who declared sitting $\geq$ 120 min/day out of school were less likely to be overweight or obese ($OR_{adj} = 0.28$, $p = 0.044$ and $OR_{adj} = 0.39$, $p = 0.004$, respectively).

Based on the above-described results, **Figure 7** shows the strength of association between the socioeconomic and PA factors on the one hand and the overweight and obesity risk on the other hand.

DISCUSSION

This study, which focused on PA in a pluri-ethnic adolescent population, showed that 66% of adolescents reported 60 min or more of daily PA with disparities driven by place of living and ethnic community. More specifically, Melanesian adolescents were generally more active and spent less sitting time than Caucasians. Melanesian adolescent boys living in rural areas presented the most active lifestyle.

Belonging to an ethnic community or SES was associated with PA and sitting time in girls but not in boys. In addition, feeling safe was a strong driver for PA in both male and female adolescents.

Finally, being active (PA $\geq$ 60 min/day) and out-of-school sitting time $\geq$ 120 min/day were not significant predictors for weight status in female adolescents, while they seemed to prevent overweight in adolescents boys.

Physical Activity

The proportion of adolescents who regularly engaged in PA in New Caledonia (66%) is fairly encouraging, when compared with results of previous studies in other Pacific Island Countries and Territories (PICTs) (10, 34). Indeed, in a previous study on adolescents 13–17 years old, Kessaram et al. (34) found that

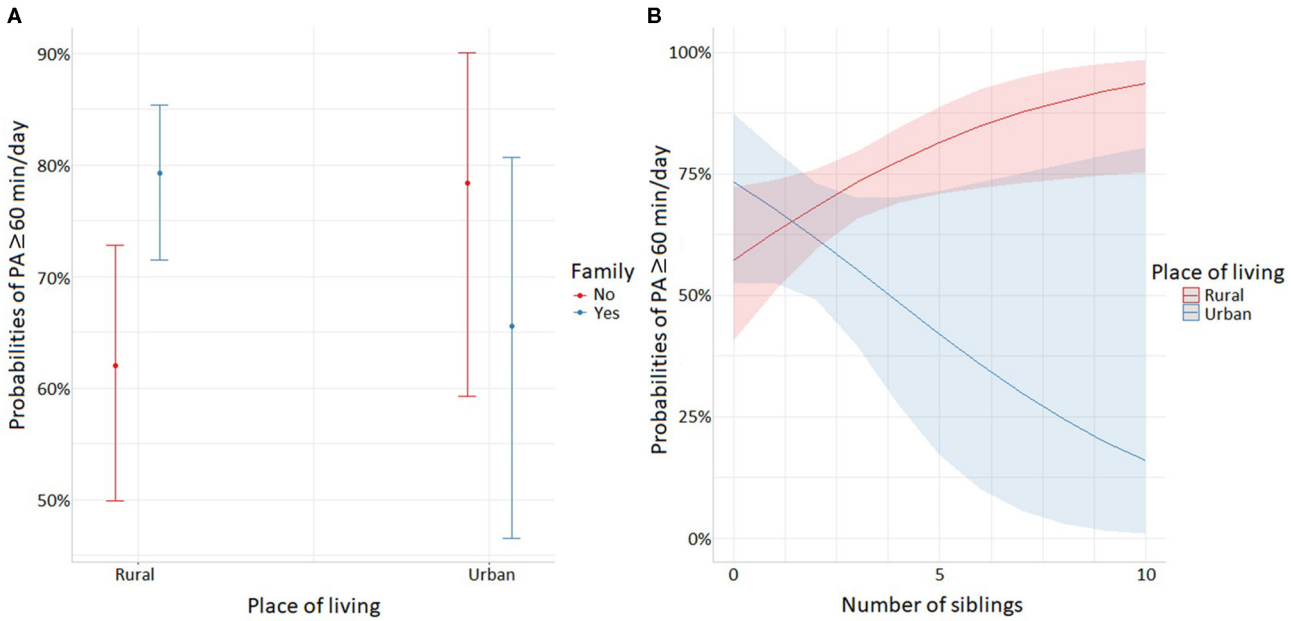

FIGURE 5 | Significant interactions for PA time: **(A)** interactions between place of living and family in female adolescents, **(B)** interactions between place of living and siblings in male adolescents.

17.5% (in Nauru) to 45.7% (in Vanuatu) of males and 12.5% (in Nauru) to 46.7% (in Vanuatu) of females were physically active, e.g., engaged in 60 min/day of PA, as assessed by the Global School-Based Student Health Survey 2011 (34). However, the more restrictive definition of an "active person" in their study, which was different from ours (at least 60 min/day of PA for at least 5 days in Kessaram et al. and at least 60 min/day on average in the current study), could explain this difference.

In this study, we did not find significant differences for the average amounts of PA in male and female adolescents except in rural Melanesians (see **Supplementary Table 1**). This is an unprecedented result in New Caledonia that contrasts with previous studies in which authors found significant differences according to gender (19, 23, 34, 69, 70).

More Melanesian adolescents reached 60 min/day of PA in rural compared to urban areas, both for girls (75.78% in rural and 46.67% in urban) and boys (74.79% in rural and 45.83% in urban). A similar association was found in populations of Turkey and the Republic of Cameroon (71, 72). Because of the industrialized urban life, schoolchildren tend to spend much more time in sedentary activities such as reading, playing video games or watching TV. Indeed, they have less chance to play outside when compared with their rural counterparts (72). In contrast, there was an inverse association in Polish adolescents between place of residence and PA, with a higher percentage of adolescents with low PA in a rural environment (48). The authors explained that the lack of PA time in the rural environment may have been due to a high dependence on driving. In our study, the higher PA level in Melanesian adolescents living in rural areas may be explained by the strong participation in community life, especially in tribes. Indeed, most of the Melanesians living in rural areas have kept a tribal lifestyle, which implies engaging in a number of physical activities such as hunting, fishing, and cultivating fields (30, 73).

Ethnicity was associated with PA and sedentary time in New Caledonia, which is in line with previous studies conducted in other countries (2, 8, 23, 49). In a rural environment, Melanesian adolescents, both females and males, were significantly more active (higher PA time and lower out-of-school sitting time) than Caucasians. Caucasians living in rural areas, as well as Melanesians, were likely to engage in activities such as hunting and fishing. However, as highlighted before, the community lifestyle followed by Melanesians may require more PA time to meet the community needs and may also provide more opportunities for pleasurable PA when extended family and friends gather for leisure time (30, 73). Moreover, practicing PA with peers has been identified by adolescents as an important component for PA participation, as discussed further below (16).

The socioeconomic and socioenvironmental factors related to PA were different according to gender in New Caledonia. In adolescent girls, ethnic community, age, peers, family and feeling safe were associated with PA $\geq$ 60 min/day. In adolescent boys, PA was related to the number of siblings, peers, feeling safe and accessibility. In addition, while engaging in PA with peers was negatively associated with a high sitting time (out-of-school sitting time $\geq$ 120 min/day) for female adolescents, siblings and safety were negatively associated with a high sitting time in male adolescents. Several studies have reported that general social support is determinant for adolescent engagement in PA (8, 18, 23, 49). However, the current study suggests that friendship had more impact on adolescent girls' sitting time than

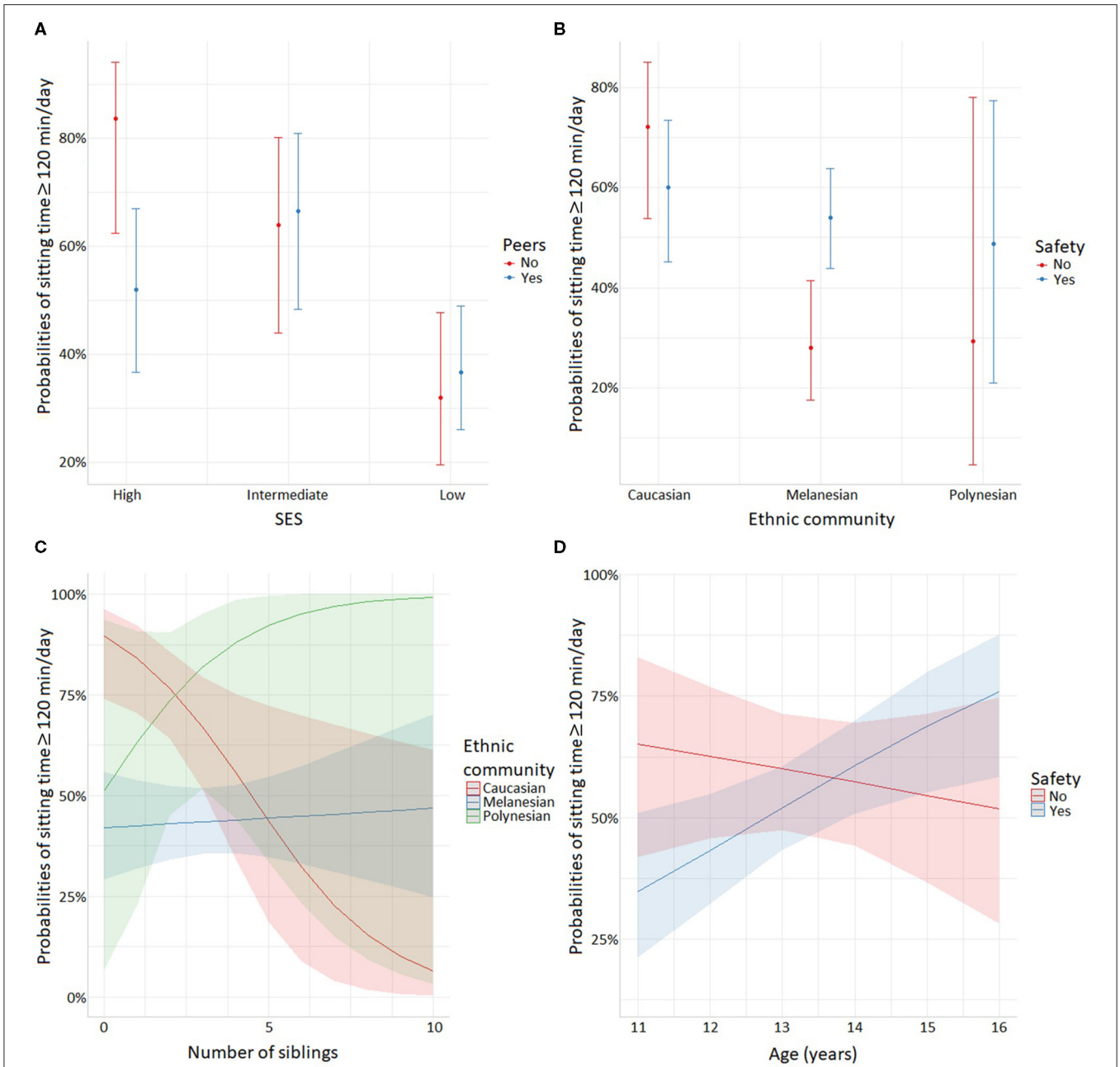

FIGURE 6 | Significant interactions for out-of-school sitting time: **(A)** interactions between SES and importance of practicing PA with peers in female adolescents, **(B)** interactions between ethnic community and safety in female adolescents, **(C)** interactions between ethnic community and siblings in male adolescents, **(D)** interactions between age and safety in male adolescents.

family ties. A study conducted in Hawaiian adolescents revealed that they were more likely to spend sedentary time with parents and active time with friends (21). Indeed, during childhood and the preadolescent years, parental modeling of PA plays a crucial role in lifestyle behavior and establishing a social norm (74). As children mature, they become more independent and the association between parental and children's lifestyles start to diverge (22). For this reason, studies have reported inconsistent associations between PA and parental behavior, modeling, and encouragement, depending on the children's age (22, 23). Other studies have pointed to the importance of friendship for engaging in PA (16, 26), particularly because it expands the number and types of PA that adolescents can participate in Rittenhouse et al. (75) and Salvy and Bowker (16). Also, with regard to our finding that family ties were associated with PA in girls but not in boys, we should take into account that the place of women is central

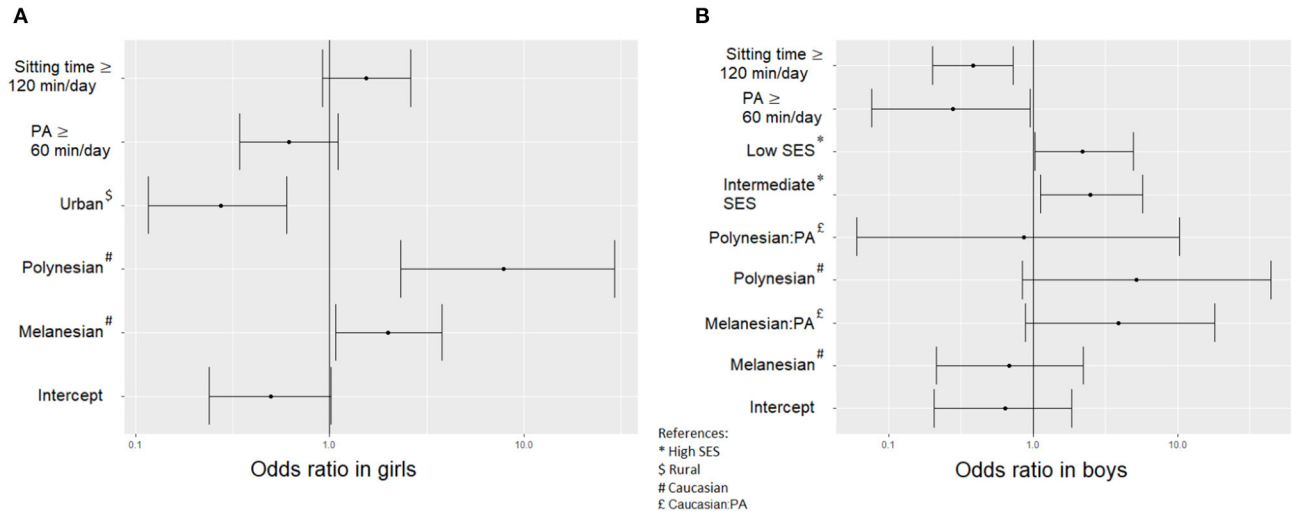

FIGURE 7 | Odds ratio and error bars for the multivariate logistic regressions estimating the chance of being overweight or obese in **(A)** female adolescents and **(B)** male adolescents.

in household activities in Pacific communities (76), whereas men carry out their customary activities outside the household, i.e., with friends and extended family members (45–47).

Although feeling safe has not always been found to be an important factor in previous studies (8, 15), it was an important driver of PA in this study independently of the place of residence or ethnicity. Our survey does not allow us to further explore the reasons behind the feeling of safety expressed by the adolescents. Did they have in mind the risk of injury, incivilities, or aggressions or the risk related to traffic? They may have been thinking about one or more of these risks or another kind of risk; this study did not provide a straight answer to this question.

Easy access to a place perceived as suitable for engaging in PA or sports was positively associated with PA in boys but not in girls. The question about accessibility was designed to elicit information about the proximity to infrastructures that would facilitate adolescent engagement, especially in PA and sports. In New Caledonia, adolescents living in the urban environment can easily access sports fields. Although most villages and tribes (in rural environments) have such infrastructures, they provide less diversity. Yet, interestingly, even though some of the rural adolescents may have lived quite far from PA infrastructures, this did not seem to limit their engagement in PA, probably because the rural community context offered other opportunities, such as open areas, beaches or marine activities. Other adolescents have reported an inconsistent relationship between PA and access to parks, recreation facilities and street connectivity (15). In the urban environment, there were probably some barriers (including safety and appropriate infrastructures) since PA was lower. Environmental and policy modifications, such as improving infrastructure and safety for walking, have been suggested as effective ways to address these barriers to PA (8, 15). Overall, WHO suggested international best practices for implementing PA programs in developing countries that could help populations increase the daily PA levels (14, 45).

PA, Sitting-Time, and Weight Status

In this study, about 38% of the girls and 34% of the boys were overweight or obese (being O). The analysis of the predictors of being O revealed that both PA and sitting time were not significantly predictive in girls, whereas they were in boys. Previous studies found that large amounts of sedentary behavior but not PA were associated with overweight and obesity (8, 77, 78). The PA in our study was not necessarily MVPA, and therefore the adolescents reaching 60 min/day of PA did not necessarily fulfill the international recommendations of 60 min/day of MVPA. It may be that on average the intensity of PA was light to moderate rather than moderate to vigorous. It may also be that the girls engaged in different types of PA compared to boys. One surprising result is that larger amounts of sitting time were not a predictor of overweight in the adolescent boys. One explanation may be that the boys engaged in more intense PA and that sitting time was more quiet time to rest. Indeed, the relationship between sitting and overweight is largely determined by gaming and recreational screen time. It is important to note that adolescents living far from school have to use public transport to go to school and then to come back home, and this too implies a fairly long sitting time.

Nevertheless, the association between PA and weight status, especially overweight and obesity, is complex. Bauman et al. (8) highlighted that the association between overweight or obesity and PA can go in both directions. While it is assumed that PA can help with weight loss in overweight individuals, some studies have challenged this assumption, pointing out that insufficient PA may lead to obesity but that obesity can be a driver of physical inactivity (8).

A study conducted in Vanuatu found negative associations between physical activities such as gardening and housekeeping and indicators of weight status including BMI, body fat mass percentage, waist circumference and the sum of skinfolds (31). The associations between sedentary activities (TV, radio, and

video) and indicators of weight status were also negative, which is consistent with our study. The authors highlighted the finding that PA was high even in the individuals who spent a lot of time in sedentary recreational activities. The study also investigated nutrition and found a positive association between obesity and consumption of non-traditional food, consistent with another study (73). A recent study including adolescents living in New Caledonia revealed that a large number of adolescents regularly consume sugar-sweetened beverages (79). The authors did not find a significant association between consumption of these beverages and weight status or PA. Although consumption of sugar-sweetened beverages or other unhealthy food products like snacks and fast foods is generally associated with sedentary time, including uninterrupted sitting times or long periods screen watching (4, 13), it might also be associated with time spent with friends or even with active time (80, 81). When adolescents spend time together, they do not necessarily sit but they could share unhealthy food and beverages (82, 83). Thus, they might consume more products favoring overweight when they are together than when they are sitting alone at home.

Limitations and Strengths

We analyzed the overall PA time and out-of-school sitting time of adolescents. However, our data did not provide information on the quantity of MVPA, and therefore we could not determine whether the adolescents fulfilled the international recommendations for MVPA. Moreover, the questionnaire did not elicit information on the type of the activities they were doing. It would be helpful and even necessary that future studies include further investigation about the adolescents' activities.

The current study collected information from adolescents *via* a self-administered survey that allowed them to self-assess their PA. While this method is widely used worldwide to collect PA information, it is influenced by the ability to make fairly accurate assessments. We acknowledge that an objective dataset collected from activity sensors or accelerometers would provide a more reliable assessment of PA in New Caledonia across ages, genders, and ethnicities (84).

The two IOTF weight status categories, underweight and normal, were pooled together even though underweight is not considered healthy. We chose to do so because of the low proportion of underweight adolescents (females in rural areas: 6 Melanesians, 2 Caucasians, and 0 Polynesians; females in urban areas: 1 Melanesian, 7 Caucasians, and 2 Polynesians; males in rural areas: 3 Melanesians, 3 Caucasians, and 0 Polynesians; males in urban areas: 1 Melanesian, 3 Caucasians, and 1 Polynesian). In addition, we found no significant difference between these categories for PA or sitting time.

This is a cross-sectional study so it cannot provide evidence of causal relationships between the factors we looked at and PA or these factors and weight status. But to our knowledge, this is the only study thus far in New Caledonia reporting data on PA and sitting time for adolescents.

Last, we acknowledge that the sample size for the Polynesian adolescents was rather small. We were not able to draw consistent conclusions about this population but we found relevant results by comparing the two largest ethnic communities, i.e., Melanesians and Caucasians.

Considerations and Implications of the Study

As noted in a study conducted in Vanuatu, traditional gardening should be considered an important protective factor against obesity in the Melanesian culture (31). The current study did not provide corroborating evidence for this because we did not specifically study how the adolescents engaged in PA. However, we found that the Melanesian adolescents living in rural environments were physically more active than those living in the urban environment, probably because in the Melanesian tribal lifestyle people regularly engage in such community activities as gardening, hunting and fishing. Adolescents are thus generally involved in these activities with extended family and friends (16, 30). Despite the lifestyle transition occurring in New Caledonia and other PICTs, promoting outdoor activities and traditional foods, especially for rural Melanesians (39), might encourage the population to maintain healthy PA levels and limit unhealthy sedentary time. Providing spaces for leisure-time PA, such as sports, is another way to promote physically active behavior in adolescents, but Bauman et al. (5), who studied energy expenditures in young adults living in China, found this kind of PA insufficient to prevent obesity. The authors suggested that an "active living" lifestyle with active transport and occupational activity could help people reach a 60 min/day quantum of PA for obesity prevention and weight loss (5). Walking or cycling to school would be an interesting way to increase the PA levels in the adolescents of New Caledonia, especially those living in urban areas. Unfortunately, these modes of transport seem unsuitable for many rural adolescents whose schools are often located at a fair distance from home. PA promotion could alternatively make these adolescents more aware of outdoor activities instead of spending too much time in sedentary activities.

CONCLUSION

This study, the first of this type in New Caledonia, shows that although 66% of adolescents were physically active, notable disparities existed in relation to place of residence, gender and ethnicity. Girls were less active on average than boys only for Melanesian adolescents living in rural areas. Melanesian adolescent boys living in rural areas were the most active group, suggesting that, in rural areas, activities related to the traditional Melanesian lifestyle may help adolescents to be active. In addition, feeling safe in the community was also identified as an important driver for PA. Although physically active adolescent boys were less likely to be overweight or obese, this was not the case for adolescent girls, perhaps because the girls engaged in different types of PA compared to boys. Additional research is warranted to better understand the barriers and facilitators driving overall engagement in PA and the role of PA and exercise on metabolic health in New Caledonian adolescents.

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 Human Research Ethics Committee of the University of New Caledonia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SF and OG conceived and designed the study. SF, CC, and OG collected data. GW conducted the statistical analyses and drafted the manuscript. CC, OG, and GW interpreted the results. All the authors participated in writing the manuscript, revised, and approved its final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.623685/full#supplementary-material

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