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Anthropogens, lifestyle and pathophysiology of chronic diseases: From mutual interplay to translational research and personalized medicine

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Anthropogens, lifestyle and pathophysiology of chronic diseases: From mutual interplay to translational research and personalized medicine

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Editorial: Anthropogens, lifestyle and pathophysiology of chronic diseases: From mutual interplay to translational research and personalized medicine

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Editorial on the Research Topic
Anthropogens, lifestyle and pathophysiology of chronic diseases: From mutual interplay to translational research and personalized medicine

Modern man-made environments, lifestyles, and behaviors (also known as anthropogens) have the ability to induce long-term low-level systemic inflammation (meta-inflammation) that may activate the inflammatory-oxidative cascade ultimately resulting in the development and progression of a wide range of chronic diseases (CD). These include cardiovascular diseases, metabolic disorders, cancer, among others, with high morbidity and mortality rates worldwide (1–3).

The interaction of magnitude and duration of exposure to anthropogens and chemicals with individuals' genetic background and/or epigenetic changes may represent the early stages of the pathogenesis of the chronic cardio-metabolic diseases mentioned earlier (4).

For effective disease management strategies, health professionals should focus on identifying all the potential risk factors associated with meta-inflammation and on tackling these through personalized health care services.

The aim of this Research Topic was to bring together the newest research results in the field of anthropogens, lifestyle (with a focus on nutrition), the pathophysiology of chronic diseases, and potential interventions for the prevention and management of chronic diseases, particularly at individual level.

Considering the high diversity of anthropogenic factors, the contributors covered a wide range of Research Topics, focusing on environmental risk factors, lifestyle, potential

interventions, and outcomes measurement, most of them providing original research results (8/9 articles).

In a population-based cohort study, He et al. analyzed pre- and perinatal risk factors, and found that even a normal Apgar score of 7–9 may predict a suboptimal brain development and higher risk of poor short and long-term outcomes, such as mental disorders, organic disorders, and neurodevelopmental disorders. The results of this study, performed with data from Danish national registries, suggested that suboptimal Apgar scores <7–9 should be also considered an alarming risk factor for subsequent mental disorders and for designing targeted public health strategies.

Giambò et al. reviewed the role played by the gut microbiota regarding the metabolism and protection mechanisms against environmental chemicals (i.e., pesticides, metals, and microplastics) to tailor personalized preventive strategies. The human gut microbiota may interact with environmental pollutants, such as pesticides, metals, and microplastics, which are incriminated as risk factors for cancer, obesity, diabetes, immune disorders, and etc. GM composition analysis, together with the determination of well-known biomarkers, could be used as a screening tool for assessing the long-time exposure to pollutants and shaping specific preventive interventions.

Eguiguren-Jiménez et al. performed a retrospective cross-sectional study focused on the risk factors associated with chronic kidney diseases (CKD) in non-institutionalized adults in Quito, Ecuador's capital city. Based on data registered between 2019 and 2021 on a randomly selected sample of 1,701 adults, out of whom 813 met the inclusion criteria, indicated a CKD prevalence of 7.2%, with about 45% in diseases stages 2–4. Systolic blood pressure, sex and residence area (probably linked to environmental factors) were the main risk factors significantly associated with estimated glomerular filtration rate (eGFR) as a proxy for CKD.

Xenos et al. analyzed the impact of Vitamin D supplements on the outcome of weight loss diets in Caucasian population, through a randomized double-blind placebo-controlled clinical study on vitamin D3 supplementation together with personalized weight-loss diet on obesity markers in overweight and obese individuals with vitamin D deficiency or insufficiency. The study found that 3,000 IU vitamin D3 oral spay decreased obesity markers, with the response being influenced by the vitamin D receptor (VDR) and adrenergic receptors (ADRs) genetic polymorphism. Edo et al. also dedicated their research to the role of dietary factors, investigating the potential association between the nutrients intake and the retinal vessels caliber in a cross-sectional survey performed in Japanese descendants living in Los Angeles. The study found a significant inverse association between vitamins A, C, and potassium intake and retinal venular caliber, indicative of a beneficial effect of these nutrients on the retinal microvascular profile and a healthier retinal micro-circulation.

Li et al. performed a cross-sectional study to examine the association between a lifestyle component—leisure time physical activity and multi-morbidity (17 chronic diseases, representing the most common non-communicable diseases among Chinese population). The study was based on 6,084 participants from a large-scale, multi-ethnic cohort in China. Results showed significant associations between low level of physical activity and chronic diseases, suggesting that health interventions should be specifically designed.

Two articles were dedicated to potential interventions addressing risk factors or symptoms of dyslipemia and chronic prostatitis. Lee and Lee presented the results of a randomized double-blind placebo-controlled trial, assessing the lipid-lowering effects of *Ulmus macrocarpa Hance extract* (UME) in adults with untreated high low-density lipoprotein cholesterol concentration. After 12 weeks, the results indicated that the lipid profile of patients treated with UME improved significantly as compared with the placebo group. Searching for an effective and safe solution for chronic prostatitis (CP)/chronic pelvic pain syndrome (CPPS), the most common chronic prostatitis in men, Wu et al. investigated the analgesic mechanism of electroacupuncture (EA), the combination between traditional acupuncture and modern electrical stimulation, in rats with CP/CPPS. Results showed that the analgesic mechanism of EA may be related to the cAMP-PKA-TRPV1/PLC-PKC-TRPV1 signaling pathway, by normalizing the altered gene expression.

Finally, Pan et al. investigated the model for end-stage liver disease (MELD) score and the changes on the dynamic changes in circulating lymphocyte subsets in Chinese patients undergoing liver transplantation. The study showed that patients with either the low MELD scores or the long-term follow-up period are in a relatively good immune condition and may receive higher doses of immunosuppressants, to prevent the potential complications.

Overall, the studies included in this Research Topic highlight how lifestyle, nutritional and environmental interventions, apart from specific pharmacologic treatments, can influence the prognosis of chronic diseases and contribute to identify new mechanisms involved in their pathogenesis.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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

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The Model for End-Stage Liver Disease Score and the Follow-Up Period Can Cause the Shift of Circulating Lymphocyte Subsets in Liver Transplant Recipients

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Little is known about the shift of lymphocytes under the condition of the model for end-stage liver disease score and the follow-up period. Then, we detected the peripheral blood from liver transplant recipients by flow cytometry and compared the results. The model for end-stage liver disease score affected the percentages of T-cell subsets and B cells during the short-term follow-up period, but failed to influence the lymphocyte subsets during the long-term follow-up period. In contrast, the follow-up period not only affected the absolute counts of T-cell subsets and natural killer (NK) cells in patients with the low model for end-stage liver disease scores, but also influenced the percentages and absolute counts of T-cell subsets in patients with the high model for end-stage liver disease scores. In the two-way ANOVA, we further revealed that the model for end-stage liver disease score was associated with the percentages of T cells and $CD4^+$ T cells and the absolute numbers of T-cell subsets and B cells, while the follow-up period was associated with the percentages of T-cell subsets and the absolute numbers of lymphocyte subsets. Therefore, patients with either the low model for end-stage liver disease scores or the long-term follow-up period are in a relatively activated immune condition.

Keywords: liver transplantation, circulating lymphocyte subsets, follow-up period, model for end-stage liver disease score, dynamic changes

INTRODUCTION

Liver transplantation is a promising procedure for patients with benign and malignant liver diseases. However, these patients are universally plagued by immunosuppression-related complications following liver transplantation, especially acute rejection during the posttransplant period (1–3). Currently, the model for end-stage liver disease (MELD) score has been widely accepted as a fair and objective method for liver transplant allocation, which is based on disease severity (4). The correlation between the preoperative MELD score and the occurrence of acute rejection is uncertain. Jia et al. (5) found that liver transplant recipients (LTR) with rejection had higher MELD scores. Similar results were described in adult-to-adult living donor liver transplantation (6). In contrast, Selzner et al. (7) reported that there were no differences between

patients with high or low MELD scores in terms of acute rejection. Actually, different circulating lymphocyte subsets have been repeatedly reported as critical components in acute graft rejection (8–11). Therefore, the correlation could be further investigated between the MELD scores and the lymphocytes. In patients with hepatitis B virus-related acute-on-chronic liver failure, the MELD score was found to be correlated positively with the regulatory T cells to T helper 17 (Th17) ratio at the peak point (12), while negatively with the ratio between circulating $CD3^+$ T cells and monocytes (13). These patients also had significantly increased $CD4^+$ T cells and decreased lymphocytes $CD3^+$ T cells than normal subjects (14). Since studies mainly focus on the relations between T cells and the MELD scores, the impact of the MELD score on lymphocytes remains to be elucidated.

Different follow-up periods might have an association with the occurrence of acute rejection, as most acute rejections reported in LTR occur within the first year, especially the first 6 months (15–17). Zhu et al. noted a group of LTR that showed acute rejection at a mean follow-up of nearly 2 months, whose interferon-γ^+ (IFN-γ^+) $CD4^+$ T cells and interleukin-2 (IL-2^+) $CD4^+$ T cells and transforming growth factor-β (TGF-β^+) $CD19^+$ B cell and granzyme B^+ $CD19^+$ B cell rose significantly compared with LTR without rejection (9). Boix et al. found that LTR, who rejected the allograft, had a statistically significant higher ratio of $CD4^+$ $CD154^+$ T cells and $CD8^+$ $CD154^+$ T cells on the 7th and 15th postoperative days (18). Nevertheless, the recommended tacrolimus trough concentrations used in immunosuppressive schemes taper during the first 6 months (19–21). In contrast, patients, who tend to develop a late opportunistic infection, are found to have lower counts of $CD3^+$, $CD4^+$, $CD8^+$ T cells, and natural killer (NK) cells at the first postoperative month (22, 23). Therefore, in an attempt to better control acute rejection following liver transplantation, it is of great importance to know the rationale of the lymphocyte subset shift over time.

Presently, little is known about the dynamic changes of circulating lymphocyte subsets in LTR with the different MELD scores and the follow-up periods. In the meantime, previous studies mainly focused on a specific cell subset and our understanding of how the circulating lymphocyte subsets as a whole respond to a transplant is lacking. Thus, characterizing the shift of circulating lymphocyte subsets in the MELD score follow-up period co-occurrence would help to understand the postoperative immune status and tailor individualized immunosuppressive therapy. We conducted this study to analyze the effects of the MELD score, the follow-up period, and their possible interaction on the lymphocyte subsets following liver transplantation.

MATERIALS AND METHODS

Study Design

This study was conducted to investigate the MELD score and the follow-up period-related dynamic changes in circulating lymphocyte subsets in LTR, who underwent a single liver transplant and were followed up at the Beijing Chaoyang Hospital between December 2017 and July 2020. To accurately evaluate the effects of the MELD score and the follow-up period on circulating lymphocyte subsets, LTR with concurrent autoimmune disease, HIV, diseases of hematopoietic and lymphoid systems, or any postoperative complications were excluded. We followed the method part of Pan et al. (24) in this study.

Immunosuppressive Therapy

Immunosuppressive therapy consisted of induction with basiliximab (20 mg on days 0 and 4) and maintenance, which was based on steroids, mycophenolate mofetil, and tacrolimus. Methylprednisolone (500 mg) was intravenously infused during the operation. After surgery, it was given by 240 mg/day and daily reduced by 40 mg till the 6th postoperative day. Then, it was changed to prednisolone (20 mg/day). Prednisolone was gradually withdrawn within 1 month. Sirolimus was used in selected patients with impaired renal function or for its antitumor effects at least 1 month after surgery.

Cell Preparation and Surface Staining

A sample of 5 ml of whole blood was taken from LTR. The separation of peripheral blood mononuclear cells (PBMCs) was performed *via* Ficoll density gradient centrifugation. After that, PBMCs were resuspended in phosphate-buffered saline (PBS). Then, PBMCs were stained with antibodies at 4°C in the dark for 20 min. The following reagents were obtained from BD Biosciences (Franklin Lakes, New Jersey, USA): fluorescein isothiocyante (FITC)-anti-CD3, CY5.5-anti-CD4, phycoerythrin (PE)-anti-CD19, allophycocyanin (APC)-anti-CD16, and PE-anti-CD56.

Flow Cytometric Measurement

After surface staining, PBMCs were washed twice with 2 ml PBS and resuspended in 400 μl PBS for flow cytometry analysis. Flow cytometry was conducted on NovoCyte D2060R (ACEA Biosciences Incorporation, San Diego, California, USA). We used the NovoExpress software (San Diego, California, USA) for analysis. The lymphocytes evaluated were T ($CD3^+$), TCD4 ($CD3^+$ $CD4^+$), TCD8 ($CD3^+$ $CD8^+$), B ($CD19^+$), and NK ($CD56^+CD16^+$). Flow cytometry characterization of circulating lymphocyte subsets is given in **Figure 1**. The lymphocyte subset counts were obtained using the percentages *via* flow cytometry and the absolute numbers of lymphocytes obtained *via* routine blood tests on the same day.

Statistical Analysis

All the statistical analyses were produced in the SPSS 19.0 computer software (IBM Corporation, Armonk, New York, USA). All the values compared were expressed as mean ± SD. Normal distribution tests were applied by the Kolmogorov–Smirnov test. The chi-square test or Fisher's exact test was employed to compare nominal variables. Student's *t*-test was applied to compare independent samples. The two-way ANOVA was used to test for the MELD score vs. the follow-up period interaction. The results were statistically significant at the 0.05 level. The prism was used for figures.

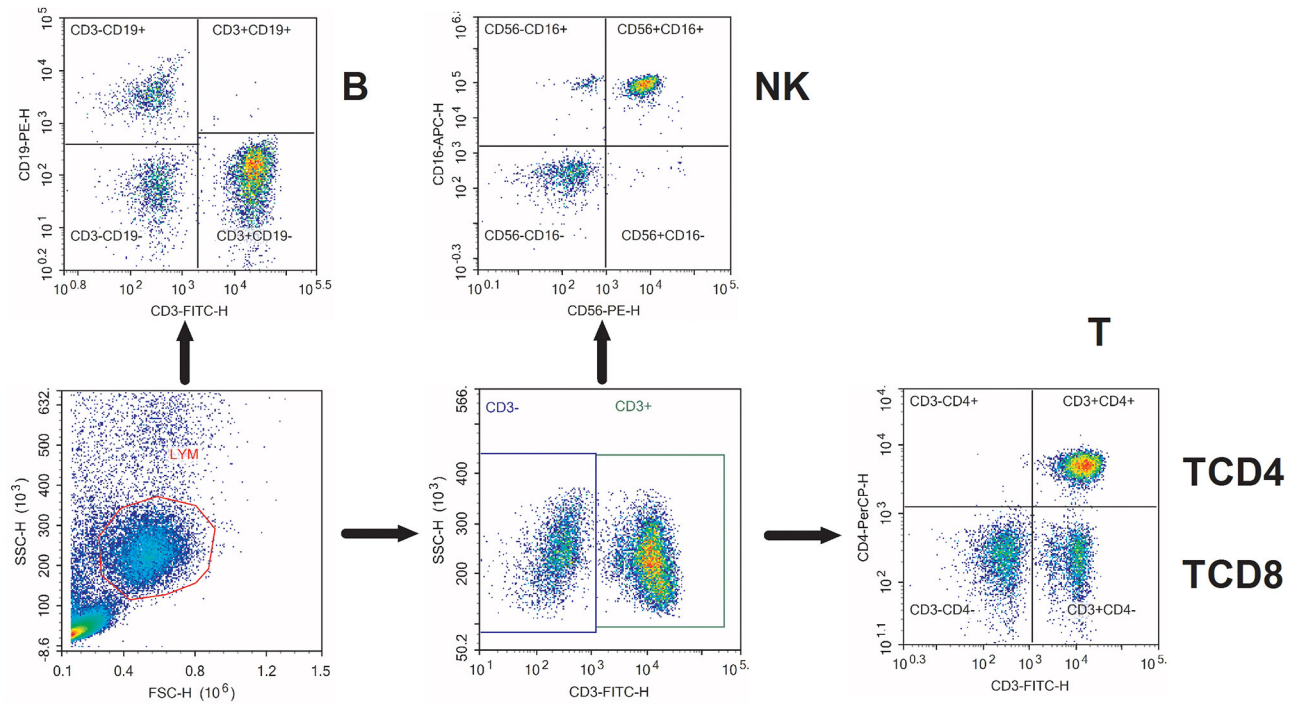

FIGURE 1 | Flow cytometry characterization of circulating lymphocyte subsets. Representative flow cytometry dot plots of the characterization of various circulating lymphocyte subsets. T, $CD3^+$ T cells; TCD4, $CD3^+$ $CD4^+$ T cells; TCD8, $CD3^+$ $CD8^+$ T cells; B, $CD19^+$ B cells; NK, $CD56^+$ $CD16^+$ natural killer cells.

TABLE 1 | Demographic data.

	LmLf (n = 18)	HmLf (n = 13)	LmSf (n = 20)	HmSf (n = 15)	P
Gender (Male)	16	12	18	13	0.830
Age	55.17 ± 7.06	53.62 ± 9.16	52.20 ± 11.65	50.07 ± 15.55	0.221
Hepatic carcinoma	9	4	8	3	0.309
MELD score	3.94 ± 2.45[a]	28.85 ± 4.67[b]	4.45 ± 2.61[c]	29.8 ± 5.94[d]	0.000
Follow-up period	56.78 ± 15.16[a]	56.38 ± 16.52[c]	16.95 ± 5.60[b]	18.07 ± 5.90[d]	0.000

Lm, low MELD score; Hm, high MELD score; Sf, short-term follow-up; Lf, long-term follow-up; MELD, a model for end-stage liver disease.
a vs. b, a vs. d, c vs. b, and c vs. d, $p < 0.001$.

RESULTS

Study Population

As circulating lymphocyte subsets have been reported to be affected under physiological and pathological conditions (25–27), we selected LTR in the absence of any postoperative complications to minimize the potential impact. A total of 66 LTRs with stable liver function were enrolled in this study. There were 7 women and 59 men with a mean age of 52 years (range, 26–73 years). Hepatic carcinoma was pathologically proven in 24 patients, while the rest had hepatitis-related cirrhosis. LTR was divided into four groups depending on the MELD score (low MELD score < 10, high MELD score ≥ 20) and the follow-up period (short-term follow-up < 28 days, long-term follow-up 28 days−3 months) including the LmLf group (low MELD score and long-term follow-up), the HmLf group (high MELD score and long-term follow-up), the LmSf group (low MELD and short-term follow-up), and the HmSf group (high MELD score and short-term follow-up). Characteristics of these patients are given in **Table 1**.

Effect of the MELD Score on Circulating Lymphocyte Subsets

First, we wanted to determine whether the MELD score would affect the postoperative circulating lymphocyte subsets. We performed the comparison between the low MELD score (<10) and long-term follow-up (28 days−3 months) (LmLf) group and the high MELD score and long-term follow-up (HmLf) group and the low MELD score and short-term follow-up (LmSf) group and the high MELD score (≥20) and short-term follow-up (<28 days) (HmSf) group. After comparison, we found that there was no statistical difference between the LmLf group and the HmLf group with respect to the percentages (**Figure 2** and **Table 2**) and

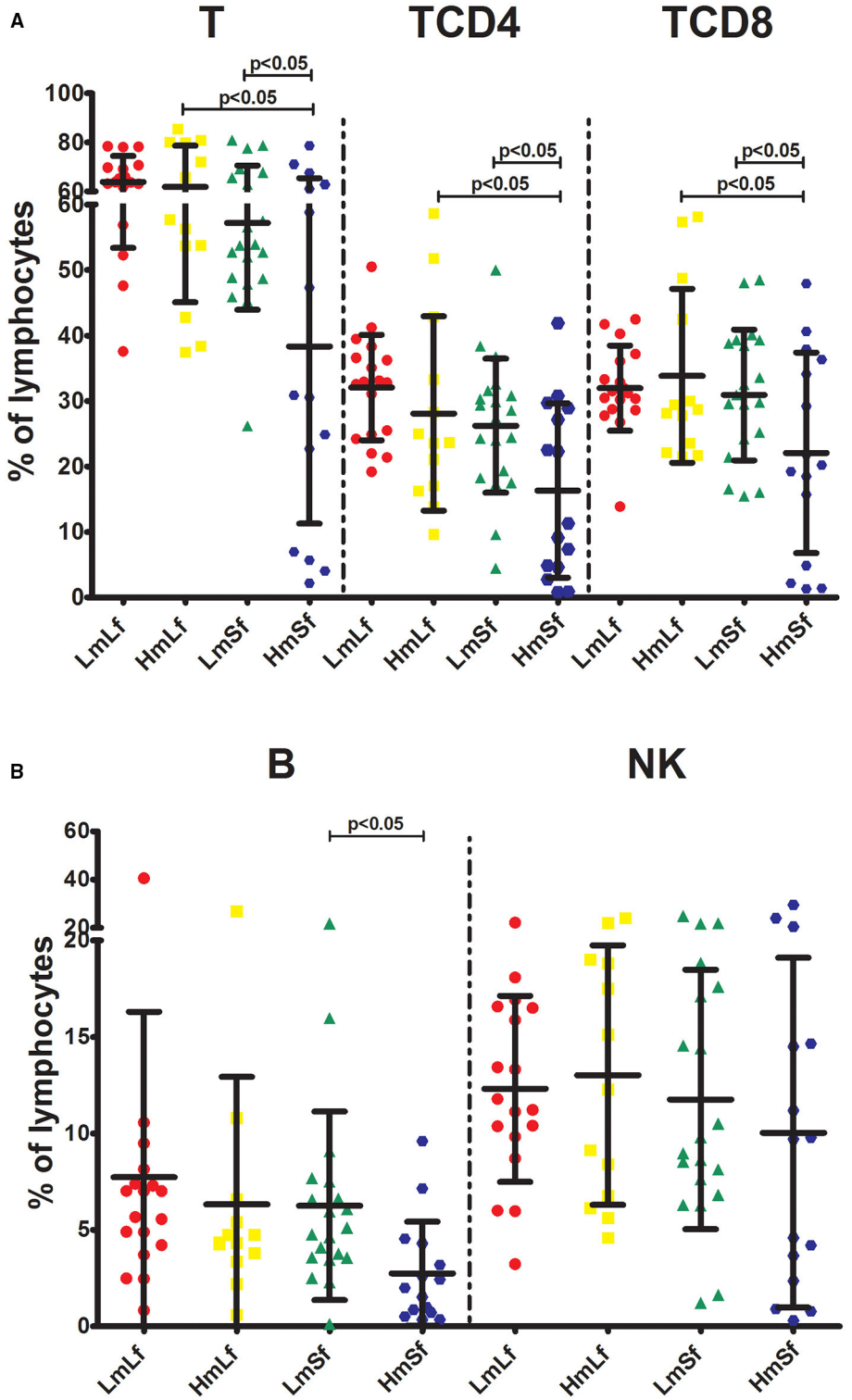

FIGURE 2 | Effects of the MELD score and the follow-up period on the percentages of lymphocyte subpopulations following liver transplantation. Comparison of the percentages of T, TCD4, and TCD8 **(A)**, B and NK **(B)** among LmLf ($n = 18$), HmLf ($n = 13$), LmSf ($n = 20$), and HmSf ($n = 15$). Bars represent mean and SD. MELD, model for end-stage liver disease; T, $CD3^+$ T cells; TCD4, $CD3^+$ $CD4^+$ T cells; TCD8, $CD3^+$ $CD8^+$ T cells; B, $CD19^+$ B cells; NK, $CD56^+$ $CD16^+$ natural killer cells; LmLf, low MELD score (<10) and long-term follow-up (28 days–3 months); HmSf, high MELD score (≥20) and short-term follow-up (<28 days); HmLf, high MELD score and long-term follow-up; LmSf, low MELD score and short-term follow-up.

TABLE 2 | Effects of the MELD score and the follow-up period on the percentages of lymphocyte subpopulations following liver transplantation.

Percentages	LmLf ($n = 18$)	HmLf ($n = 13$)	LmSf ($n = 20$)	HmSf ($n = 15$)
T	63.90 ± 10.52	61.89 ± 16.81*	57.22 ± 13.29*	38.35 ± 27.06
TCD4	32.06 ± 8.06	28.09 ± 14.85*	26.24 ± 10.23*	16.33 ± 13.30
TCD8	31.97 ± 6.52	33.84 ± 13.28*	30.93 ± 9.98*	22.09 ± 15.30
B	7.73 ± 8.56	6.32 ± 6.62	6.25 ± 4.88*	2.74 ± 2.69
NK	12.31 ± 4.82	13.02 ± 6.73	11.76 ± 6.73	10.03 ± 9.06

MELD, model for end-stage liver disease; T, $CD3^+$ T cells; TCD4, $CD3^+$ $CD4^+$ T cells; TCD8, $CD3^+$ $CD8^+$ T cells; B, $CD19^+$ B cells; NK, $CD56^+$ $CD16^+$ natural killer cells; Lm, low MELD score; Hm, high MELD score; Sf, short-term follow-up; Lf, long-term follow-up.
*HmLf vs. HmSf, LmSf vs. HmSf, *$p < 0.05$.*

absolute counts (**Figure 3** and **Table 3**) of T, TCD4, TCD8, B, and NK ($p > 0.05$), suggesting that the MELD score did not affect the lymphocyte subsets during long-term follow-up.

In contrast, we observed higher percentages of T, TCD4, TCD8, and B (**Figure 2** and **Table 2**) in the LmSf group ($p < 0.05$), but the similar percentage of NK (**Figure 2** and **Table 2**) and absolute counts (**Figure 3** and **Table 3**) of T, TCD4, TCD8, B, and NK ($p > 0.05$) when the LmSf group was compared with the HmSf group. The results mean that the MELD score could affect the lymphocyte subsets during short-term follow-up.

Effect of the Follow-Up Period on Circulating Lymphocyte Subsets

Next, we checked whether the follow-up period played an important role in the shift of circulating lymphocyte subsets. We compared the LmLf group with the LmSf group and subsequently found that the comparison failed to reach significance although the percentages (**Figure 2** and **Table 2**) of T, TCD4, TCD8, B, and NK were a little higher in the LmLf group ($p > 0.05$). However, the absolute counts (**Figure 3** and **Table 3**) of T-cell subsets and NK cells rather than B cells were significantly different between the groups ($p < 0.05$). These results showed that T-cell subsets and NK cells from patients with mild liver disease proliferated significantly over the follow-up period.

Surprisingly, the HmSf group presented lower percentages (**Figure 2** and **Table 2**) and lower absolute numbers (**Figure 3** and **Table 3**) of T-cell subsets ($p < 0.05$), but no significant differences in percentages (**Figure 2** and **Table 2**) and absolute numbers (**Figure 3** and **Table 3**) of B and NK ($p > 0.05$) were observed when compared with the HmLf group. These data reflected that T-cell subsets from patients with severe liver disease also proliferated significantly over the follow-up period.

Interaction of the MELD Score and the Follow-Up Period on Circulating Lymphocyte Subsets

From the above analysis, we noticed that the MELD score and the follow-up period could affect the circulating lymphocyte subsets. Therefore, we, then investigated the combined effect of the MELD score and the follow-up period on circulating lymphocyte subsets. To strengthen the analysis of the combined effects and to test their possible interaction, we performed the two-way ANOVA with four groups in a two-by-two factorial design. The independent variables were the MELD score (high vs. low) and the follow-up period (short term vs. long term).

After comparison, we found that there was no synergetic effect on the percentages or absolute counts of lymphocyte subsets (**Table 4**). In this analysis, the MELD score influenced the percentages of T and TCD4 and the absolute numbers of T, TCD4, TCD8, and B ($p < 0.05$), while the follow-up period had an impact on the percentages of T, TCD4, and TCD8 and the absolute numbers of T, TCD4, TCD8, B, and NK ($p < 0.05$). The results found in the two-way ANOVA are a little different from the above findings.

DISCUSSION

In this study, data from LTR with the different MELD scores at the different follow-up periods were collected and analyzed to determine the function of the two factors on the shift of circulating lymphocyte subsets. We found that the MELD score and the follow-up period did not have a synergetic effect on the percentages or absolute counts of lymphocyte subsets. The MELD score affected the percentages of T and TCD4 and the absolute numbers of T-cell subsets and B cells. The follow-up period was in relation to the percentages of T-cell subsets and the absolute numbers of lymphocyte subsets.

Currently, extensive reports have described the shift of a specific cell subpopulation in various diseases (28–30). Nevertheless, there are only limited studies on the changes in lymphocyte subsets under the condition of the MELD score following the liver transplantation. It was found that patients with acute-on-chronic liver failure had high Th17 frequency since the onset point (12) and the decreased T-cell repertoire (31). Freitas et al. reported that patients with benign renal diseases had lower absolute counts of T-cell subsets and B cells when compared with healthy controls (32). These studies reflect that patients with high MELD scores have suppressed immunity. In this study, we also confirmed that LTR with the high MELD scores had lower absolute counts of T-cell subsets and B cells. Since liver failure can lead to a significant decrease in the percentage of $CD4^+$ T cells (33), we detected a rather lower percentage of $CD4^+$ T cells in LTR with the high MELD scores. In addition, Tanimine et al. (34) demonstrated that chronic liver disease could also impair the potential of intrahepatic NK cells, which is not in agreement with our finding. Of note, dysfunction of NK cells rather than

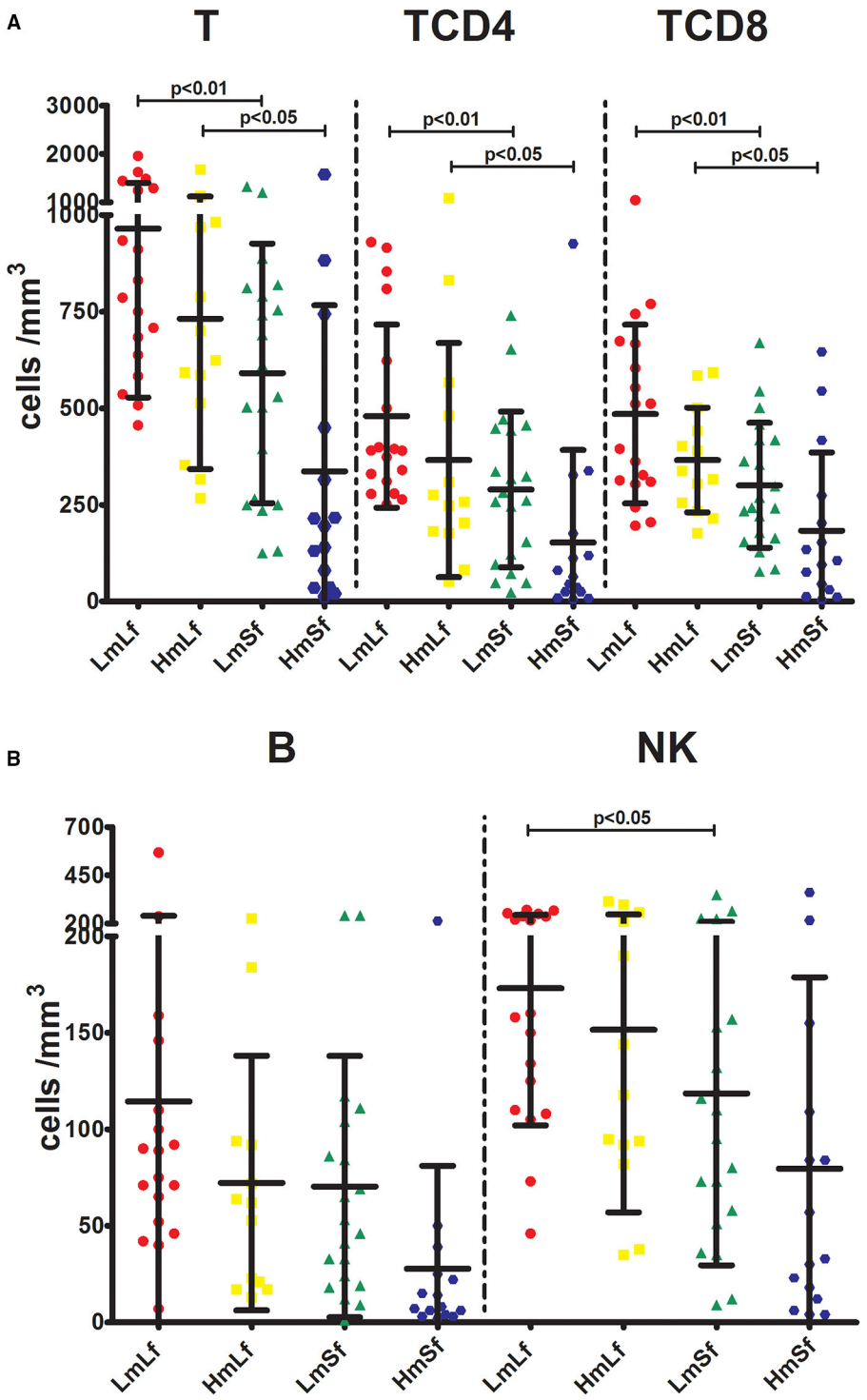

FIGURE 3 | Effects of the MELD score and the follow-up period on the absolute counts of lymphocyte subpopulations following liver transplantation. Comparison of the absolute counts of T, TCD4, and TCD8 **(A)**, and NK **(B)** among LmLf ($n = 18$), HmLf ($n = 13$), LmSf ($n = 20$), and HmSf ($n = 15$). Bars represent mean and SD. MELD, model for end-stage liver disease; T, $CD3^+$ T cells; TCD4, $CD3^+$ $CD4^+$ T cells; TCD8, $CD3^+$ $CD8^+$ T cells; B, $CD19^+$ B cells; NK, $CD56^+$ $CD16^+$ natural killer cells; LmLf, low MELD score (<10) and long-term follow-up (28 days–3 months); HmSf, high MELD score (≥20) and short-term follow-up (<28 days); HmLf, high MELD score and long-term follow-up; LmSf, low MELD score and short-term follow-up.

TABLE 3 | Effects of the MELD score and the follow-up period on the absolute counts of lymphocyte subpopulations following liver transplantation.

Absolute counts	LmLf ($n = 18$)	HmLf ($n = 13$)	LmSf ($n = 20$)	HmSf ($n = 15$)
T	964.72 ± 437.14**	731.62 ± 388.89*	590.60 ± 335.74	336.40 ± 429.80
TCD4	479.61 ± 236.73*	365.69 ± 302.93*	289.95 ± 201.47	153.00 ± 239.11
TCD8	485.22 ± 231.35**	365.92 ± 135.42*	300.65 ± 161.45	183.47 ± 201.86
B	114.44 ± 124.70	72.15 ± 65.96	70.25 ± 67.68	27.80 ± 53.15
NK	173.11 ± 71.15*	151.54 ± 94.55	118.60 ± 89.08	79.67 ± 99.06

MELD, model for end-stage liver disease; T, $CD3^+$ T cells; TCD4, $CD3^+$ $CD4^+$ T cells; TCD8, $CD3^+$ $CD8^+$ T cells; B, $CD19^+$ B cells; NK, $CD56^+$ $CD16^+$ natural killer cells; Lm, low MELD score; Hm, high MELD score; Sf, short-term follow-up; Lf, long-term follow-up.
*HmLf vs. HmSf, LmSf vs. HmSf, *$p < 0.05$, **$p < 0.01$.*

TABLE 4 | The two-way ANOVA for the MELD score, the follow-up period, and the MELD score × the follow-up period interaction.

Lymphocyte subsets	Percentages			Absolute counts		
	MELD score	Follow-up period	MELD score x follow-up period	MELD score	Follow-up period	MELD score x follow-up period
T	0.020	0.001	0.057	0.017	0.000	0.916
TCD4	0.019	0.003	0.305	0.042	0.001	0.849
TCD8	0.224	0.028	0.063	0.014	0.000	0.982
B	0.112	0.103	0.496	0.049	0.040	0.997
NK	0.770	0.309	0.481	0.174	0.006	0.694

MELD, model for end-stage liver disease; T, $CD3^+$ T cells; TCD4, $CD3^+$ $CD4^+$ T cells; TCD8, $CD3^+$ $CD8^+$ T cells; B, $CD19^+$ B cells; NK, $CD56^+$ $CD16^+$ natural killer cells.

the percentage or the cell number is favored by poor clinical outcomes in liver cancer (35, 36). In this study, circulating NK cells were detected and LTR with both the malignant and benign liver diseases was enrolled. Therefore, this might, at least in part explain that the MELD score failed to affect the percentages and absolute counts of NK cells.

At present, the impact of the follow-up period on circulating lymphocyte subsets following liver transplantation is unclear. Zhuang et al. detected that the cell counts of T, TCD4, TCD8, B, and NK dropped profoundly shortly after stereotactic body radiation therapy and gradually recovered 2 months later (37). Similarly, we observed that the absolute numbers of lymphocyte subsets were reduced in LTR with short-term follow-up. Moreover, we found that the follow-up period affected the percentages of T-cell subsets instead of B cells and NK cells. At our center, basiliximab was used for induction and steroids and tacrolimus were used for maintenance. After the administration of prednisone and tacrolimus, there was a profound lymphocytopenia, a selective decrease in T cells (38–40). Nevertheless, there was no relation between prednisone or tacrolimus trough level and B cells, as mycophenolate mofetil has an impact on the suppression of B-cell functions (41, 42). Therefore, the percentages of T-cell subsets are relatively lower during short-term follow-up. With the rapid recovery of circulating lymphocyte subsets over time, there was a significant rise in the percentages of T-cell subsets.

From the above analysis, we know both the MELD score and the follow-up period can affect the circulating lymphocyte subsets, which show the clinical role in regulating the dose of immunosuppressive drugs. Since the MELD score is calculated based on the parameters of liver function and renal function, LTR with the high MELD scores usually has deteriorated renal function. Additionally, calcineurin inhibitors can further worsen renal function. Exposure to high serum levels of calcineurin inhibitors can result in infection, although the occurrence rate of acute rejection is scarce. Therefore, it is possible to employ a lower dose of calcineurin inhibitors in patients with high MELD scores following the liver transplantation. On the other hand, with the patients' recovery over time, their immunity improves, which makes the patients vulnerable to acute rejection. Hence, the dose of calcineurin inhibitors should increase accordingly. In contrast, current guidelines recommend that tacrolimus trough concentrations are high shortly after surgery, while it is maintained at a relatively low level over time (19–21), which might account for the higher occurrence rate of acute rejection within the first 6 months (15, 16). Taken together, based on the individual immune status instead of empiric therapy, the tailored treatment for each recipient is more favored.

The main limitation of this study is the relatively small number of patients enrolled that may limit the accuracy of our assessment. However, to make the data among different groups comparable, we excluded LTR with any complications. Second, the results represented the experience of a single center in selected patients. Future studies, preferably larger patient cohorts from multicenters, are needed to further confirm our preliminary outcomes.

CONCLUSION

Liver transplant recipients with either the low MELD scores or the long-term follow-up period are in a

relatively activated condition and should be exposed to higher levels of immunosuppressive drugs to prevent immunosuppression-related complications.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Beijing Chaoyang Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-QZ and QH contributed conception and design of the study. FP, R-lW, and SC organized the database. X-LL and Y-nJ performed the statistical analysis. FP and SC wrote the first draft of the manuscript. The rest wrote sections of the manuscript. All the authors contributed to manuscript revision, read, and approved the submitted version. They were accountable for all the aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Five-Minute Apgar Score and the Risk of Mental Disorders During the First Four Decades of Life: A Nationwide Registry-Based Cohort Study in Denmark

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Objectives: The associations of long-term risks of the full spectrum of mental disorders with clinically reassuring but suboptimal score range 7–9 remain unclear. This study investigated these associations during up to 38 years of follow-up.

Methods: In a nationwide cohort study of 2,213,822 singletons born in Denmark during 1978–2015, we used cox regression to estimate the hazard ratio (HR) of mental disorders with a 95% CI.

Results: A total of 3,00,679 (13.6%) individuals were diagnosed with mental disorders. The associations between suboptimal Apgar score 7–9 and mental disorders differed by attained age. In childhood (≤18 years), declining Apgar scores were associated with increased risks of overall mental disorders with HRs (95% CI) of 1.13(1.11-1.15), 1.34 (1.27–1.41), and 1.48 (1.31–1.67) for Apgar scores of 7–9, 4–6, and 1–3, respectively, compared with a score of 10. A dose-response association was seen even within the score range from 9 to 7 (HR 1.11 [95% CI: 1.08–1.13], 1.14 [1.10–1.18], and 1.20 [1.14–1.27], respectively). Of note, individuals with scores of 7–9 had increased risks of organic disorders (HR: 1.27, 95% CI: 1.05–1.53), neurotic disorders (HR: 1.07, 95% CI: 1.03–1.11), and a wide range of neurodevelopmental disorders, such as intellectual disability (1.87, 1.76–1.98), childhood autism (1.13, 1.05–1.22) and attention deficit hyperactivity disorder (1.10, 1.06–1.15). In early adulthood (19–39 years), suboptimal Apgar scores 7–9 were not associated with the risks of overall and specific mental disorders.

Conclusion: Infants born with clinically reassuring but suboptimal 5-min scores 7–9 are at increased risks of a wide spectrum of mental disorders in childhood.

Keywords: 5-min Apgar score, mental disorders, childhood, early adulthood, cohort study

INTRODUCTION

The Apgar score is based on five components (skin color, heart rate, reflex irritability, muscle tone, and respiration). Each item is scored from 0 to 2 with a total score of 7–10 as normal and the highest score of 10 representing the optimal condition (1, 2). The Apgar score has been used worldwide as a vitality index for almost every newborn immediately after birth (1). Although its use has been criticized due to the problems of accuracy, reproducibility, and universality, the American Academy of Pediatrics and the American College of Obstetrics and Gynecology endorsed its continued use in their 2015 statement (3) because of its abilities to assess the need for and response to resuscitation and robust associations with the risks of infant morbidity and mortality (4, 5).

A low Apgar score represents combined effects of various pre- and perinatal risk factors, which may predict non-optimal brain development (6–10). There is mounting evidence that a low Apgar score, commonly defined as a score <7, has been linked with certain mental disorders in later life, such as Autism Spectrum Disorder (ASD), Obsessive-Compulsive Disorder (OCD), and Attention Deficit Hyperactivity Disorder (ADHD) (10–15). However, the evidence on the associations with other mental disorders is lacking. More importantly, most prior studies considered an Apgar score of 7–9 to be "normal" and did not investigate whether the scores of 7–9 would be associated with a higher risk of mental disorders. Quantifying such associations is important as suggested by recent findings that even the scores of 7–9 were associated with a higher risk of adverse short- and long-term outcomes, such as neonatal mortality, morbidity, cerebral palsy, and epilepsy in childhood (16, 17).

In this population-based study, we hypothesized that the risk of mental disorders would increase with decreasing Apgar scores, even within the 'normal' range 7–9. Taking advantage of a Danish register-based cohort with up to 38 years of follow-up, we investigated the associations between 5-min Apgar scores and the risks of a full spectrum of mental disorders using population-based analyses.

METHODS

Study Population

We conducted a population-based cohort study using data from Danish national registers (18). A total of 2,272,473 live singletons were identified during 1978–2015 from the Danish Medical Birth Registry (19). We excluded 1,001 births with missing information on sex and 41,252 births with no valid information on 5-min Apgar scores (including the score of 0) (11). We further excluded 16,398 infants who died or emigrated from Denmark before the age of 1 year. The final cohort comprised 2,213,822 births.

Abbreviations: ASD, Autism Spectrum Disorder; OCD, Obsessive-Compulsive Disorder; ADHD, Attention Deficit Hyperactivity Disorder; PCRR, Psychiatric Central Research Register; NPR, National Patient Register; ICD, International Classification of Diseases; ODD/CD, Oppositional Defiant Disorder/Conduct Disorder; HR, Hazard Ratio; CI, confidence interval.

Main Exposures

Apgar scores at 5 min were the main exposures. We categorized them into three groups of compromised scores (1–3, 4–6, 7–9) and one optimal score (10). In addition, we analyzed Apgar scores in the way of each score (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10).

Outcomes of Interest

Information on mental disorders was obtained from the Danish Psychiatric Central Research Register (PCRR) and the National Patient Register (NPR) (20, 21). The PCRR contains all admissions to psychiatric inpatient facilities since 1969 and contacts to outpatient psychiatric departments and emergency care units since 1995. The NPR includes hospital discharge diagnoses since 1977 and outpatient and emergency diagnoses since 1995. The diagnostic system used was the Danish modification of the International Classification of Diseases, Eighth Revision (ICD-8) from 1969 to 1993 and Tenth Revision (ICD-10) from 1994 onwards. Details of the specific diagnoses included in each group of disorders are presented in **Supplementary Table S1** (22–24). For each mental disorder, the date of onset was defined as the first day of the first contact. Individuals with more than one disorder were included in the numerator for each specific disorder.

Potential Confounders

We included the following potential confounders based on causal diagrams using directed acyclic graphs (**Supplementary Figure S1**): sex (male/female), calendar period of birth (1978–1985/1986–1994/1995–2005/2006–2015), gestational age at childbirth (≤31/32–33/34–36/37–38/39–40/41/≥42 weeks), birth weight percentiles (<10th/10th−90th/>90th centile) using the distribution of sex and year of delivery of the entire study population as the standard, parental psychiatric history at delivery (yes/no), and other maternal characteristics (parity [1/2/≥3 children], age at birth [<20/20–24/25–29/30–34/ ≥35 years], smoking during pregnancy [yes/no], highest attained level of education [0–9/10–14/≥15 years], cohabitation with a partner [yes/no], residence [Copenhagen/cities with 1,00,000 or more inhabitants/other], birth country [Denmark/others]). Missing data for each variable were coded using a missing data indicator.

Statistical Analysis

All included children were followed up from the earliest possible age at onset of the disorder (for each disorder separately) until the date of the first diagnosis, death, emigration, or December 31, 2016, whichever came first. Data were analyzed from December 2019 through June 2020. Cox regression was used to estimate hazard ratios (HRs) with a 95% CI to assess the associations between Apgar score and mental disorders, with participant's age as the time scale. Because there was evidence of non-proportional hazards, we split person time by attained age such that associations were allowed to vary over time. Age groups were set with childhood (≤18 years) and early adulthood (19–39 years). Regarding the analyses for early adulthood, we excluded some categories of mental disorders not commonly diagnosed in adulthood, such as autism.

In sensitivity analyses, we investigated potential sex specificity of risks of mental disorders by repeating the analyses in male or female individuals. We restricted the analyses to individuals without congenital malformations of the nervous system and chromosomal abnormalities to preclude the potential consequences of those defects on mental disorders and Apgar score. Owing to ICD code changes in 1994 and integration of registered data from three departments (inpatient, outpatient, and emergency) in 1995, we performed sub-analyses restricted to individuals born after 1994. In addition, we stratified the participants by gestational age at birth or birth weight percentile to assess whether the risk pattern was modified by fetal maturity or growth status *in utero*. All analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC).

Ethical Approval

The study was approved by the Data Protection Agency (No. 2013-41-2569). By Danish law, no informed consent is required for a registry-based study using anonymized data.

RESULTS

Of the 2,213,822 singleton live newborn infants, 1,47,984 (6.68%) were assigned compromised 5-min Apgar score (scores of 1–3:0.09%, 4–6:0.49%, 7–9: 6.10%). Compared with those with a score of 10, infants with compromised scores are more likely to be male and to have a parental history of mental disorders. Mothers of newborns with compromised scores are more likely to be primiparous, to bear a child before age 20 or after 35, to smoke during pregnancy, to live alone, and to have a low education level (**Table 1**).

During up to 38 years of follow-up, 3,00,679 individuals received a diagnosis of mental disorders (356, 1886, 19 565, and 278 872 in groups of score at 1–3, 4–6, 7–9, and 10 respectively) (**Figure 1**). Throughout the childhood period, we found a dose-dependent pattern between compromised scores and the overall risks of mental disorders with HRs (95% CI) of 1.48 (1.31–1.67), 1.34 (1.27–1.41), and 1.13 (1.11–1.15) for scores of 1–3, 4–6, and 7–9, respectively, compared with the score of 10. Importantly, a dose-response association was also observed within the clinically "normal" score range of 7, 8, and 9: 1.20 (1.14–1.27), 1.14 (1.10–1.18), and 1.11 (1.08–1.13), respectively.

The relative risks of all studied subcategories of mental disorders in childhood were presented (**Figure 2** and **Supplementary Table S2**). In the population analyses, dose-response gradients were observed across the entire score strata for intellectual disability (HR range: 1.87 to 5.33), organic disorders (HR range: 1.27 to 4.26), pervasive developmental disorders (HR range: 1.15 to 1.63), ADHD (HR range: 1.10 to 1.38), and for Oppositional Defiant Disorder/Conduct Disorder (ODD/CD) (HR range: 1.07 to 1.68), meanwhile, a higher risk (HR, 1.31) of neurotic disorders was seen in individuals with a score of 1–3. Of note, individuals with clinically "normal" score range of 7–9 have an 87% higher risk of intellectual disability (95% CI, 1.76–1.98) and 7∼30% increased risks of other specific subcategories: organic disorders (1.27 [1.05–1.53]), neurotic disorders (1.07 [1.03–1.11]), pervasive developmental disorders (1.15 [1.10–1.20]), childhood autism (1.13 [1.05–1.22]), and ADHD (1.10 [1.06–1.15]).

During early adulthood, we did not find that compromised 5-min Apgar scores were associated with overall mental disorders (**Figure 1**). Nevertheless, higher risks but with low statistical precisions in the population cohort were found for some subtypes: schizophrenia (1.11 [0.66–1.88]), neurotic disorders (1.22 [0.94–1.58]), and personality disorders (1.34 [0.90–1.99]) in individuals with a score of 1–3, and organic disorders (1.65 [1.00–2.72]) in individuals with a score of 4–6 (**Figure 2** and **Supplementary Table S3**).

In the sensitivity analysis, effect estimates from analyses stratified by gestational age at birth or birth weight percentile for gestational age were basically consistent with those observed in the main analysis (**Supplementary Figures S2**, **S3**). Stratified analyses by sex in the entire cohort showed no clear evidence of differences in associations between male and female individuals (**Supplementary Tables S4**, **S5**). Results from separate analyses restricted to individuals without diagnoses of congenital malformations of the nervous system or chromosomal abnormalities, or born after 1994, or without neonatal brain lesions were similar to those obtained in the primary analyses (**Supplementary Tables S6**–**S9**).

DISCUSSION

Main Findings

In this large population-based cohort study, during childhood, we found individuals with even clinically "normal" Apgar score range of 7–9 still had higher risks of overall mental disorders and some specific diagnoses: organic disorders and a series of neurodevelopmental disorders (intellectual disability, pervasive developmental disorders, childhood autism, and ADHD). It is also interesting to observe that compromised Apgar scores were at elevated risks of developing organic disorders and neurotic disorders, which were reported for the first time. During early adulthood, compromised 5-min Apgar scores were not found to be associated with mental disorders.

Comparisons With Other Studies

To our knowledge, this is the first study to examine the association of the full spectrum of mental disorders with the 5-min Apgar score. Our findings indicate the strongest associations for intellectual disability in childhood, which corroborates the results from previous studies (4, 15, 25, 26). Most of previous studies were based on the results of different non-standardized intelligence tests, and cross-sectional or descriptive designs (15, 25, 26), except a recent Swedish study, by virtue of clinically confirmed diagnosis and cohort design, reporting that term infants with low 5-min Apgar score had a higher risk of severe neurologic morbidity, including a 9-fold risk of intellectual disability (4). However, the Swedish study only captured cases before 14 years of age and only adjusted for year of birth, maternal age, parity, and smoking. Similarly, we observed 3∼5-fold risks of intellectual disability in childhood (until 18 years of age), and we were able to adjust for not only the aforementioned confounders but also parental psychiatric

TABLE 1 | Baseline characteristics according to Apgar score at 5 min, live singleton births in Denmark from 1978 to 2015.

Characteristics	Apgar 1–3 (n = 2083)	Apgar 4–6 (n = 10833)	Apgar 7–9 (*n* = 135,068)	Apgar 10 (*n* = 2,065,838)
Sex				
Male	1,149 (55.2)	6,168 (56.9)	76,258 (56.5)	1,052,202 (50.9)
Female	934 (44.8)	4,665 (43.1)	58,810 (43.5)	1,013,636 (49.1)
Calender year of birth				
1978–1985	291 (14.0)	1,944 (18.0)	20,592 (15.3)	398,633 (19.3)
1986–1994	477 (22.9)	2,656 (24.5)	31,622 (23.4)	504,430 (24.4)
1995–2005	611 (29.3)	3,453 (31.9)	45,530 (33.7)	632,435 (30.6)
2006–2015	704 (33.8)	2,780 (25.7)	37,324 (27.6)	530,340 (25.7)
Maternal parity				
1	1,139 (54.7)	6,258 (57.8)	73,061 (54.1)	911,448 (44.1)
2	612 (29.4)	3,019 (27.9)	41,458 (30.7)	778,448 (37.7)
≥3	332 (15.9)	1,556 (14.4)	20,549 (15.2)	375,942 (18.2)
Maternal age at birth (years)				
<20	35 (1.7)	252 (2.3)	2,499 (1.9)	36,413 (1.8)
20–24	349 (16.8)	1,881 (17.4)	22,110 (16.4)	337,310 (16.3)
25–29	683 (32.8)	3,803 (35.1)	48,320 (35.8)	739,226 (35.8)
30–34	677 (32.5)	3,156 (29.1)	41,258 (30.6)	647,121 (31.3)
≥35	339 (16.3)	1,741 (16.1)	20,881 (15.5)	305,768 (14.8)
Maternal smoking during pregnancy[a]				
Yes	316 (20.4)	1,576 (21.0)	18,505 (18.9)	273,737 (19.5)
No	1,152 (74.2)	5,547 (73.9)	75,597 (77.0)	1,078,105 (76.9)
Missing	84 (5.4)	386 (5.1)	4,022 (4.1)	49,771 (3.6)
Maternal education at childbirth (years)				
0–9	600 (28.8)	3,205 (29.6)	35661 (26.4)	556962 (27.0)
10–14	893 (42.9)	4,766 (44.0)	60,357 (44.7)	892,458 (43.2)
≥15	539 (25.9)	2,649 (24.5)	36,875 (27.3)	579,258 (28.0)
Missing	51 (2.5)	213 (2.0)	2,175 (1.6)	37,160 (1.8)
Maternal cohabitation at childbirth				
Yes	1,020 (49.0)	5,355 (49.4)	68,410 (50.7)	1,144,783 (55.4)
No	1,060 (50.9)	5,472 (50.5)	66,613 (49.3)	920,495 (44.6)
Missing	3 (0.1)	6 (0.1)	45 (0.0)	560 (0.0)
Maternal residence at childbirth				
Copenhagen	188 (9.0)	1,052 (9.7)	14,601 (10.8)	231,791 (11.2)
Big cities ≥100,000 inhabitants	238 (11.4)	1,354 (12.5)	19,238 (14.2)	261,971 (12.7)
Others	1,657 (79.6)	8,427 (77.8)	101,229 (75.0)	1,572,076 (76.1)
Maternal country of birth				
Denmark	1,846 (88.6)	9,672 (89.3)	121,930 (90.3)	1,836,244 (88.9)
Others	233 (11.2)	1,137 (10.5)	12,961 (9.6)	226,544 (11.0)
Missing	4 (0.2)	24 (0.2)	177 (0.1)	3,050 (0.2)
Maternal history of mental disorders				
Yes	192 (9.2)	944 (8.7)	11,134 (8.2)	140,696 (6.8)
No	1,891 (90.8)	9,889 (91.3)	123,934 (91.8)	1,925,142 (93.2)
Paternal history of mental disorders				
Yes	158 (7.6)	708 (6.5)	8,133 (6.0)	114,468 (5.5)
No	1,919 (92.1)	10,096 (93.2)	126,542 (93.7)	1,946,511 (94.2)
Missing	6 (0.3)	29 (0.3)	393 (0.3)	4,859 (0.2)

Expressed as frequency (percentage). Percentages have been rounded and may not total to 100.

[a]*Maternal smoking during pregnancy was available since 1991.*

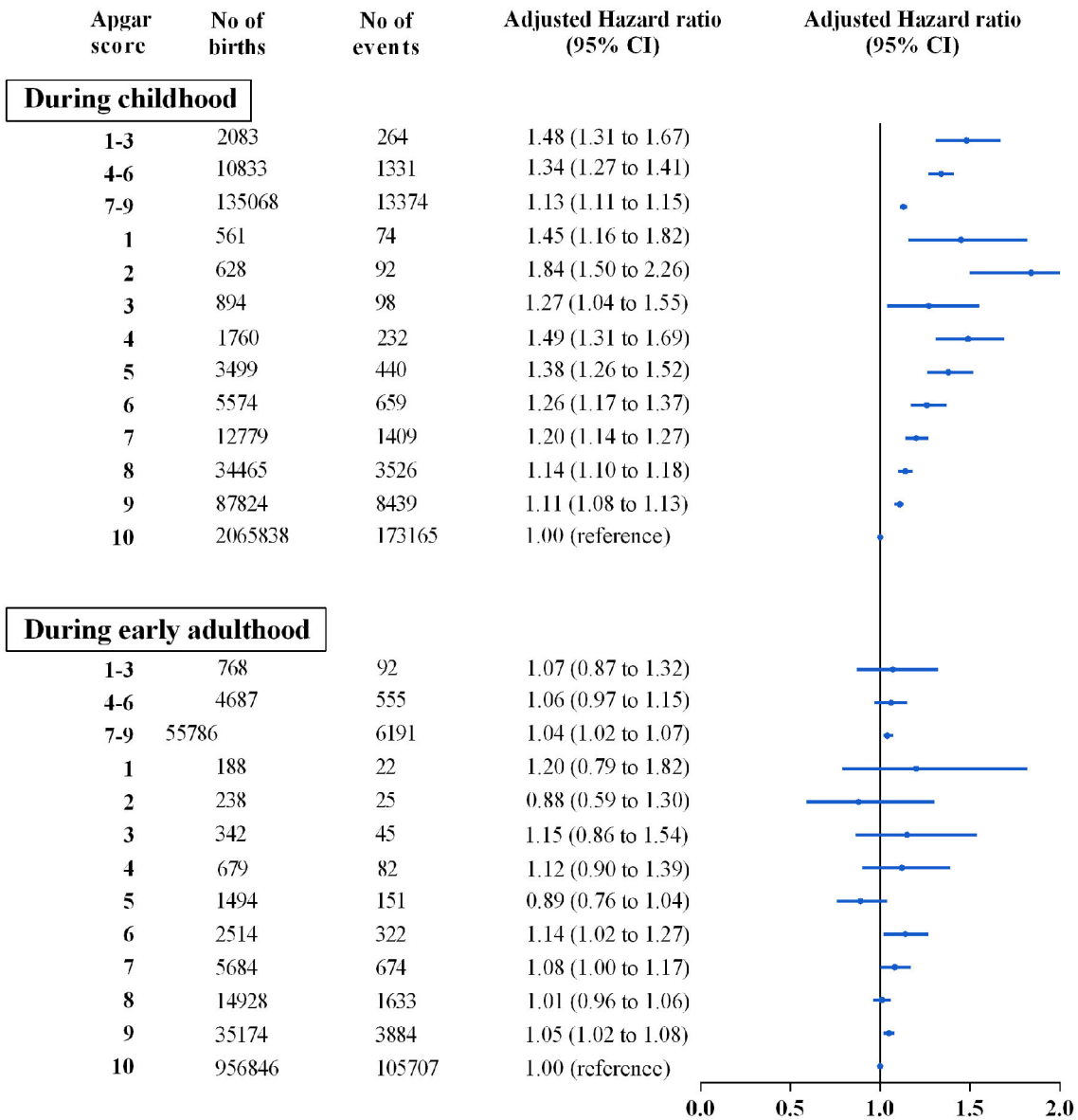

FIGURE 1 | Associations between 5-min Apgar score and overall mental disorders by age periods during the first four decades of life. Hazard ratios are presented in the Cox regression models adjusted for parental psychiatric history, maternal characteristics (parity, age at birth, smoking during pregnancy, highest education level, cohabitation with a partner, residence, birth country), and birth characteristics (participant's sex, calendar year of birth, gestational age at birth and birth weight percentiles).

history and socioeconomic status, indicating a more robust association. In addition, ADHD and autism were another two widely studied neurodevelopment disorders in relation to Apgar score during childhood, but the existing results were inconsistent (13, 27–31), which may be due to heterogeneity of methodology, in particular categorizations of Apgar scores and definition of outcomes. For example, some studies used pervasive developmental disorder (ICD-10 codes: F84) as a proxy to define autism (27, 28), which includes but is not limited to autism. To reduce the possibility of misclassification, we only focused on childhood autism–the typical and most severe type of autism–to explore the association. Our findings further support that compromised 5-min Apgar scores were associated with childhood autism.

Furthermore, we observed that individuals with a compromised 5-min Apgar score had higher risks of organic disorder and neurotic disorder during childhood, which have not been reported previously. These findings imply that less-than-optimal Apgar scores at birth may be an indicator for a broad scope of mental disorders in childhood, not merely neurodevelopmental disorders.

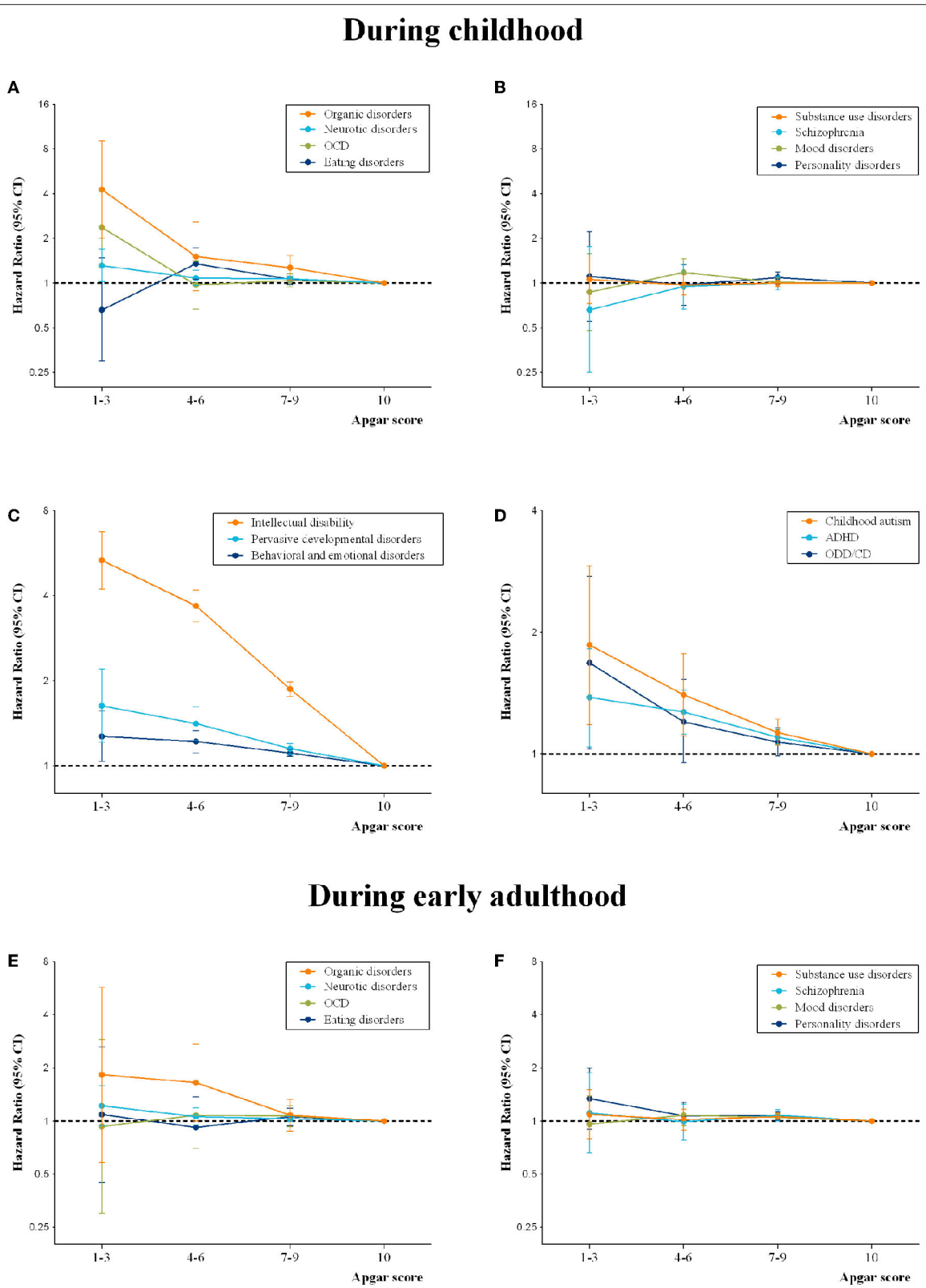

FIGURE 2 | Associations between 5-min Apgar score and specific mental disorders during childhood **(A–D)** and early adulthood **(E,F)**. Hazard ratios are presented in the Cox regression models adjusted for parental psychiatric history, maternal characteristics (parity, age at birth, smoking during pregnancy, highest education level, cohabitation with a partner, residence, birth country), and birth characteristics (participant's sex, calendar year of birth, gestational age at birth and birth weight percentiles).

There have been scarce studies examining the association between low Apgar score and adulthood mental health. We did not find that compromised 5-min Apgar scores were associated with mental disorders during early adulthood, which may be attributed to the incomplete records of Apgar scores during the initial establishment of the Danish Medical Birth Register (MBR). We observed that participants with suboptimal Apgar scores at 5 min tended to have higher risks of organic disorders, schizophrenia, neurotic disorders, and personality disorders, and the low statistical precisions may probably be due to limited cases in the low Apgar score groups. Considering that the maximum attained age in our study was only up to 39 years, the follow-up between 19 and 39 years was not long enough to detect some late-onset mental disorders (e.g., dementia), therefore, future studies with extended follow-up to late adulthood are warranted.

Current guidelines recommend Apgar scores of 7 or higher to be reassuring, hence, infants with these scores are often assumed to constitute a homogeneous group. Nevertheless, recent studies showed that even reassuring Apgar scores of 7–9 are associated with higher risks of neonatal mortality, neonatal morbidity, and adverse long-term neurological outcomes, compared with an Apgar score of 10 (16, 17). We found a dose-response increasing the overall risk of mental disorders with decreasing Apgar score of 9 toward 7. Furthermore, individuals with "normal" scores of 7–9 carried increased risks of a wide range of neurodevelopmental disorders, such as intellectual disability, pervasive developmental disorders, childhood autism, and ADHD. Similarly, prior studies based on developmental screening scales found children aged 5 years with 5-min Apgar scores of 7–9 were more vulnerable on the emotional or physical health domain of the Early Development Instrument (14, 32). Recently, a large transnational study also suggested that low Apgar scores of 7–9 were associated with a higher risk of autistic disorder but without controlling for socioeconomic status and paternal psychiatric history (11). Our findings are in line with those of previous studies by showing that reassuring Apgar scores 7–9 are associated with various neurodevelopmental disorders in childhood. These findings support that 5-min Apgar scores routinely available in contemporary neonatal settings, even within the normal range 7–9, are not totally reassuring.

The causes of mental disorders are multifactorial. Adverse prenatal events (e.g., gestational diabetes mellitus, preterm, and restricted fetal growth) are important risk factors and could have a programming effect on fetal brain development, resulting in increased risk for psychopathology later in life (24, 33, 34). In this study, adjusting for gestational age at birth and fetal growth status did not substantially change the risks, indicating that preterm birth or restricted fetal growth do not strongly modify the relations between low Apgar scores at birth and subsequent mental disorders. Although Apgar scores are not clear on any causal pathway of pathogenesis, less-than-optimal Apgar scores at birth may be a potential sign of the cumulative effect of those adverse prenatal events. Especially, the clinically reassuring but suboptimal score range 7–9 may indicate subtle but still detrimental intrauterine insults which will act negatively on fetal brain development. In clinical settings, a distressed infant will receive resuscitation well before the 5-min Apgar score is assigned, so the score 7–9 could not well reflect severe conditions prior to the assessment (3). That may be one of the reasons we observed exposure to the scores 7–9 was associated with an increased risk of mental disorders. In this study, a novel finding that a compromised 5-min Apgar score was linked to increased risks of organic disorder and neurotic disorder was reported. As is known, organic disorder comprises a range of mental disorders based on a demonstrable etiology in cerebral disease, brain injury, or other insults leading to cerebral dysfunction (35). Increased risk of organic disorder with low Apgar score implies adverse prenatal insults (e.g., hypoxia-ischemia, white matter injury, reduced blood flow, malnutrition) exert a long-lasting impact on brain function in later life. With regard to neurotic disorder, its prevalence is relevant to low levels of socioeconomic status (SES) (36). In this study, we found individuals with compromised Apgar scores tended to be born in families with worse SES (e.g., mothers live alone and have a low education level). It is, therefore, possible that SES factors at least partially mediate the observed association between compromised Apgar scores and neurotic disorder. However, further elucidation of other potential mechanisms is needed, also whether Apgar scores can be used for predicting and screening at-risk infants needs further research, probably also in combination with other diagnostic tests.

Strengths and Limitations

The study has several strengths. First, prospectively collected registry data including all live births in Denmark minimized the potential selection bias and recall bias. Second, a large sample size of over 2 million populations provided sufficient statistical power to perform detailed subgroup analyses that have not been studied previously. Last, the availability of rich sociodemographic and clinical information-enabled considerations of a wide range of important confounding factors.

Our study also has some limitations. First, we lacked information on interventions, which will be given to a distressed newborn before the first Apgar score is assigned and accordingly influence the Apgar score values (3). Whereas, failure to achieve an optimal score after resuscitation may signify some intrinsic defects and poor health status, hence, interventions during resuscitation are unlikely to affect the observed associations substantially. Also, we do not have the information on respiratory care (e.g., O_2 cannula) and whether the patients were admitted to a sick baby room, which may be potential confounders. Second, on one hand, the Apgar score can be assessed subjectively without quality control and is prone to inter-observer variability. Whereas, the Apgar score has been shown to have good internal validity and could provide valid information on nation-level trends about newborn health (37). On the other hand, concerning large international variations in evaluating the Apgar score (37), the findings in this Danish cohort study are limited to generalize to other countries. Third, the study period spanned almost four decades, and advances in medical care over time and alterations in the diagnostic criteria may have influenced the exposures and outcomes. However, the inclusion of calendar time in the analyses would partially alleviate the effects of temporal changes in medical care. Last, the absence of information from primary care as well as the delayed inclusion of outpatient records in

the PCRR and the NPR might result in an underestimation of the associations. This concern was partially relieved in our sensitivity analyses restricting to individuals born after 1994 when inpatient and outpatient records were integrated together, revealing similar results.

Conclusions and Implications

Infants born with declining 5-min Apgar scores have incremental risks of a wide range of mental disorders, mainly during childhood but probably in adulthood, too. We found individuals with even clinically "normal" Apgar score range of 7–9 still had higher risks of overall mental disorders and some specific diagnoses: organic disorders and a series of neurodevelopmental disorders. Our findings suggested suboptimal Apgar score of 7–9 should be also considered as an alarming risk factor for subsequent mental disorders, which may help to identify and monitor at-risk neonates to minimize the risks of adverse psychiatric outcomes in their later life. The 5-min Apgar score should be taken into account in designing public health strategies that target populations at increased risks of specific mental disorders in both childhood and adulthood.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Data Protection Agency (No. 2013-41-2569). By Danish law, no informed consent is required for a registry-based study using anonymized data.

AUTHOR CONTRIBUTIONS

JL had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. FL conceptualized, designed, and reviewed the study. HH carried out the initial analyses, drafted the initial manuscript, and revised the manuscript. YY checked the statistical results and reviewed the manuscript. CO gave administrative, technical, or material support. All authors critically reviewed the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.796544/full#supplementary-material

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Dietary Vitamins A, C, and Potassium Intake Is Associated With Narrower Retinal Venular Caliber

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Introduction: The retinal vasculature, a surrogate for the systemic microvasculature, can be observed non-invasively, providing an opportunity to examine the effects of modifiable factors, such as nutrient intake, on microcirculation. We aimed to investigate the possible associations of dietary nutrient intake with the retinal vessel caliber.

Methods: In this cross-sectional study, a total of 584 participants in a medical survey of Japanese descendants living in Los Angeles in 2015 underwent a dietary assessment, fundus photographic examination, and comprehensive physical and blood examinations. Retinal vessel caliber was measured using fundus photographs with a semi-automated computer system and summarized as central retinal artery and vein equivalents (CRAE and CRVE). The association between dietary nutrient intake and retinal vessel caliber was analyzed using a multivariate linear regression model adjusted for two models including potential confounders. The first model was adjusted for age and sex. The second model was adjusted for age, sex, smoking status, body mass index, hypertension, diabetes, dyslipidemia, history of coronary heart disease, and history of stroke.

Results: After adjustment of potential confounders, compared to the quartile with the lowest intake, the difference in CRVE for the highest quartile was −5.33 μm [95% confidence interval (CI): −9.91 to −0.76, *P* for trend = 0.02] for vitamin A, −4.93 μm (95% CI: −9.54 to −0.32, *P* for trend = 0.02) for vitamin C and −3.90 μm (95% CI: −8.48 to 0.69, *P* for trend = 0.04) for potassium.

Conclusions: A significant association was observed between higher vitamins A, C and potassium intakes and narrower retinal venular caliber.

Keywords: retinal vascular caliber, nutrient, vitamin A, vitamin C, potassium

INTRODUCTION

The retinal microvasculature is the easiest and most widely used vascular bed that can be directly visualized *in vivo* and may provide a non-invasive, surrogate method to study early structural changes and pathological features of the human microcirculation (1–3). Over the past two decades, a computerized method of measuring the retinal vascular caliber using retinal photographs has been developed (4). Deviations from the optimal structure of the retinal vasculature have been shown to involve narrower retinal arterial caliber and wider retinal venular caliber, which have been demonstrated to independently predict coronary heart disease (CHD) and stroke (2, 5, 6).

Modificable dietary factors are presumably associated with cardiovascular disease; meta-analyses have shown that higher intake of fish, nuts, fruits, and vegetables is inversely associated with the development of CHD (7–9). It has been reported that dietary nutrition intake of antioxidants, vegetable proteins, potassium, magnesium, and fiber might be partially effective in reducing the risk of CHD and stroke, independent of cardiovascular risk factors (10–14). Similarly, the relationship between retinal microvascular caliber and dietary factors is under investigation. Gopinath et al. (15) have reported that the consumption of a high-quality diet, reflecting high compliance with published dietary guidelines or recommendations, is associated with an advantageous retinal microvascular profile, that is, a wider retinal arteriolar caliber and narrower retinal venular caliber. Keel et al. (16) reported that lower intake of vegetables and fish is associated with wider retinal venular caliber in children and adolescents with type 1 diabetes. Some studies have shown that higher dietary fiber (17), yogurt (18), and fish (19) consumption is associated with a wider retinal arterial caliber and narrower retinal venular caliber. However, these reports are limited and sufficient evidence has not been established.

Since 1970, we have conducted medical surveys of Japanese Americans who migrated from Japan to the United States and their descendants, an epidemiological study called the Hawaii–Los Angeles–Hiroshima Study, to investigate the effects of environmental factors on disease structures among Japanese people (20). We hypothesized that there may be an association between various nutrient intakes and retinal microvascular caliber, just as there is an association between dietary factors and cardiovascular disease. To substantiate this hypothesis, we conducted a study in 2015 using fundus photographic examination and dietary assessment together with a computer-assisted menu suggestion system for Japanese Americans living in Los Angeles, California. The purpose of this study was to investigate the association between retinal vascular caliber and dietary nutritional intake.

METHODS

Study Design and Population

As part of the Hawaii–Los Angeles–Hiroshima Study (20), medical examinations were conducted in Los Angeles for Japanese Americans in August 2015. The medical examinations were announced through the local Japanese newspaper "Rafu Shimpo" and radio advertisements in Los Angeles; a total of 584 Japanese-Americans participated. First-generation Japanese immigrants from Japan and their descendants born and raised in the United States (second-generation and later) were included. Individuals who had mixed/non-Japanese ethnicity were excluded. All participants received an explanation of the study procedures and provided written informed consent. Participants underwent physical, dietary, and fundus photographic examination by a team of well-trained internists, optometrists, dietitians, and nurses. This study was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Hiroshima University (No. E-139).

Dietary Assessment

Dietary intake and dietary habits of all participants were assessed by a food frequency method, as previously described (21–23). First, a paper questionnaire concerning the frequency of food intake was given to the participants. Then, two trained dietitians conducted detailed interviews regarding the frequency of food intake, amount consumed per meal, and preparation method for each food group; they used food models and real foods, while observing the results in personal interviews. They calculated the values for daily total energy and intake for individual nutritional elements [i.e., animal protein, vegetable protein, animal fat, vegetable fat, saturated fatty acids (SFA), polyunsaturated fatty acids (PUFA), cholesterol, carbohydrates, fiber, vitamins A, B1, B2 and C, calcium, iron, potassium and salt]. The average daily intake of each food group was calculated as (average intake per meal) × (frequency of intake per day); the nutrient intake from each food group was calculated as (nutrient value per 1 gram of each food) × (average daily intake of each food group) (21–23). The nutritional value of each food group was determined on the basis of the US Department of Agriculture Nutritive Value of American Foods in Common Units (24).

Retinal Vascular Caliber Measurement

Bilateral fundus photographs were captured with a 45 degree non-mydriatic retinal camera (NIDEK AFC-300, NIDEK CO., LTD., Gamagori, Japan). Retinal vascular caliber was measured using a semi-automated computer imaging program (Retinal Analysis-IVAN, University of Wisconsin, Madison, WI) by a trained ophthalmologist masked to participants' clinical data (4, 25, 26). Images were presented randomly to a grader. Two circular grids with radii of 0.5 and 1.0 disc diameters were semi-automatically drawn from the edge of the disc; the calibers of all arterioles and venules passing completely through the region of 0.5–1.0 disc diameter were measured. Using the calibers of the six widest arterioles and venules, the central retinal artery and vein equivalents (CRAE and CRVE) were summarized according to the formulae described by Parr-Hubbard (25) and revised by Knudson (27). For the reproducibility of retinal vascular measurements, the intraclass correlation coefficient was high (>0.9). For this study, data of the right eye were included. When the right eye was ungradable, we used data from the left eye.

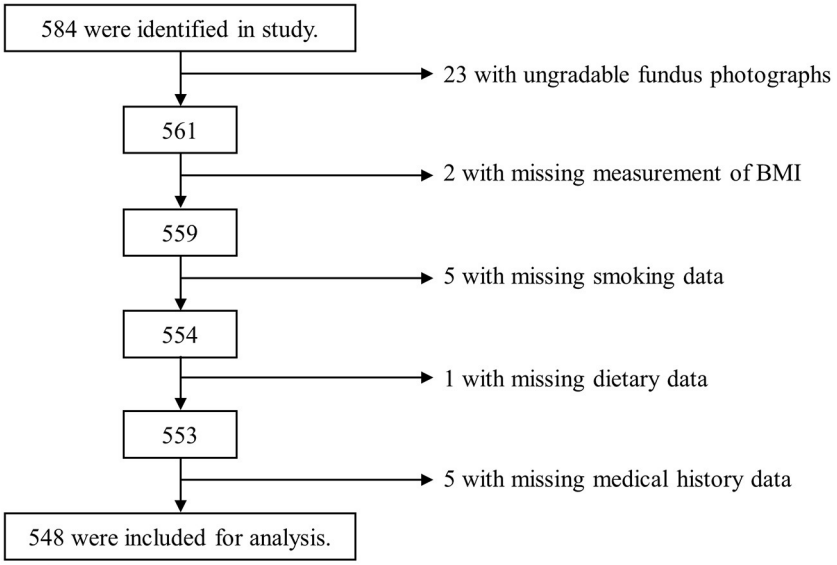

FIGURE 1 | Flow chart showing participant selection in the present study. BMI, body mass index.

Assessment of Covariates

All participants underwent an interview and comprehensive physical examination, and each provided a blood sample after an overnight fast. Venous blood was collected to measure high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG) and blood glucose levels. These measurement methods are described elsewhere (28). Height and weight were measured using a digital scale with a stadiometer. Body mass index (BMI) was then calculated as weight divided by height squared (kg/m^2). Information regarding smoking history and a previous diagnosis of hypertension, diabetes, dyslipidemia, CHD, and stroke was obtained in personal interviews. According to participants' self-reports, smoking history was categorized as never, former, and current smoker. Hypertension was determined as a history of hypertension or using mean arterial blood pressure (MABP), calculated as one-third of the systolic blood pressure plus two-thirds of the diastolic blood pressure; hypertension was defined as MABP $\geq$105.68 mmHg (29). Each participant without diabetes underwent fasting serum glucose measurement and a 75-g oral glucose tolerance test (OGTT). In line with American Diabetes Association guidelines (30), diabetes was defined as either a previous diabetes diagnosis, a fasting serum glucose level of $\geq$126 mg/dL or a 2-h serum glucose level of $\geq$200 mg/dL after an OGTT. Dyslipidemia was defined as a history of dyslipidemia diagnosis, HDL-C $<$40 mg/dL, LDL-C $\geq$140 mg/dL, or TG $\geq$150 mg/dL (31).

Statistical Analysis

In this study, CRAE and CRVE were examined as continuous dependent variables. Continuous variables are expressed as mean $\pm$ standard deviation (SD). First, the mean values of CRAE and CRVE were compared according to participants' background data. After application of the Anderson–Darling test for each variable, the Wilcoxon rank-sum test was used for comparisons between two groups, and the Kruskal–Wallis test was used for comparisons between three or more groups (smoking status and BMI). Next, we used multivariate linear regression model to evaluate the association between retinal vascular caliber (CRAE and CRVE) and dietary nutrient intake. In model 1, we adjusted for age (years, continuous) and sex (male/female). To subsequently assess the effect of confounders, model 2 was additionally adjusted for several known potential confounding factors: smoking status (current/former/never), BMI (kg/m^2, continuous), hypertension (yes/no), diabetes (yes/no), dyslipidemia (yes/no), history of CHD (yes/no), and history of stroke (yes/no) (1). Spearman's rank correlation test was performed to examine correlations regarding nutrient intake. Each dietary nutrient intake was adjusted for total energy intake using the residual method described by Willett et al. (32) and was categorized into quartiles, with the first quartile indicating lower intake. Differences in retinal vascular caliber per quartile of nutrient intake were estimated using the first quartile as a reference. *P*-values for trend were estimated using nutrient intake as a continuous variable. All statistical analyses were performed using JMP Pro statistical software 15.0.0 (SAS Institute Inc., Cary, NC, USA). All *P*-values were two-sided, and a P-value < 0.05 was considered significant.

RESULTS

Characteristics of Study Participants

Of the 584 participants, we excluded 23 with ungradable fundus photographs and 13 with missing data of BMI, smoking status, dietary intake, and medical history, leaving 548 participants included in the analyses (**Figure 1**).

TABLE 1 | Demographic characteristics of study participants[a].

Characteristics	*N* = 548
Age, mean ± SD, years	61.7 ± 13.3
Sex, *N* (%)	
Male	210 (38.3%)
Female	338 (61.7%)
BMI, mean ± SD, kg/m^2	23.3 ± 3.6
Smoking status, *N* (%)	
Never	324 (59.1%)
Former	161 (29.4%)
Current	63 (11.5%)
Diabetes, *N* (%)	72 (13.1%)
Hypertension, *N* (%)	95 (17.3%)
Dyslipidemia, *N* (%)	323 (58.9%)
HDL-C <40 mg/dL	24 (4.4%)
LDL-C ≥140 mg/dL	181 (33.0%)
TG ≥150 mg/dL	139 (25.3%)
Previous CHD, *N* (%)	13 (2.4%)
Previous stroke, *N* (%)	9 (1.6%)
CRAE, mean ± SD, μm	131.8 ± 15.5
CRVE, mean ± SD, μ m	192.0 ± 19.8

[a]*Data are presented as number (percentile) unless otherwise indicated. SD, standard deviation; N, number; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; CHD, coronary heart disease; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent.*

TABLE 2 | Relationship between participants' background data and retinal vascular caliber (CRAE and CRVE).

		CRAE		CRVE	
Variables	***N***	**Mean ± SD**	***P*-value[a]**	**Mean ± SD**	***P*-value[a]**
Age, years			<0.01		<0.01
<65	284	134.5 ± 14.8		195.3 ± 18.9	
≥65	264	129.0 ± 15.8		188.5 ± 20.2	
Sex			<0.01		0.31
Male	210	129.3 ± 16.6		192.8 ± 20.3	
Female	338	133.4 ± 14.6		191.5 ± 19.5	
BMI, kg/m^2			<0.01		0.08
<18.5	49	139.7 ± 17.5		195.6 ± 18.3	
18.5–24.9	340	132.0 ± 15.0		190.4 ± 20.1	
≥25	159	129.0 ± 15.2		194.3 ± 19.4	
Smoking status			0.16		<0.01
Never	324	132.2 ± 15.7		190.5 ± 19.5	
Former	161	130.0 ± 15.9		191.4 ± 19.8	
Current	63	135.0 ± 13.0		201.0 ± 19.6	
Hypertension			<0.01		0.16
Yes	95	125.1 ± 14.9		189.6 ± 19.3	
No	453	133.3 ± 15.3		192.5 ± 19.9	
Diabetes			0.35		0.39
Yes	72	130.5 ± 14.8		194.0 ± 22.3	
No	476	132.0 ± 15.6		191.7 ± 19.4	
Dyslipidemia			0.28		0.38
Yes	323	131.5 ± 15.4		192.5 ± 19.3	
No	225	132.3 ± 15.7		191.2 ± 20.5	
HDL-C <40 mg/dL			0.53		0.53
Yes	24	128.5 ± 19.1		195.5 ± 19.2	
No	524	132.0 ± 15.3		191.8 ± 19.8	
LDL-C ≥140 mg/dL			0.27		0.13
Yes	181	133.4 ± 15.2		194.0 ± 19.0	
No	367	131.0 ± 15.6		191.0 ± 20.2	
TG ≥150 mg/dL			0.71		0.06
Yes	139	131.2 ± 15.2		194.7 ± 20.0	
No	409	1 for 32.0 ± 15.6		191.1 ± 19.7	
Previous CHD			0.32		0.88
Yes	13	129.5 ± 6.4		190.5 ± 21.1	
No	535	131.9 ± 15.7		192.0 ± 19.8	
Previous stroke			0.04		0.65
Yes	9	121.9 ± 12.4		190.1 ± 17.4	
No	539	132.0 ± 15.5		192.0 ± 19.9	

[a]*Analyzed with Wilcoxon rank-sum test to compare two groups (age, sex, hypertension, diabetes, dyslipidemia, HDL-C <40 mg/dL, LDL-C ≥140 mg/dL, TG ≥150 mg/dL, previous CHD and previous stroke) and Kruskal–Wallis test to compare three groups (smoking status and BMI). P < 0.05 was considered significant. SD, standard deviation; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; CHD, coronary heart disease.*

Table 1 gives the demographic characteristics of the included participants and mean retinal vessel caliber. Of the overall participants, 38.3% were men and the mean age ± SD was 61.7 ± 13.3 years. The mean CRAE and CRVE was 131.8 ± 15.5 μm and 192.0 ± 19.8 μm, respectively. In univariate analysis, narrower CRAE was related to older age, male sex, hypertension, previous stroke, and obesity. Wider CRVE was related to younger age and current smoking (**Table 2**). The mean nutrient intake for each quartile is shown in **Table 3**.

Association of Nutrient Intake With Retinal Vessel Diameters

Table 4 shows the association of dietary nutrient intake with retinal arterial caliber in multivariate linear regression analysis. There was no significant linear association between CRAE and intake of animal protein, vegetable protein, animal fat, vegetable fat, SFA, PUFA, cholesterol, carbohydrates, fiber, vitamins A, B1, B2 and C, calcium, iron, potassium and salt in both model 1 (adjusted for age and sex) and model 2 (adjusted for age, sex, smoking status, BMI, hypertension, diabetes, dyslipidemia, history of CHD, and history of stroke).

Table 5 demonstrates the association of nutrient intake in multivariate linear regression analysis for retinal venular caliber. After adjustment for age and sex (model 1), there were significant inverse associations of retinal venular caliber with vitamins A, C, and potassium [mean difference for the second, third, and highest quartiles: −2.35 (95% CI: −6.94, 2.24), −2.27 (−6.88, 2.33), and −5.70 (−10.29 to −1.11), *P*-value for trend = 0.01 for vitamin A; −2.12 (−6.74, 2.50), −0.39 (−5.04, 4.25), and −5.20 (−9.82 to −0.58), *P*-value for trend = 0.02 for vitamin C; −2.67 (−7.27, 1.94), −4.61 (−9.23, 0.02), and −3.95 (−8.56, 0.66), *P*-value for trend = 0.04 for potassium]. We found that

TABLE 3 | Mean daily energy-adjusted nutrient intake, by quartile.

Nutrient	Q1	Q2	Q3	Q4
Animal protein, g	25.0 ± 4.6	33.5 ± 1.8	39.5 ± 1.6	51.9 ± 8.0
Vegetable protein, g	26.2 ± 4.2	32.9 ± 1.2	37.4 ± 1.3	44.2 ± 3.7
Animal fat (g)	9.9 ± 11.8	28.1 ± 3.2	39.0 ± 3.9	72.7 ± 30.8
Vegetable fat (g)	25.1 ± 4.6	32.9 ±1.5	37.9 ± 1.4	46.6 ± 5.4
SFA, g	12.4 ± 3.9	18.8 ± 1.0	22.5 ± 1.1	31.7 ± 7.7
PUFA, g	10.8 ± 1.3	13.3 ± 0.4	14.8 ± 0.6	18.2 ± 2.3
Cholesterol, mg	179.7 ± 37.3	244.0 ± 13.5	290.4 ± 16.0	400.6 ± 84.3
Carbohydrates, g	216.8 ± 55.5	282.0 ± 8.4	308.6 ± 8.4	357.4 ± 32.0
Fiber, g	10.7 ± 1.9	14.1 ± 0.8	17.2 ± 1.0	24.0 ± 4.2
Vitamin A, μgRAE	341.4 ± 79.2	466.6 ± 30.2	644.0 ± 62.6	961.9 ± 207.7
Vitamin B1, mg	0.6 ± 0.1	0.8 ± 0.04	1.0 ± 0.06	1.3 ± 0.2
Vitamin B2, mg	0.8 ± 0.1	1.1 ± 0.06	1.3 ± 0.07	1.7 ± 0.2
Vitamin C, mg	95.1 ± 21.8	139.1 ± 10.9	180.9 ± 15.1	267.0 ± 51.5
Calcium, mg	384.9 ± 63.5	537.7 ± 36.8	661.0 ± 36.2	858.7 ± 99.4
Iron, mg	4.7 ± 0.6	6.1 ± 0.38	7.4 ± 0.4	9.8 ±1.3
Potassium, mg	2,151.8 ± 250.3	2,613.0 ± 111.4	3,088.5 ± 151.3	3,978.5 ± 517.1
Salt, g	2.9 ± 1.1	4.7 ± 0.4	6.3 ± 0.5	8.8 ± 1.1

SD, standard deviation; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; RAE, retinol activity equivalent; Q, quartile.

higher intake of vitamins A, C and potassium had a significant inverse association with venular caliber after adjusting for age, sex, smoking status, BMI, hypertension, diabetes, dyslipidemia and history of diagnosed CHD and stroke (model 2); mean difference for the second, third, and highest quartiles: −2.55 (95% CI: −7.15, 2.05), −1.83 (−6.45, 2.78), and −5.33 (−9.91 to −0.76), *P*-value for trend = 0.02 for vitamin A; −2.18 (−6.83, 2.47), −0.27 (−4.92, 4.37), and −4.93 (−9.54 to −0.32), *P*-value for trend = 0.02 for vitamin C; −2.64 (−7.26, 1.97), −4.58 (−9.24, 0.07), −3.90 (−8.48, 0.69), *P*-value for trend = 0.04 for potassium. There were no significant associations of CRVE with animal protein, vegetable protein, animal fat, vegetable fat, SFA, PUFA, cholesterol, carbohydrates, fiber, vitamin B1, vitamin B2, calcium, iron, or salt in either model.

Correlations between the intake of these nutrients (vitamins A, C, and potassium) are shown in **Figure 2**. Intake of vitamin A and vitamin C, vitamin A and potassium, and vitamin C and potassium were significantly correlated with each other (vitamin A vs. vitamin C, $R = 0.86$, $P < 0.01$; vitamin A vs. potassium, $R = 0.87$, $P < 0.01$; vitamin C vs. potassium, $R = 0.91$, $P < 0.01$).

DISCUSSION

In the present study, we found that vitamins A, C and potassium intake had an inverse association with retinal venular caliber after accounting for cardiovascular disease factors. The nutritional intake of the population in this study generally did not greatly deviate from the U.S. Dietary Reference Intakes (33). With respect to vitamin C, the recommended intake of vitamin C is 75–90 mg/day (33); the majority of participants in this study met the recommended intake. Previous studies have shown that intake of vitamins A and C, which have antioxidant properties, contributes to a reduction in the risk of CHD and stroke (10, 12, 14). Adequate dietary potassium reduces the risk of CHD and stroke (34). Retinal vessel diameter, which reflects systemic microvascular status, has been shown to be associated with CHD and stroke outcomes, and epidemiological studies have shown that wider CRVE is independently associated with future risk of CHD and stroke (1, 5, 6). Although the association between these individual nutrient intake and the structure of the retinal microvasculature has not been reported, our results are supported by Gopinath et al. (15). They reported that a high-quality diet rich in fruits and vegetables was associated with narrower retinal venular caliber, indicating better retinal microvascular health. Vitamins A, C and potassium come largely from fruits and vegetables (34), and our results are consistent with their report.

We consider that the antioxidant effects of vitamins A and C might be involved in our finding that higher intake of vitamins A and C prevented retinal venular caliber enlargement. It has been suggested that retinal venular caliber widening underlies destruction of the endothelial surface layer (ESL) (35). Indeed, Wong et al. reported that wider retinal venular caliber is related to higher levels of soluble intercellular adhesion molecule-1 and plasminogen activator inhibitor-1, biomarkers of endothelial dysfunction (26). Tamai et al. reported that oxidative stress caused by lipid hydroperoxide injection into the vitreous of rats resulted in an increase in the number of leukocytes in the retinal microvasculature and an enlarged retinal venular caliber (36). Epidemiological studies have also reported that systemic inflammatory markers such as white blood cell count, erythrocyte sedimentation rate, high-sensitivity c-reactive protein (CRP), interleukin-6, and serum amyloid A are associated with wide retinal venular caliber (35, 37).

Antioxidants, such as vitamins A and C, are considered to protect against oxidant-mediated inflammation by virtue

TABLE 4 | Mean CRAE (μm) differences across quartiles of energy-adjusted nutrient intake compared with the lowest quartiles.

Nutrient	Model	Q2 vs. Q1	Q3 vs. Q1	Q4 vs. Q1	*P* for trend
Animal protein	1	4.61 (1.09, 8.14)	2.07 (−1.46, 5.59)	1.61 (−1.92, 5.13)	0.45
	2	4.41 (0.93, 7.89)	1.50 (−2.02, 5.01)	1.93 (−1.59, 5.45)	0.38
Vegetable protein	1	2.46 (−1.08, 6.00)	−1.85 (−5.41, 1.70)	−0.97 (−4.54, 2.60)	0.30
	2	2.14 (−1.37, 5.66)	−2.22 (−5.73, 1.29)	−0.88 (−4.40, 2.65)	0.38
Animal fat	1	1.09 (−2.48, 4.67)	2.32 (−1.28, 5.91)	1.99 (−1.59, 5.57)	0.32
	2	0.32 (−3.24, 3.87)	1.36 (−2.24, 4.95)	1.56 (−1.99, 5.11)	0.41
Vegetable fat	1	0.50 (−3.07, 4.08)	−1.83 (−5.43, 1.76)	0.95 (−2.59, 4.50)	0.98
	2	0.64 (−2.89, 4.17)	−1.41 (−4.99, 2.17)	1.08 (−2.42, 4.59)	0.85
SFA	1	2.65 (−0.95, 6.25)	2.52 (−1.13, 6.17)	2.68 (−0.91, 6.26)	0.20
	2	1.88 (−1.69, 5.46)	1.75 (−1.89, 5.39)	2.31 (−1.27, 5.89)	0.24
PUFA	1	0.28 (−3.28, 3.83)	−0.05 (−3.67, 3.57)	0.69 (−2.86, 4.24)	0.73
	2	0.43 (−3.08, 3.94)	0.18 (−3.42, 3.79)	0.78 (−2.74, 4.30)	0.53
Cholesterol	1	1.08 (−2.47, 4.63)	1.29 (−2.26, 4.83)	1.73 (−1.81, 5.27)	0.16
	2	1.08 (−2.43, 4.58)	0.76 (−2.74, 4.27)	2.29 (−1.22, 5.81)	0.15
Carbohydrates	1	0.98 (−2.56, 4.52)	2.31 (−1.23, 5.85)	0.15 (−3.39, 3.70)	0.97
	2	0.76 (−2.77, 4.28)	2.31 (−1.19, 5.82)	0.58 (−2.94, 4.11)	0.90
Fiber	1	1.16 (−2.40, 4.72)	1.62 (−1.93, 5.18)	−0.15 (−3.70, 3.41)	0.35
	2	0.70 (−2.83, 4.24)	1.22 (−2.31, 4.75)	−0.02 (−3.53, 3.48)	0.62
Vitamin A	1	1.15 (−2.41, 4.70)	1.05 (−2.52, 4.62)	0.14 (−3.42, 3.71)	0.44
	2	0.68 (−2.87, 4.23)	1.00 (−2.57, 4.56)	0.36 (−3.17, −3.89)	0.62
Vitamin B1	1	−0.36 (−3.86, 3.13)	2.26 (−1.26, 5.79)	−0.75 (−4.29, 2.79)	0.73
	2	−0.23 (−3.70, 3.24)	2.31 (−1.24, 5.85)	0.36 (−3.17, 3.89)	0.73
Vitamin B2	1	0.63 (−2.90, 4.16)	1.60 (−1.95, 5.14)	1.27 (−2.24, 4.79)	0.80
	2	0.52 (−2.96, 4.01)	1.89 (−1.62, 5.41)	1.35 (−2.17, 4.88)	0.74
Vitamin C	1	−2.22 (−5.78, 1.35)	0.63 (−2.95, 4.22)	−2.87 (−6.43, 0.70)	0.28
	2	−2.10 (−5.67, 1.48)	0.61 (−2.96, 4.18)	−2.41 (−5.95, 1.13)	0.43
Calcium	1	1.69 (−1.89, 5.26)	1.61 (−2.00, 5.22)	1.20 (−2.41, 4.81)	0.82
	2	1.80 (−1.72, 5.32)	2.22 (−1.35, 5.79)	1.31 (−2.26, 4.88)	0.69
Iron	1	0.84 (−2.71, 4.39)	−0.66 (−4.21, 2.89)	−1.61 (−5.15, 1.93)	0.24
	2	0.38 (−3.14, 3.90)	−0.76 (−4.29, 2.76)	−1.33 (−4.83, 2.17)	0.34
Potassium	1	−0.52 (−4.08, 3.04)	−0.95 (−4.53, 2.63)	−1.02 (−4.59, 2.55)	0.32
	2	−1.12 (−4.68, 2.44)	−1.30 (−4.89, 2.29)	−0.81 (−4.35, 2.72)	0.45
Salt	1	−2.33 (−5.87, 1.22)	−3.11 (−6.64, 0.43)	−0.87 (−4.43, 2.68)	1.00
	2	−2.05 (−5.58, 1.47)	−2.88 (−6.39, 0.64)	−0.63 (−4.17, 2.90)	0.95

The 95% confidence interval is in parentheses. Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, smoking status, body mass index, hypertension, diabetes, dyslipidemia, history of coronary heart disease and stroke. CRAE, central retinal artery equivalent; Q, quartile; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

of their capacity to scavenge reactive oxygen species (ROS) and inhibit the activation of nuclear factor kappa-B (NF-κB), a transcription factor that promotes the expression of genes that induce inflammation (38, 39). Previous studies have shown that blood concentrations of vitamins A and C have a negative association with CRP, a marker of inflammation (40–42). Additionally, Hermersson et al. showed that higher dietary β-carotene and vitamin C intake significantly reduced formation of F2-isoprostanes, a marker of oxidative stress (43). Experimental studies have also reported that vitamins A and C inhibit the activation of NF-κB, which regulates the promotion of inflammation (44, 45). The detailed mechanism by which antioxidants prevent the enlargement of retinal venular caliber remains to be elucidated. However, according to the evidence presented above, we consider that higher intake of vitamins A and C prevented ESL destruction by reducing oxidative stress and inhibiting the development of inflammation, thereby preventing retinal venular caliber widening.

Hypertension is a risk factor for CHD and stroke. It has been considered that the antihypertensive effect of potassium is responsible for the reduced risks of CHD and stroke associated with high potassium intake (46). However, Tobian et al. reported that the incidences of stroke and death were drastically reduced in rats with high potassium intake, regardless of identical blood pressure (47). These findings suggest that potassium has a beneficial effect on blood vessels through mechanisms other than its antihypertensive effect. The mechanism has not yet been fully elucidated; however, the antioxidant effect may be a

TABLE 5 | Mean CRVE (μm) differences across quartiles of energy-adjusted nutrient intake compared with the lowest quartiles.

Nutrient	Model	Q2 vs. Q1	Q3 vs. Q1	Q4 vs. Q1	*P* for trend[a]
Animal protein	1	2.86 (−1.73, 7.45)	3.48 (−1.11, 8.06)	1.33 (−159, 5.91)	0.84
	2	2.53 (−2.02, 7.07)	1.91 (−2.69, 6.50)	0.27 (−4.33, 4.88)	0.80
Vegetable protein	1	−0.09 (−4.70, 4.52)	−0.96 (−5.59, 3.67)	−2.41 (−7.06, 2.24)	0.24
	2	−0.22 (−4.82, 4.38)	−0.56 (−5.16, 4.03)	−2.40 (−7.01, 2.21)	0.29
Animal fat	1	0.89 (−3.74, 5.52)	3.30 (−1.36, 7.96)	0.69 (−3.95, 5.32)	0.95
	2	0.22 (−4.40, 4.04)	3.06 (−1.61, 7.73)	0.51 (−4.11, 5.13)	0.95
Vegetable fat	1	−1.92 (−6.56, 2.73)	−2.41 (−7.08, 2.26)	−0.72 (−5.32, 3.89)	0.45
	2	−2.35 (−6.94, 2.25)	−3.00 (−7.66, 1.67)	−1.40(−5.97, 3.16)	0.28
SFA	1	0.60 (−4.07, 5.28)	1.99 (−2.75, 6.73)	−0.03 (−4.69, 4.63)	0.95
	2	0.55 (−4.10, 5.21)	1.72 (−3.02, 6.46)	−0.08 (−4.75, 4.58)	0.90
PUFA	1	0.41 (−4.20, 5.01)	−0.57 (−5.26, 4.12)	−0.17 (−4.78, 4.43)	0.38
	2	0.23 (−4.34, 4.80)	−1.48 (−6.17, 3.21)	−1.19 (−5.77, 3.39)	0.21
Cholesterol	1	−0.34 (−4.93, 4.25)	3.33 (−1.24, 7.91)	−1.08 (−5.66, 3.49)	0.52
	2	−0.43 (−4.98, 4.12)	3.38 (−1.18, 7.93)	−0.97 (−5.53, 3.59)	0.59
Carbohydrates	1	3.56 (−1.03, 8.14)	2.49 (−2.09, 7.07)	0.50 (−4.08, 5.09)	0.56
	2	3.34 (−1.25, 7.92)	2.45 (−2.10, 7.01)	0.92 (−3.67, 5.51)	0.46
Fiber	1	−1.55 (−6.17, 3.06)	−0.49 (−5.10, 4.12)	−4.82 (−9.44, −0.19)	0.05
	2	−0.57 (−5.16, 4.02)	0.33 (−4.25, 4.91)	−3.37 (−7.92, 1.19)	0.06
Vitamin A	1	−2.35 (−6.94, 2.24)	−2.27 (−6.88, 2.33)	−5.70 (−10.29, −1.11)	0.01
	2	−2.55 (−7.15, 2.05)	−1.83 (−6.45, 2.78)	−5.33 (−9.91, −0.76)	0.02
Vitamin B1	1	0.37 (−4.15, 4.89)	3.91 (−0.65, 8.48)	−1.23 (−5.81, 3.34)	0.88
	2	−0.76 (−5.27, 3.76)	2.39 (−2.215, 7.00)	−2.58 (−7.18, 2.01)	0.42
Vitamin B2	1	−0.67 (−5.25, 3.91)	0.51 (−4.08, 5.11)	−1.17 (−5.74, 3.39)	0.69
	2	−1.06 (−5.60, 3.48)	0.17 (−4.41, 4.75)	−2.13 (−6.72, 2.46)	0.44
Vitamin C	1	−2.12 (−6.74, 2.50)	−0.39 (−5.04, 4.25)	−5.20 (−9.82, −0.58)	0.02
	2	−2.18 (−6.83, 2.47)	−0.27 (−4.92, 4.37)	−4.93 (−9.54, −0.32)	0.02
Calcium	1	−1.68 (−6.30, 2.94)	−4.91 (−9.57, −0.24)	−2.41 (−7.07, 2.26)	0.40
	2	−1.02 (−5.60, 3.55)	−4.40 (−9.04, 0.24)	−2.61 (−7.25, 2.03)	0.31
Iron	1	1.46 (−3.13, 6.04)	−2.38 (−6.97, 2.21)	−3.38 (−7.96, 1.20)	0.05
	2	1.60 (−2.97, 6.16)	−2.63 (−7.19, 1.94)	−3.04 (−7.58, 1.49)	0.05
Potassium	1	−2.67 (−7.27, 1.94)	−4.61 (−9.23, 0.02)	−3.95 (−8.56, 0.66)	0.04
	2	−2.64 (−7.26, 1.97)	−4.58 (−9.24, 0.07)	−3.90 (−8.48, 0.69)	0.04
Salt	1	−0.38 (−4.98, 4.22)	2.12 (−2.47, 6.70)	−2.07 (−6.68, 2.55)	0.52
	2	−0.64 (−5.23, 3.94)	1.87 (−2.71, 6.44)	−2.62 (−7.21, 1.98)	0.35

The 95% confidence interval is in parentheses. Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, smoking status, body mass index, hypertension, diabetes, dyslipidemia, history of coronary heart disease and stroke. [a]*P < 0.05 considered significant. CRVE, central retinal vein equivalent; Q, quartile; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.*

contributing factor. Although vitamins A and C are the typical antioxidant nutrients, a potassium diet also has antioxidant effect, and it reduces the free radical formation (34). He et al. showed that without changing blood pressure, 64 mmol of potassium, whether as the chloride or bicarbonate salt, improved vascular endothelial function in 42 adults (48). Our result might be explained by the antioxidant effects described above. However, in this study, there were significant correlations between the intake of vitamins A, C and potassium. Vitamins A, C, and potassium are abundant in fruits and vegetables; therefore, it might not be possible to simply conclude that high potassium intake is related to narrower CRVE.

In this study, no significant association was found between dietary nutrient intake and retinal arterial caliber. In particular, there was no significant association of arterial caliber with vitamins A, C and potassium which were found to be associated with venous caliber. In previous animal studies, administration of lipid hydroperoxide did not result in narrowing of the retinal arterial caliber (36), which is consistent with our results. Although the mechanism explaining this observation is unclear, these findings suggest that ROS/antioxidants might only affect the retinal venous caliber and not the arterioles. As there are still few reports on the relationship between antioxidants and retinal blood vessel diameter, it is possible that confounding factors not analyzed in this study may have influenced the results. Both arterial caliber narrowing and venous caliber enlargement have been considered to indicate deterioration of the systemic microvascular circulation; however, in recent years, the role of

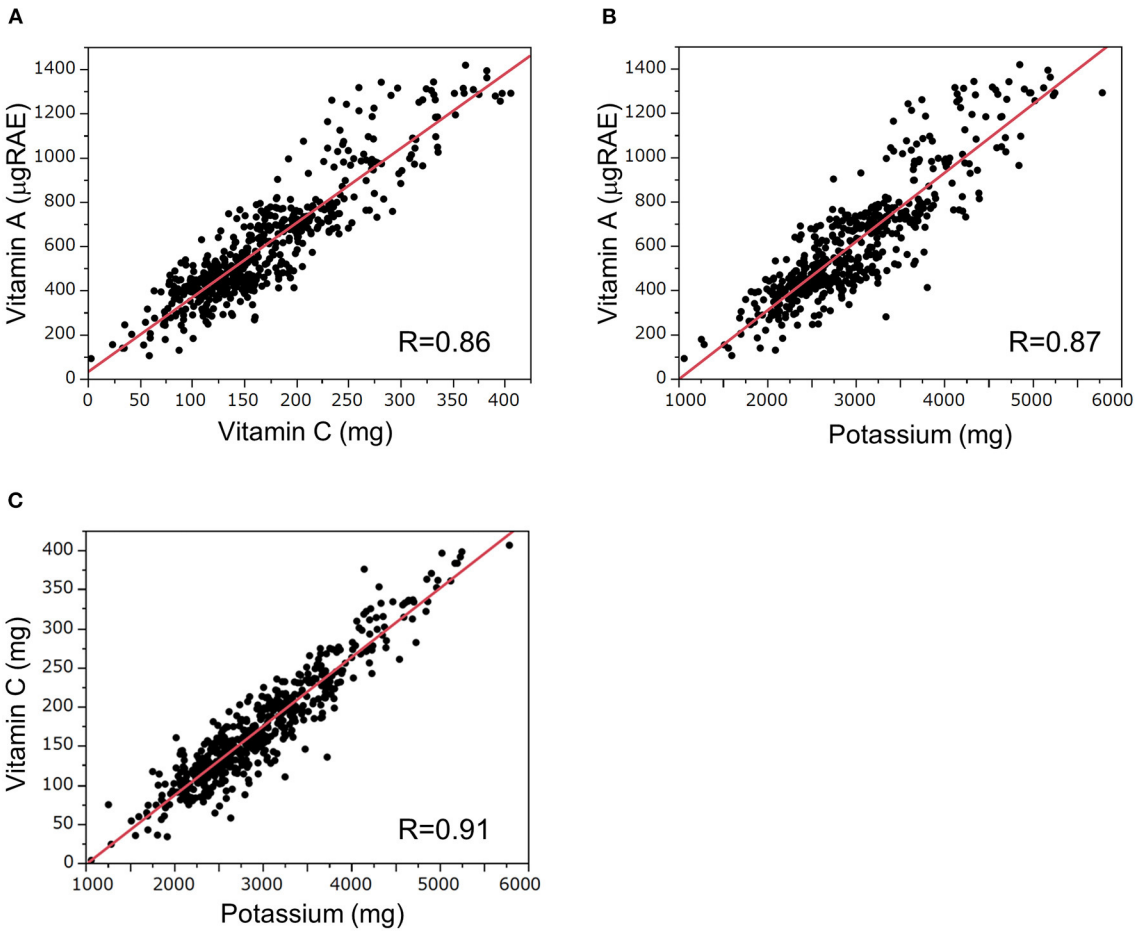

FIGURE 2 | Correlations of dietary daily intakes of vitamins A, C, and potassium. Correlations between dietary intake of **(A)** vitamin A and vitamin C, **(B)** vitamin A and potassium, and **(C)** vitamin C and potassium. The correlations were analyzed by Spearman's rank correlation test. RAE, retinol activity equivalent.

venules, independent of arterioles, has been attracting attention. In particular, wider venular caliber has been shown to be a potentially important marker of micro-vascular disease (35). Smoking has also been shown to be associated with only a wider venular caliber but not arterial caliber (1, 49). Further research is needed to fully elucidate all aspects of the effects of nutrient intake on the microcirculatory system.

There are several limitations in this study. First, the number of participants was limited. Second, the participants in this study were not representative of the general population. Hence, our results might not be relevant to the general population as the current study only included Japanese American individuals. Third, our study used a cross-sectional design; thus, it was impossible to determine the directional associations. Fourth, physical activity, socioeconomic factors, eye condition, and serum levels of vitamins and minerals were not examined. In myopic eyes, retinal vessel caliber is reportedly narrower because of longer ocular axis (50). To clarify the causal relationships between vitamins A, C, and potassium with the microvasculature, prospective studies with larger sample sizes drawn from the general population are needed; such studies should examine serum levels of vitamins and minerals, ocular conditions (e.g., refractive status and ocular axial length), and physical and social factors.

In conclusion, we showed that vitamins A, C and potassium intake was inversely associated with retinal venular caliber. This suggests that dietary intake of vitamins A, C and potassium might be beneficial for a healthy retinal microvascular profile. The retinal vasculature provides a non-invasive window into the status of systemic microvascular (1). This study provides insights for clarifying the effects of dietary nutrition on microvasculature. We would like to emphasize that this is the first time that the association of dietary vitamins A, C and potassium intake with retinal vascular status has been demonstrated; therefore, further prospective studies are needed to assess whether the evidence is consistent.

DATA AVAILABILITY STATEMENT

The data analyzed in this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Hiroshima University (approval No. E-139). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YK: conceptualization. HO and MY: data curation. AE: formal analysis. DI, RT, MK, AN, and HO: investigation. AE and DI: writing—original draft preparation. KH, RK, MY, and YK: writing—review and editing. KH, MY, and YK: supervision. All authors contributed to the article and approved the submitted version.

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Role-Playing Between Environmental Pollutants and Human Gut Microbiota: A Complex Bidirectional Interaction

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There is a growing interest in the characterization of the involvement of toxicant and pollutant exposures in the development and the progression of several diseases such as obesity, diabetes, cancer, as well as in the disruption of the immune and reproductive homeostasis. The gut microbiota is considered a pivotal player against the toxic properties of chemicals with the establishment of a dynamic bidirectional relationship, underlining the toxicological significance of this mutual interplay. In fact, several environmental chemicals have been demonstrated to affect the composition, the biodiversity of the intestinal microbiota together with the underlining modulated metabolic pathways, which may play an important role in tailoring the microbiotype of an individual. In this review, we aimed to discuss the latest updates concerning the environmental chemicals–microbiota dual interaction, toward the identification of a distinctiveness of the gut microbial community, which, in turn, may allow to adopt personalized preventive strategies to improve risk assessment for more susceptible workers.

Keywords: microbiota, environmental pollutants, occupational medicine, chronic diseases, occupational toxicology

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INTRODUCTION

During the entire lifespan, since the conception and whole fetal development, we are constantly exposed to the so-called exposome, defined as the totality of the environmental exposures, which are dynamic in their quality and quantity over time (1). Such environmental factors may include air pollutants, radiations, chemicals present in soil, food, and water, but also individual factors associated with the personal lifestyle, such as tobacco smoking, food consumption, specific use of drugs, and xenobiotics, altogether representing the external exposome (2).

Another constant source of exposure is considered our internal exposome, mainly accounting for the effects mediated by the microbiota, a heterogeneous consortium of microorganisms that populates all the exposed surfaces of the body, and having with the host a relationship of mutual advantage (3). For many chemicals, the health impact associated with exposure (and the corresponding exposure pathways) remains yet poorly understood (4). To find novel biomarkers of exposure, as well as to characterize the real associations existing between exposures and the development of a certain disease, both represent goals of pivotal importance, especially for the most exposed occupational categories (including agriculture,

construction plant manufacturing, and mining) (5). Given its role as a key link between the external exposome and the human health, the internal exposome, and in particular our microbiota represents a promising source of novel functional correlations and biomarkers of exposure (6).

Among the different microbiota, the most characterized is the gastro-intestinal one, which plays a main role as a dynamical interface between the host and the external exposome (7). Intestinal dysbiosis has been associated with marked structural changes in the mucosa, including permeability and inflammation. The dysbiosis may, in turn, favor either the insurgence or the worsening of several chronic, non-communicable diseases, including cardiovascular diseases, diabetes, neurological diseases, and cancer (8, 9). Furthermore, dysbiosis has been associated with multiple extraintestinal diseases such as neurological or behavioral outcomes (10–14), respiratory dysfunctions (15, 16), metabolic/endocrine impairment (17–19), and inflammatory/autoimmune diseases (20–26).

Additionally, it is now commonly recognized that stress actively modulates both structure and activity of the Gut Microbiota (GM) community and it may be a critical factor in causing dysbiosis (27–30). There is a growing evidence that a healthy and resilient GM can contribute to optimize the health and performance of the general host. However, developing responses for this goal requires elucidating the impact of stressors on the GM. Environmental pollutants such as microplastics or synthetic compounds interact with GM through several toxicokinetic and toxicodynamic pathways and, in turn, GM may alter the bioavailability and toxicity of xenobiotic metabolites. This bidirectional interaction can modulate the physiological homeostasis, finally leading to health disorders (31–33).

This review explores all the up-to-date studies regarding the role played by the GM in xenobiotics metabolism and protection mechanisms, especially for what concerns the main groups of environmental chemicals (i.e., pesticides, metals, and microplastics) to tailor personalized preventive strategies, as well as to improve the risk assessment for more susceptible workers.

FEATURES OF GUT MICROBIOTA

GM includes bacteria, archaea, viruses, yeasts, and other fungi whose population density progressively increases from 10^3 to 10^4 cells/ml within the gastric acidic environment to about 10^{11} cells/ml within the colon (34). The dominant phyla are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. Overall, a healthy human adult hosts over 100 different bacterial species in the gastrointestinal tract, with a marked interindividual variation in genus and species compositions (35).

The so-called gut microbiome includes the whole genome of the GM, encoding for over 100-fold more genes than the human genome (36). The recent advent of metagenomics, which combines the next-generation sequencing (NGS) technology with the computational analysis of the 16S ribosomal RNA (rRNA) amplicons, helps in the characterization of both diversity and abundance of the gut microbiome (37). Metagenomics, together with metatranscriptomics, metaproteomics, and metabolomics, is currently allowing us to understand the impact of each individual bacterial species on the health of the host (38).

Despite the existence of several definitions of healthy GM, a number of endogenous and exogenous factors may cause the microbiota to shift from eubiotic to dysbiotic; in general, a more diverse GM, both in terms of diversity and abundance of taxa, is considered a healthier GM (39). Additionally, a healthy GM can resist or overcome perturbations by returning to a state of balance or eubiosis.

Currently, species belonging to the *Eubacterium*, *Roseburia*, and *Faecalibacterium* genera are included among the beneficial taxa, given their ability to secrete butyrate, a short-chain fatty acid (SCFA) with several health effects, such as to improve the integrity of the intestinal barrier, as well as to reduce the gut oxidative stress or inflammatory status (40).

Potentially harmful bacteria are considered those belonging to the *Enterobacteriaceae* family that includes the intestinal commensals *Escherichia*, *Shigella*, *Proteus*, and *Klebsiella* with pronounced pro-inflammatory effects (41). Several bacterial species are able to actively secrete toxins, including CagA from *Helicobacter pylori*, colibactin and cytolethal distending toxin (CDT) from *Escherichia coli*, inositol phosphate phosphatase D (IpgD), and cysteine protease-like virulence gene A (VirA) from *Shigella flexneri*, that in turn damage the intestinal epithelial integrity (9). These toxins may induce direct damage to the epithelial cellular DNA, trigger proproliferative pathways (including Akt serine/threonine kinase family and Wnt/β-Catenin signaling), or stimulate the local secretion of reactive oxygen species (ROS). Overall, these toxins may promote a proinflammatory milieu and even trigger the local cellular neoplastic transformation (9).

The proinflammatory environment may also have a counterintuitive health effect on the human host. For example, the lipopolysaccharide (LPS, also known as endotoxin), the main component of the outer membrane in gram-negative bacteria, may activate the pattern recognition receptors (PRRs) of host, including the toll-like receptor 4 (TLR4), thereby activating the immune T cell-mediated response, which activates a solid proinflammatory reaction. Although the inflammation may often promote colitis or mucositis, a proinflammatory status may also be protective in certain conditions, such as during tumor development (42).

Specific intestinal taxa are able to secrete essential micronutrients, such as vitamins (i.e., vitamin K and vitamin B) or the linoleic acid, which is an antidiabetic compound. Also, specific bacteria can catabolize secondary bile acids and phenolic compounds (43). Finally, certain gut-resident taxa, upon fermentation of dietary fibers in the large intestine, may produce hormone-like metabolites, such as the SCFAs.

Additionally, the gut microbial functions are tightly interconnected and directly affect the host immune response, both locally and systemically (44). This topic is deeply analyzed elsewhere, and, although out from the scope of this review, it is important here to underline that the microbial dysbiosis deriving by both environmental exposure and genetic susceptibility may

be associated with aberrant mucosal immune responses, such as the upregulation of the Th17, Th1, and Th2 immune phenotypes, the downregulation of the T regulatory cells, and dysregulated humoral immunity. Overall, this immune shift may result in chronic intestinal inflammation and a generally altered immune response to pathogens and insults (45).

Given the role as a barrier, metabolic, and immune interface, the GM is extremely important as the joining link between the external exposome and the human host (46). In details, the GM plays a pivotal role in xenobiotics metabolism, including toxicants and chemical pollutants (47) (**Figure 1**).

Once ingested, the toxicants, which are efficiently adsorbed in the intestine, through the bloodstream, arrive at the liver where they are oxidized, forming conjugates with glucuronic acid, sulfate, or glutathione that can be excreted in the bile and enter the intestine again, where the GM may interfere with their excretion (47). The GM can additionally directly metabolize the chemicals which are poorly adsorbed and hence transported to the large intestine through the action of several bacterial enzymes (such as beta-glucosidases, beta-glucuronidases, sulfatases, azoreductases, nitroreductases, and transferases) (48). Overall, the GM-mediated metabolism can lead to: (1) inactivation, (2) activation, or (3) reactivation (upon liver inactivation) of the specific compound (49). In the case of toxicants such as chemical pollutants, the GM-mediated inactivation can neutralize the hazard of the exposure. On the contrary, the activator outcome may be detrimental and increase the risk of developing associated pathologies, including cancer (50). In parallel, the GM composition may be actively shaped by the chemicals, as it was demonstrated in a number of preclinical studies (51). Many examples will be further discussed in the following section.

Consequently, the maintenance of a healthy GM may be protective against the toxicity of such chemicals and the occurrence of associated chronic diseases (52). This could be very relevant in specific occupational settings with a high rate of

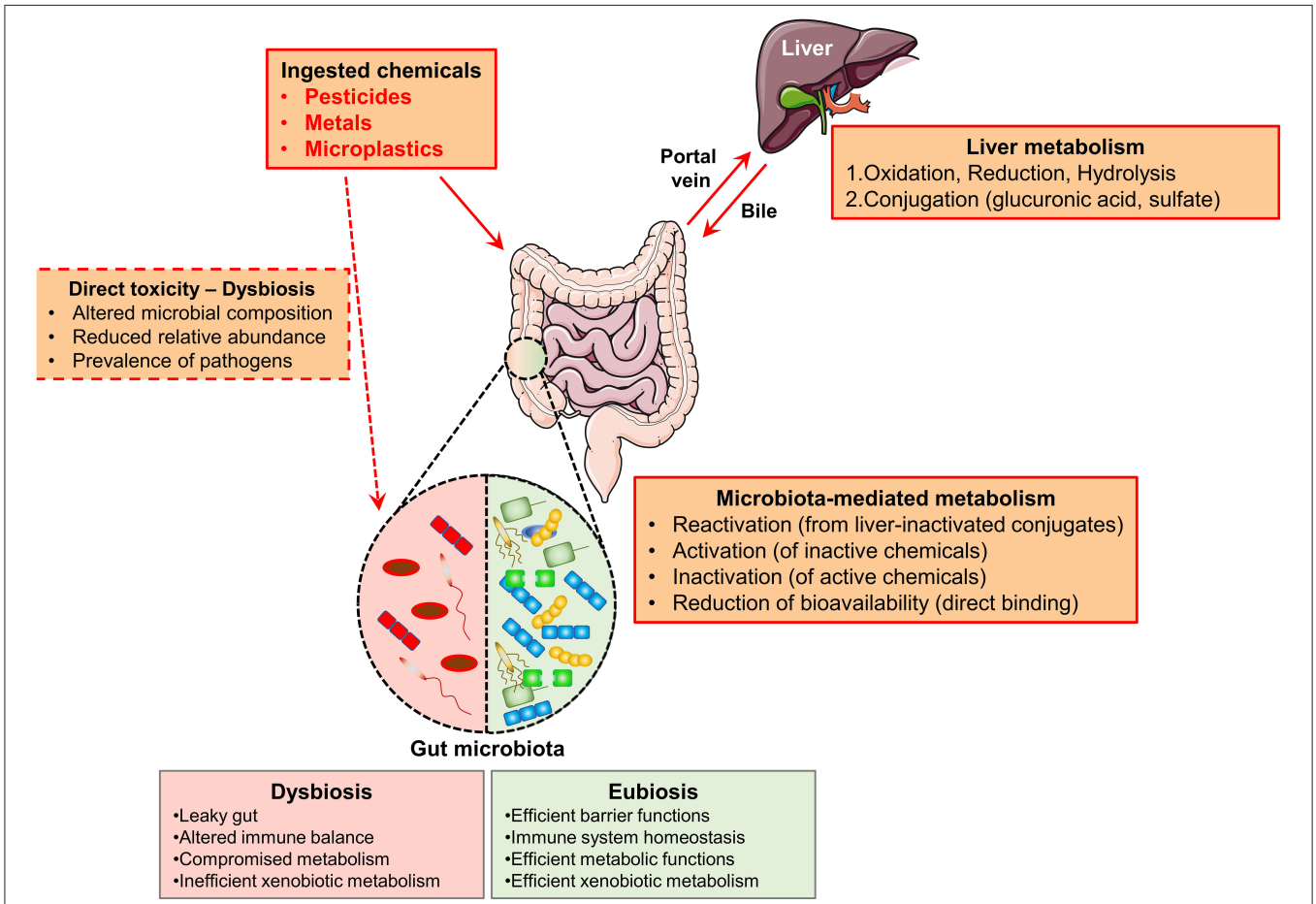

FIGURE 1 | Schematic representation of the biotransformation routes of ingested chemicals. Ingested chemicals (pesticides, metals, microplastics), arrive in the intestine through the oral route. Well-adsorbed compounds are transported to the liver through the portal vein. In the liver, such compounds may be metabolized (through the action of liver enzymes, the compounds are oxidized, reduced, or hydrolysed and finally they are conjugated) and hence released in the intestine within the bile. The gut microbes can: reactivate conjugated chemicals, directly metabolize non-adsorbed chemicals (activation or inactivation), or direct bind such compounds (reducing their bioavailability). Importantly, several chemicals might directly induce microbial dysbiosis (red dotted box). The features of a dysbiotic or eubiotic intestinal microbiota are summarized, respectively, in the red and green boxes.

specific exposures (53). Overall, the modulation of the intestinal gut microbes, through active strategies such as the assumption of specific nutrients or also specific beneficial probiotics, with the aim to repristinate the eubiosis, may be protective against the development of the linked diseases and it can be suggested as a preventive intervention method.

BIDIRECTIONAL INTERACTION BETWEEN ENVIRONMENTAL CHEMICALS AND GUT MICROBIOTA

The concern is about the adverse health effects deriving from occupational exposure to toxic substances and environmental pollutants. Several xenobiotic chemicals have been described to interact with the biological activity of GM affecting the microbial composition and global homeostasis, with dangerous alterations to the host (48, 49). Noteworthy are occupational exposures to pesticides, used for the control of pests, which could affect human GM (54). Heavy metals (HMs) including cadmium, lead, arsenic, and other metals can contaminate soils and reach the human GM through the food chain (55). Exposures to environmental toxicants have been studied primarily for long-term systemic health effects on respiratory disease and cognition, among others, but there is growing evidence that these components also affect the GM. The molecular mechanisms leading to these interactions are not well-known. GM composition depends on several factors and significant changes in this composition, even if minor, are very expected to happen in studies involving animals and chemicals, but it is not clear if these alterations lead to biologically relevant outcomes in the host (56). Establishing such causal relationships should be a priority to elucidate this topic. Now we discuss recent literature findings of interaction between main environmental chemicals (pesticides, metals, and microplastics) and GM. The main findings are shown in **Table 1**.

Pesticides

Many studies have focused on the mechanisms underlying the relationship between pesticides and GM (47, 77). The GM can metabolize pesticides after absorption and reciprocally, active metabolites can affect GM homeostasis with adverse effects for the host. Glyphosate (Gly) is the most widely used herbicide worldwide and its use has been related to several adverse outcomes in humans (48, 49). Gly-induced GM alteration has been hypothesized to be related to neurological impairment such as autism spectrum disorders (78, 79). Several studies showed that Gly can alter the abundances of gut microbial species both *in vivo* (57, 58, 80–82), *in vitro* (59), and also through bioinformatics tools (83). It has been estimated that more than half of species living in the central human GM are sensitive to Gly (84). In addition to altering the microbial composition, a possible mechanism of action could be the modification of microRNAs (miRNAs) expression and immunomodulation as suggested by several studies (85–87). The mRNA expression levels of several inflammatory mediators [Nuclear factor kappa B (NF-κB), Tumour necrosis factor α (TNF-α), Caspase-3, MAPK3, IL-1β, and IL-6] resulted in increase after exposure to Gly, highlighting a notable decrease in the abundance of Firmicutes and enhancement of pathogenic bacteria (60, 61). Nielsen et al. showed that the presence of aromatic amino acids could relieve the antimicrobial effect of Gly (62). Another commonly used pesticide is Chlorpyrifos (Cpf), an organophosphate insecticide effective against fruit and vegetable pests. *In vivo*, it was observed that a chronic exposure to Cpf could cause an increment in Proteobacteria phylum and a decrease in Bacteroidetes phylum (63). The effect of chlorpyrifos has been explored using an *in vitro* simulator mimicking the human intestinal environment. It was observed a reduction of the *Lactobacillus* and the *Bifidobacterium* counts and alteration of the epithelial barrier integrity (64, 88) although other authors described an increment in the cultured *Enterococcus* spp. and *Bacteroides* spp. counts (65). Honeybees seem to be severely affected by neonicotinoid insecticides such as Imidacloprid and many studies suggested that a chronic exposure can alter normal GM composition with a decrement in the global bacterial count, mainly due to Firmicutes reduction (66, 89). Both Gly and Cpf can modulate mucosal-associated invariant T-cells activity in humans, leading to a pro-inflammatory immune response (90).

Metals

Heavy metals include naturally occurring chemicals with high atomic weight and density. Typically, these chemicals can be conveyed with particulate matter especially in urban areas and then reach the water and the soil (91). Several studies on humans or *in vitro* models suggest that HMs exposure can alter the composition and integrity of the GM (92–95). The changes of diversity and composition profile of GM composition resulted altered after a chronic exposure to several metals, such as arsenic (As), cadmium (Cd), cuprum (Cu), lead (Pb), and zinc (Zn). The authors observed an increase in the counts of some families (*Porphyromonadaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, and *Acidaminococcaceae*) vs. a reduction of the Prevotellaceae family. Moreover, it was found a gender difference because microbiota alterations of men were associated with work activity (mining and smelting) in polluted areas (96). As is common chemicals in nature, defined as a human carcinogen since 2012 (97). It can be found in water and soil, as both the organic and inorganic structures. It has been demonstrated to shape the GM depleting gut commensals and enriching pathogenic bacteria (98). In children exposed to As the GM alteration resulted in an abundance of *Proteobacteria*, highlighting changes in genes involved in multidrug resistance (67). As exposure not only affects GM composition but can also alter immune response increasing inflammatory cytokines such as IL-17, TNF-α, and interferon-γ (IFN-γ) (99). As can induce shifts in the earthworm GM, increasing the counts of *Proteobacteria*; these effects are amplified in a synergistic manner in a mixture exposure of As and microplastics (MPs) (68). Other authors described this combined effect of MPs and HMs including Cd, Pb, and Zn underlining GM perturbation and gonadal development in aquatic organisms (100). Pb exposure has been associated with alterations in the composition of the adult GM in humans. Increased urinary Pb level was related to GM richness and α and β-diversity, remarking an increment of *Proteobacteria* (69). Cd determines

TABLE 1 | Main results of the studies included in this review.

References	Experimental model	Pesticides	Microbiota changes
Mao et al. (57)	Rats	Gly	↑ *Prevotella* ↑ *Muscispirillum* ↓ *Lactobacillus* ↑ *Aggregatibacter*
Ruuskanen et al. (58)	Japanese quails	Gly	↓ *Firmicutes* ↑ *Actinobacteria*
Krause et al. (59)	*In vitro*	Gly	*Not evident effects*
Ding et al. (60)	Zebrafish	Gly	↑ *Fusobacteria* ↓ *Proteobacteria*
Tang et al. (61)	Rats	Gly	↓ *Firmicutes*
Nielsen et al. (62)	Rats	Gly	Not evident effects
Liang et al. (63)	Mice	Cpf	↑ *Proteobacteria* ↓ *Bacteroidetes*
Joly Condette et al. (64)	Rats	Cpf	↓ *Lactobacillus* ↓ *Bifidobacterium*
Reygner et al. (65)	SHIME	Cpf	↑ *Enterococcus* ↑ *Bacteroides*
Alberoni et al. (66)	Honeybee	Imidacloprid	↓ *Firmicutes*
		Metals	
Dong et al. (67)	Human	As	↑ *Proteobacteria*
Wang et al. (68)	Earthworm	As	↑ *Proteobacteria*
Eggers et al. (69)	Human	Pb	↑ *Proteobacteria*
Yu et al. (70)	*In vitro* and mice	Pb	↓ *Coprococcus* ↓ *Oscillospira* ↑ *Lactobacillus*
Podany et al. (71)	Mice	Zn	↑ *Pseudomonadales* ↑ *Campylobacter*
		Microplastics	
Xie et al. (72)	Zebrafish	Mps	↑ *Proteobacteria*
Zhu et al. (73)	Soil animal	Mps	↓ *Bacteroides* ↑ *Firmicutes*
Li et al. (74)	Mice	Polyethylene mps	↑ *Staphylococcus* ↓ *Parabacteroides*
Wang et al. (75)	Bees	Polystyrene mps	↓ *α-diversity*
Cheng et al. (76)	Earthworm	Polypropylene mps	↑ *Aeromonadaceae* ↑ *Pseudomonadaceae* ↓ *Nitrososphaeraceae* ↓ *Proteobacteria*

a reduction in GM richness and SCFAs production in mice; additionally, it can change the expression of genes involved in several metabolic pathways (101). In amphibians Cd has been demonstrated to reduce GM biodiversity and arrangement, disclosing significant gut histological alteration at Cd exposure (100 and 200 μg/l) (102). Mercury (Hg) toxicity on GM is confirmed by several *in-vivo* studies. The main described effects are intestinal injury, dysbiosis, enhanced expression of apoptosis genes, and neurotoxic effects (103, 104). Similarly, Pb exposure may act as a disruptor in GM homeostasis, inducing structural intestinal injuries and perturbing GM diversity both *in vivo* and *in vitro* (70). A disproportionate diet introit of Zn in mice, has been shown to cause oxidative stress in the intestinal trait, accompanied by significant shifts in GM, such as enrichment in pathogenic taxa (71).

Microplastics

Since plastics appeared, the MPs represent a novel occupational and environmental hazard (105). Although there is no scientifically agreed definition of MPs, they are usually defined as plastic particles <5 mm in diameter. Its toxicity has been broadly debated (106, 107), even though knowledge about MPs effects on gut microbiota still lacks. The accumulation of different forms of microplastics can cause several effects in the intestinal tract, damaging mucosa, and increasing permeability. Some authors have hypothesized that MPs could carrier and release phthalate esters into intestinal traits with consequential toxic effects (108). Also, MPs also can induce GM dysbiosis and specific bacteria alterations (109, 110). In the aquatic organism, MPs can affect the GM causing several harmful effects. Authors showed that low chronic MP exposure in mussel GM can

lead to an increased abundance of human pathogens (111). In the zebrafish gut, it was observed a significant alteration in the microbial community after exposure to MPs. These alterations were likely mediated by inflammation and oxidative stress (112–114). At the phylum level, the increased count of *Proteobacteria* was accompanied by a significant reduction in the count of *Fusobacteria*, *Firmicutes,* and *Verrucomicrobiota* after 21-day exposure to 1 mg/L of MPs (72). MPs exposure seems to enhance the expression of immune cytokines (TNF-α, IFN-γ, TLR4, and IL-6) as well as inducing microbiota dysbiosis (115). Besides aquatic animals, also terrestrial ecosystems seem to be affected by MPs. In soil animals exposed to MPs, it has been observed a remarkable reduced bacterial diversity; particularly a reduction of the abundance of *Bacteroides* and an increment of the abundance of *Firmicutes* (73). Several studies have shown that polystyrene MP can gut dysbiosis in GM and intestinal barrier dysfunction (promoting inflammation) besides metabolic disorders, including hepatic lipid metabolism, in the mice model (116, 117). In the same *in-vivo* model, polyethylene MPs were suggested to cause intestinal dysbiosis and inflammation. Particularly it was observed a significant increment in *Staphylococcus* abundance and a significant reduction in Parabacteroides abundance. Furthermore, serum levels of interleukin-1α were found significantly raised (74). Maternal MPs exposure resulted associated with GM dysbiosis and gut barrier impairment in mice, with long-term metabolic penalties in offspring (118). It was observed that polystyrene microplastics could decrease α-diversity of GM of bees and alter the expression of antioxidative, detoxification, and immune system-related genes (75). In soil containing MPs, the gut of earthworms can be damaged. Polypropylene MPs exposure can reduce diversity and alter microbial community in earthworms GM. Specifically, an increment in *Aeromonadaceae* and *Pseudomonadaceae* was observed with a decline of *Nitrososphaeraceae* and *Proteobacteria* (76). MPs can reach the intestine trait and accumulate interacting with GM and altering its composition. This toxicology assessment may represent a new target to evaluate the health hazards for humans in the future.

CONCLUSION AND PERSPECTIVES

The GM plays a multifaceted role in the exposure to toxic compounds. It represents the first interface between an exogenous chemical. Hence, the GM could represent a key factor in the toxicity of environmental pollutants and this may become really relevant for the identification of both novel biomarkers of exposure and molecular pathways underneath, therefore representing an indication of the true association of the exposure to the health effect observed. In this review, it has been discussed the emerging dual role played by the GM in the metabolisms of toxicants. The active modulation of the GM (e.g., with the administration of specific probiotics) to preserve the beneficial species able to neutralize the toxicity of such chemicals, may be explored in the future as a preventive therapeutic integrated approach to actively counteract exposure damages and detrimental health consequences.

In occupational medicine, a toxicological approach should be considered to elucidate GM alterations relating to environmental exposure to pollutants (119). GM composition together with the determination of well-known biomarkers could be helpful tools to assess susceptibility for disease. The risk assessment should consider that common human exposures to toxic compounds occur at low doses for a long time, while in most experimental studies, the exposure occurs for a short time at acute or sub-acute doses. It could be a new useful approach to analyze microbiome composition from a fecal sample as a screening tool, to assess the individual microbiome signature in order to address the specific intervention for preventive measures amelioration toward specific risk factors. Therefore, from a translational point-of-view, the GM may represent an important indicator for toxicological assessment, and future studies in clinics, especially in exposed cohorts of individuals, could identify the human GM as a helpful tool for the early surveillance of host health.

AUTHOR CONTRIBUTIONS

CF, FG, and MT contributed to the conceptualization. CC and CF contributed to the methodology and supervision. FG and MT contributed to the data curation and writing—original draft preparation. CC contributed to writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Vitamin D Supplementation and Genetic Polymorphisms Impact on Weight Loss Diet Outcomes in Caucasians: A Randomized Double-Blind Placebo-Controlled Clinical Study

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Vitamin D deficiency or insufficiency is common in obese people, with some studies suggesting that low vitamin D level might be an independent predictor of obesity. Thus, the purpose of the present randomized, double-blind, placebo-controlled study was to investigate the effect of oral spray vitamin D_3 3000 IU supplementation along with personalized weight-loss diet on obesity markers in overweight and obese Caucasians with vitamin d deficiency or insufficiency. The impact of vitamin D receptor (VDR) and adrenergic receptors (ADRs) genetic variants on vitamin D levels and weight loss diet outcomes was also investigated. After signing informed consent, a total of 125 eligible volunteers were randomly assigned into vitamin D (vitamin D_3 3000 IU/d oral spray supplementation, $n = 76$) or placebo (xylitol, water, mint, $n = 49$) group following a weight loss program (600 calories less than the total energy expenditure of each volunteer) for 3 months. Fat mass, BMI, REE and 25(OH)D serum level were monitored on baseline and each month. DNA samples were extracted from buccal swabs and genotyped for the rs2228570 (*VDR*), rs1544410 (*VDR*), rs731236 (*VDR*), rs1800544 (*ADRA2A*), rs1801252 (*ADRB1*), rs1042713 (*ADRB2*), and rs4994 (*ADRB3*) polymorphisms. Statistical analysis was performed using SPSS package (v.23). Between group comparisons revealed significant improvement in serum 25(OH)D level and greater reduction in weight, BMI and fat percentage in the vitamin D group compared to placebo group ($p < 0.05$). In the vitamin D group, carriers of the rs2228570T allele tended to have greater vitamin D level improvement compared with the homozygous C allele ($p = 0.067$). Furthermore, heterozygous (CT) for the rs731236 tended to have lesser weight loss ($p = 0.068$) and for the rs1042713, a lower decline in fat percentage was observed for homozygous AA carriers compared to the heterozygous ($p = 0.051$).

In the control group, differences in weight loss ($p = 0.055$) and BMI ($p = 0.045$) were observed between rs1544410 AA and GG homozygous. In conclusion, vitamin D oral spray supplementation seems to improve vitamin D status and decrease obesity markers during a weight-loss intervention in overweight/obese Caucasians with vitamin D deficiency or insufficiency. Also, the results of the present study indicate that *VDR* and *ADRs* genetic polymorphisms seem to influence vitamin D supplementation response and obesity markers.

Keywords: vitamin D, weight loss diet, obesity, 25(OH)D, VDR, adrenergic receptors, single nucleotide polymorphisms

INTRODUCTION

Vitamin D is a fat-soluble vitamin synthesized from 7-dehydrocholesterol in the skin after exposure to sunlight or obtained from diet and dietary supplements. Vitamin D comes in two forms; vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) (1, 2). Two sequential hydroxylations convert vitamin D into its biologically active form; 25-hydroxylation in the liver, which produces 25-hydroxyvitamin D_3 (25 $(OH)D_3$, calcidiol), the major vitamin D_3 circulating form in the body used as a valid vitamin D status biomarker, followed by the second 1α-hydroxylation in the kidney, which converts $25(OH)D_3$ to 1,25 dihydroxyvitamin D (1,25 $(OH)_2D_3$, calcitriol) (3–6). $1,25(OH)_2D_3$ is the most active vitamin D metabolite and a steroid hormone with multiple skeletal and extraskeletal biological roles, mediated by the vitamin D receptor (VDR), that controls over several hundreds of genes (7, 8).

Worldwide data indicate that the prevalence of hypovitaminosis D is a serious global health problem in all ages, even in countries with sun exposure throughout the year (9). Level of serum 25(OH)D below 20 ng/ml (50 nmol/L) are defined as vitamin D deficiency, whereas 21–29 ng/ml (525–725 nmol/L)serum 25(OH)D level as vitamin D insufficiency (10). Hypovitaminosis D is common in obese individuals, while recommendations for obese individuals suggest higher doses of vitamin D (10). In addition, BMI and fat mass are factors inversely related with 25(OH)D level (11–15).

An indirect association between obesity and vitamin D deficiency is possible due to the less sunlight exposure lifestyle of obese individuals (16). Moreover, obesity-related hypovitaminosis D could be attributed to the decreased bioavailability of the fat soluble vitamin D in the circulation of obese individuals due to the greater storage of vitamin D in fat tissues (15, 17, 18). Contrariwise, studies suggest that vitamin D may regulate body composition (19, 20), while a recent meta-analysis indicates that vitamin D supplementation in overweight and obese individuals may serve as a possible therapeutic option for weight l oss interventions (21). Low 25(OH) D level results in an increased parathyroid hormone (PTH) concentration which subsequently stimulates calcium influx into adipocytes and thereby promotes adipogenesis. Another hypothesis that supports the involvement of vitamin D deficiency in the pathophysiology of obesity is that $1,25(OH)_2D$ and VDR are implicated in adipocyte differentiation (19, 22). Nevertheless, causality direction and the underlying mechanism are still uncertain.

Obesity is a complex multifactorial disease affected by genetic, environmental, socioeconomic, and behavioral factor confluence, which raises remarkably the risk of debilitating morbidity and mortality. Overweight and obesity prevalence is alarmingly increasing, affecting over one-third of the world's population (23, 24). By the year 2030, if current trends continue, up to 57.8% of the world's adult population will be overweight and obese, as estimated by Kelly et al. (25). Since obesity and vitamin D deficiency and insufficiency are progressively widespread (26, 27), uncovering the casualty direction between them and identifying vitamin D supplementation treatment that has a beneficial impact on obesity and obesity-related disorders remains a crucial area of investigation. Therefore, the purpose of the present study was to investigate the effect of oral spray vitamin D_3 3000 IU/d supplementation along with personalized weight-loss diet on obesity markers in overweight and obese Caucasians with vitamin d deficiency or insufficiency. In addition, the impact of genetic polymorphisms in VDR and adrenergic receptors (ADRs) in vitamin D levels and weight loss diet outcomes was also investigated.

MATERIALS AND METHODS

Study Design

The present double-blind placebo controlled parallel group designed clinical study was conducted in "Athens Euroclinic" Hospital, Athens, Greece between January and April 2017. The study was reviewed and approved by the Ethics Committee of "Athens Euroclinic" Hospital and all volunteers provided signed informed consent.

Participants were primarily recruited through advertisements in "Athens Euroclinic" Hospital. The target population for the project included overweight and obese (BMI > 25 kg/m^2) Southeastern European Caucasians with vitamin D deficiency (serum $25(OH)D_3$ <20 ng/ml) or insufficiency (serum $25(OH)D_3$ = 20–30 ng/ml) (10), aged 18–59 years. Exclusion criteria were pregnancy and lactation; being a professional

Abbreviations: $25(OH)D_3$, 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$,1,25 dihydroxyvitamin D_3; VDR, vitamin D receptor; ADRs, adrenergic receptors; PTH, parathyroid hormone; REE, Resting Energy Expenditure; SNPs, single nucleotide polymorphisms.

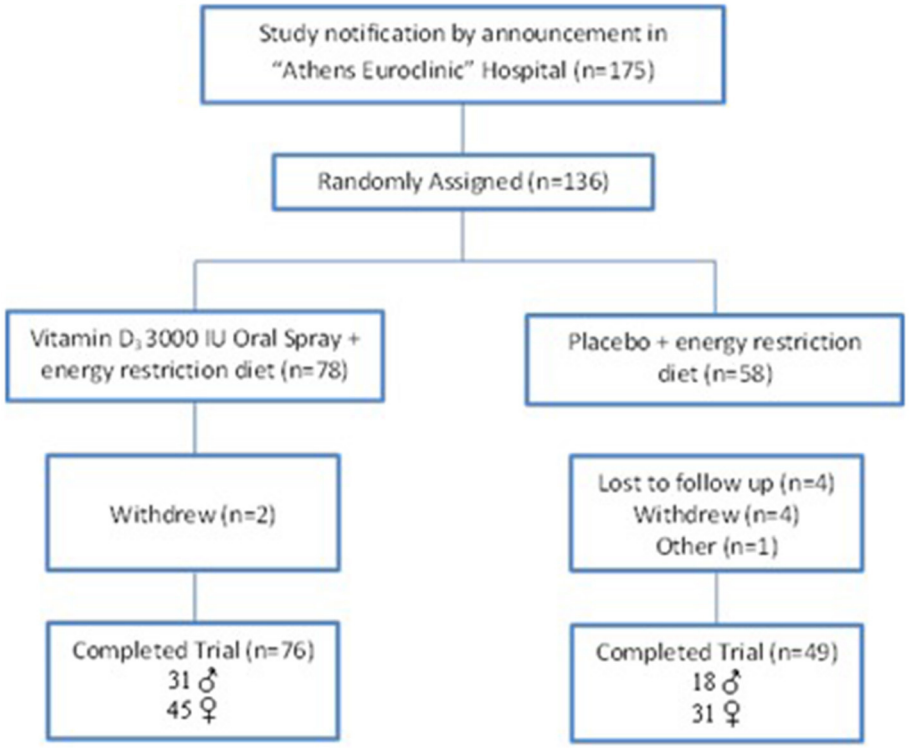

FIGURE 1 | Flow diagram of the study.

TABLE 1 | Baseline characteristics of study participants.

Variable	Placebo (*N* = 49)	Vitamin D (*N* = 76)	*P*
Age (years)*	42.96 ± 11.07	40.14 ± 8.84	0.119***
Male, *n* (%)	18 (36.7)	31 (40.8)	0.650**
Female, *n* (%)	31 (63.3)	45 (59.2)	
Weight (kg)*	98.11 ± 21.94	103.02 ± 23.95	0.250***
Height (m)*	1.69 ± 0.08	1.70 ± 0.08	
BMI (kg/m^2)*	34.00 ± 6.37	35.26 ± 7.33	0.327***
Body fat (%)*	38.04 ± 7.42	37.54 ± 7.77	0.721***
REE (kcal)*	1536.59 ± 383.66	1604.76 ± 435.99	0.373***
Serum 25(OH)D (ng/mL)*	14.16 ± 5.00	14.61 ± 4.86	0.624***

**Values are mean ± SD.*
***P-value for chi-square test.*
****P-value for Independent-Samples T-Test between means.*

athlete; participation in weight loss diet intervention 3 months before the study; taking medications e.g., Hydroxychloroquine and Cholestyramine that could influence D absorption; diabetes mellitus and other pathologies besides obesity and having increased sun exposure lifestyle.

Eligible volunteers were randomized into either weight loss program and vitamin D_3 3000 IU/d oral spray supplementation (Dlux 3000-Better You LTD) or weight loss program and placebo (oral spray containing xylitol, water, peppermint oil-Better You LTD) once daily, for 12 weeks. The goal of weight loss diet was a daily caloric restriction of 600 kcal less than the total energy expenditure of each volunteer, with reference points the Resting Energy Expenditure (REE) and the level of physical activity (PAL). Diet macronutrients were considered based on total caloric consumption as follows: 55 carbohydrates, 15 protein, and 30% fat. The Nutritionist Pro software (version 5.1.0, 2014, Axxya Systems, San Bruno, CA) enriched with recipes of the Greek traditional cuisine was used for weight loss diet design and analysis of dietary intake data and the energy and macronutrients' intakes calculation. Vitamin D intake from diet ranged from 170–250 IU/d. Participants were randomly assigned to each study group by the nutritionist, who was blind to the randomization status. Eligible volunteers had low level of physical activity (light walking). The design of the study is shown in **Figure 1**.

Subjects met the study dietitian at the initiation of the program, followed by monthly individual meetings for weight monitoring. Phone contact was performed on a weekly basis to assess the compliance with the study intervention, whereas compliance with the diet intervention was assessed with weekly food journals. Training of the subjects by an experienced dietitian using food model replicas was preceded. Each subject physical activity evaluation was carried out using smartphone's built-in applications provided by iOS and android operating systems. The primary outcomes of the study included changes in obesity markers (BMI, REE, %fat) and serum 25(OH)D level. Secondary outcome was the impact of *VDR* and *ADR* gene polymorphisms on obesity markers and serum 25(OH)D level changes.

TABLE 2 | Serum 25(OH)D levels and obesity markers at baseline and after intervention.

Variables		Placebo ($N = 49$)	Vitamin D ($N = 76$)	P^*
25(OH)D (ng/ml)	Baseline	14.16 ± 5.01	14.61 ± 4.86	0.624
	3 months	14.62 ± 4.78	34.09 ± 3.58	
	MD	0.46 ± 1.45	19.49 ± 4.66	**<0.001**
	% MD	5.23 ± 13.01	163 ± 106.57	**<0.001**
	P**	**0.031**	**<0.001**	
Weight (kg)	Baseline	98.11 ± 21.94	103.02 ± 23.95	0.250
	3 months	88.50 ± 20.88	91.89 ± 22.19	
	MD	9.61 ± 2.91	11.13 ± 2.57	**0.003**
	% MD***	−9.93 ± 2.63	−10.92 ± 1.97	**0.027**
	P**	**<0.001**	**<0.001**	
BMI (kg/m^2)	Baseline	34.00 ± 6.37	35.26 ± 7.32	0.327
	3 months	30.63 ± 5.99	31.44 ± 6.80	
	MD	3.36 ± 1.03	3.81 ± 0.85	**0.009**
	% MD***	−9.93 ± 2.63	−10.92 ± 1.97	**0.027**
	P**	**<0.001**	**<0.001**	
REE (kcal)	Baseline	1536.59 ± 383.66	1604.76 ± 435.99	0.373
	3 months	1416.69 ± 38357	1486.03 ± 442.78	
	MD	119.90 ± 22.60	118.74 ± 21.32	0.772
	% MD***	−8.28 ± 2.63	−8.06 ± 2.95	0.674
	P**	**<0.001**	**<0.001**	
Fat Mass (%)	Baseline	38.04 ± 7.42	37.54 ± 7.77	0.721
	3 months	33.14 ± 6.79	32.07 ± 7.61	
	MD	4.90 ± 0.96	5.47 ± 1.03	**0.002**
	% MD***	−13.05 ± 2.21	−15.10 ± 3.71	**<0.001**
	P**	**<0.001**	**<0.001**	

MD, Mean differences. Values are presented as mean ± SD.
P-value for Independent sample t-test.
****P-value for Paired t-test.***
*****100*(After-Baseline)/Baseline.***

TABLE 3 | Genotype distribution frequencies of *VDR*, *ADRA2A*, *ADRB1*, *ADRB2*, *ADRB3* gene polymorphisms in the study groups.

Gene	SNP	Genotype	Placebo, *n* (%) $N = 49$	Vitamin D, *n* (%) $N = 76$	P^*
VDR	rs2228570	TT	1 (2.0)	6 (7.9)	0.293
		TC	27 (55.1)	44 (57.9)	
		CC	21 (42.9)	26 (34.2)	
	rs731236	TT	22 (44.9)	35 (46.1)	0.989
		CT	18 (36.7)	28 (36.8)	
		CC	9 (18.4)	13 (17.1)	
	rs1544410	AA	7 (14.3)	6 (7.9)	0.518
		GA	20 (40.8)	34 (44.7)	
		GG	22 (44.9)	36 (47.4)	
ADRA2A	rs1800544	GG	1 (2.0)	1 (1.3)	0.940
		CG	16 (32.7)	24 (31.6)	
		CC	32 (65.3)	51 (67.1)	
ADRB1	rs1801252	AA	43 (87.8)	69 (90.8)	0.447
		AG	5 (10.2)	7 (9.2)	
		GG	1 (2.0)	0 (0.0)	
ADRB2	rs1042713	AA	5 (10.2)	6 (7.9)	0.786
		GA	23(46.9)	33 (43.4)	
		GG	21 (42.9)	37 (48.7)	
ADRB3	rs4994	TT	43 (87.7)	66 (86.8)	0.881
		CT	6 (12.3)	10 (13.2)	

**P-value for chi-square test.*

Measurements

Weight, height, REE, fat percentage, and serum 25(OH)D level measures were taken at baseline, whereas weight, REE, fat percentage and serum 25(OH)D level were measured after the intervention (12 weeks). Weight, REE and fat percentage were also calculated monthly. Weight and height were measured wearing only underwear, using the scale Tanita WB 110-A (Tanita, Tokyo, Japan) and Tanita HR200 Height Measuring Rod (Tanita, Tokyo, Japan), respectively. BMI was calculated as weight divided by the square of height (kg/m^2). Individual total energy expenditure was estimated with indirect calorimetry method using Cosmed's FitMate (COSMED Srl Rome 3700041 Albano Laziale, Italia). Body composition was measured with Quadscan 4,000 device (Bodystat, Douglas, Isle of Man, UK).

Serum 25(OH)D levels were measured with LC-MS/MS (Liquid Chromatography with tandem mass spectrometry) using the Triple Quadrupole Mass Spectrometer (LC/MS/MS) from Agilent Technologies (Santa Clara, CA 95051, United States). LC-MS/MS vitamin D assays offer better accuracy at medical decision levels to correctly classify patients as vitamin-D deficient and sufficient.

Epithelial cells from the oral cavity of each participant were collected at baseline using sterile buccal swabs. DNA was extracted from the epithelial cells using commercial nucleic acid isolation kit (Tissue Nucleospin; Macherey-Nagel GmbH & Co., KG, Düren, Germany) and analyzed using the LightSnip kit (TIB MOLBIOL Germany) according to manufacturer's recommendations on the LightCycler 480 (LC480)-Instrument platform (Roche-Diagnostics, Mannheim, Germany). The following single nucleotide polymorphisms (SNPs) were analyzed: rs2228570, rs1544410, and rs731236 (Vitamin D Receptor -*VDR*), rs1800544 (Adrenergic Receptor Alpha 2A - *ADRA2A*), rs1801252 (Beta-1 Adrenergic Receptor - *ADRB1*), rs1042713 (Beta-2 Adrenergic Receptor - *ADRB2*), and rs4994 (Beta-3 Adrenergic Receptor - *ADRB3*).

Statistical Analysis

Continuous variables were presented as mean ± standard deviation (SD) whereas categorical variables were presented as frequencies (*n*, %). Normal distribution of the data was tested by Kolmogorov-Smirnov test, thus parametric tests were performed. Post-intervention changes i.e., post-intervention minus baseline values, for each group were compared to examine the intervention responsiveness. Paired samples *t*-test was used for the assessment of intra-group mean values changes in continuous variables (serum 25(OH)D, weight, BMI, REE, fat percentage) before and after the intervention, while independent samples *t*-test was applied for between-groups differences. Pearson's chi-square (x^2) test was performed for

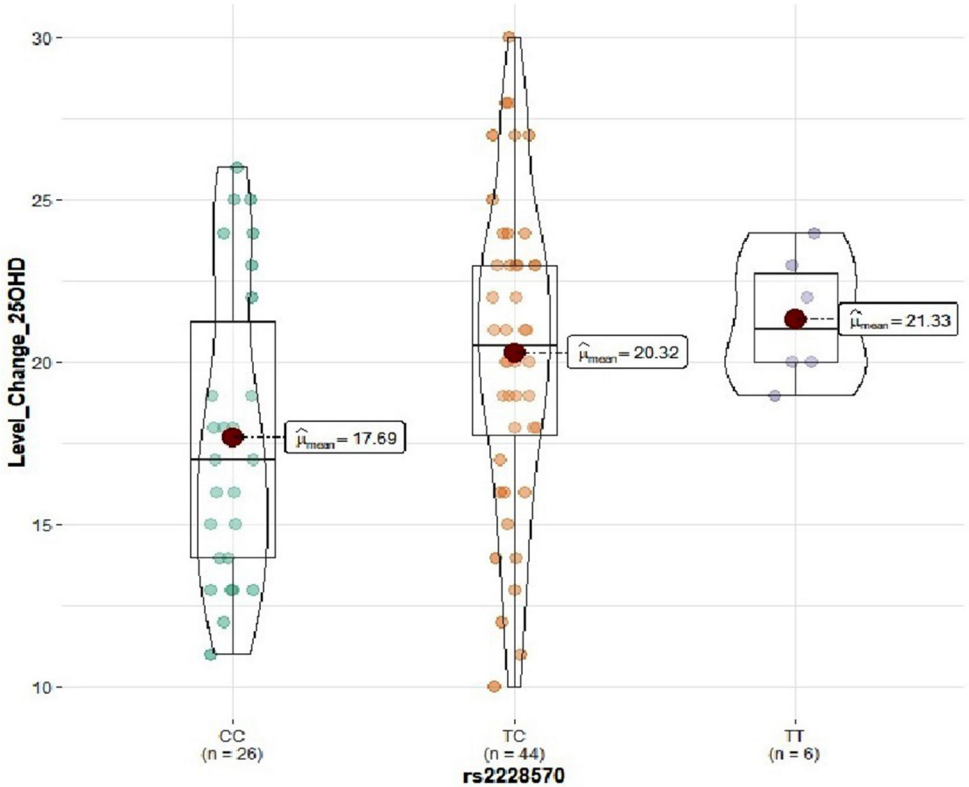

FIGURE 2 | Violin plots displaying the mean 25(OH)D serum level change before and after the intervention in the vitamin D group according to rs2228570 genotype profile.

categorical variables (gender, distribution of genotypes). One-way ANOVA analysis was first applied to detect specific effects of the examined SNPs genotype status on serum 25(OH)D level, weight, BMI, REE, and fat percentage changes before and after the intervention. For the SNPs significantly associated (one-way ANOVA analysis $p \leq 0.05$) with the examined variables, Bonferroni correction for multiple testing was then performed. The statistical power of the study was found to surpass 74.7% ($\beta = 0.747$). All statistical analyses were performed at a significance level of $\alpha = 0.05$, using IBM (Athens, NTUA, Greece) SPSS Statistics Ver. 20.0 package.

RESULTS

In total, 136 overweight and obese Southeastern European Caucasians with vitamin D deficiency or insufficiency were initially recruited in the study. 125 participants completed the study, of whom 49 were men (39.2%) and 76 women (60.8%). The baseline characteristics of the study participants are given in **Table 1**. No differences in baseline characteristics between the groups were observed (**Table 1**). Within group analyses showed a statistically significant increase in serum 25(OH)D concentration (14.61 ± 4.86 vs. 34.09 ± 3.58, $p < 0.001$) in the vitamin D group. In addition, a statistically significant decrease in weight (103.02 ± 23.95 vs. 91.89 ± 22.19, $p < 0.001$), BMI (35.26 ± 7.32 vs. 31.44 ± 6.80, $p < 0.001$), fat percentage (37.54 ± 7.77 vs. 32.07 ± 7.61, $p < 0.001$), and REE (1604.76 ± 435.99 vs. 1486.03 ± 442.78, $p < 0.001$) was observed. Accordingly, in the placebo group, a much lower increase in serum 25(OH)D levels (14.16 ± 5.01 vs. 14.62 ± 4.78, $p = 0.031$) was observed, while a statistically significant decrease in weight (98.11 ± 21.94 vs. 88.50 ± 20.88, $p < 0.001$), BMI (34.00 ± 6.37 vs. $30.63 \pm 5,99$ vs. 32.07 ± 7.61, $p < 0.001$), fat percentage (38.04 ± 7.42 vs. 33.14 ± 6.79, $p < 0.001$), and REE (1536.59 ± 383.66 vs. 1416.69 ± 383.57, $p < 0.001$) was also observed. In between-group comparisons, statistically significant differences in serum 25(OH)D levels, weight, BMI, and fat percentage changes before and after the intervention were observed. Significant improvement in vitamin D status (0.46 ± 1.45 vs. 19.49 ± 4.66, $p < 0.001$) and reduction in weight (9.61 ± 2.91 vs. 11.13 ± 2.57, $p = 0.003$), BMI (3.36 ± 1.03 vs. 3.81 ± 0.85, $p = 0.009$), and fat percentage (4.90 ± 0.96 vs. 5.47 ± 1.03, $p = 0.002$) were observed in the vitamin D group compared to the placebo group (**Table 2**).

Genotype distribution frequencies of rs2228570 (*VDR*), rs1544410 (*VDR*), rs731236 (*VDR*), rs1800544 (*ADRA2A*), rs1801252 (*ADRB1*), rs1042713 (*ADRB2*), and rs4994 (*ADRB3*) genetic polymorphisms in the study groups are shown in **Table 3**. No differences in genotype distribution frequencies were observed between the groups (**Table 3**). In the vitamin D group, carriers of the rs2228570 T allele tended to have

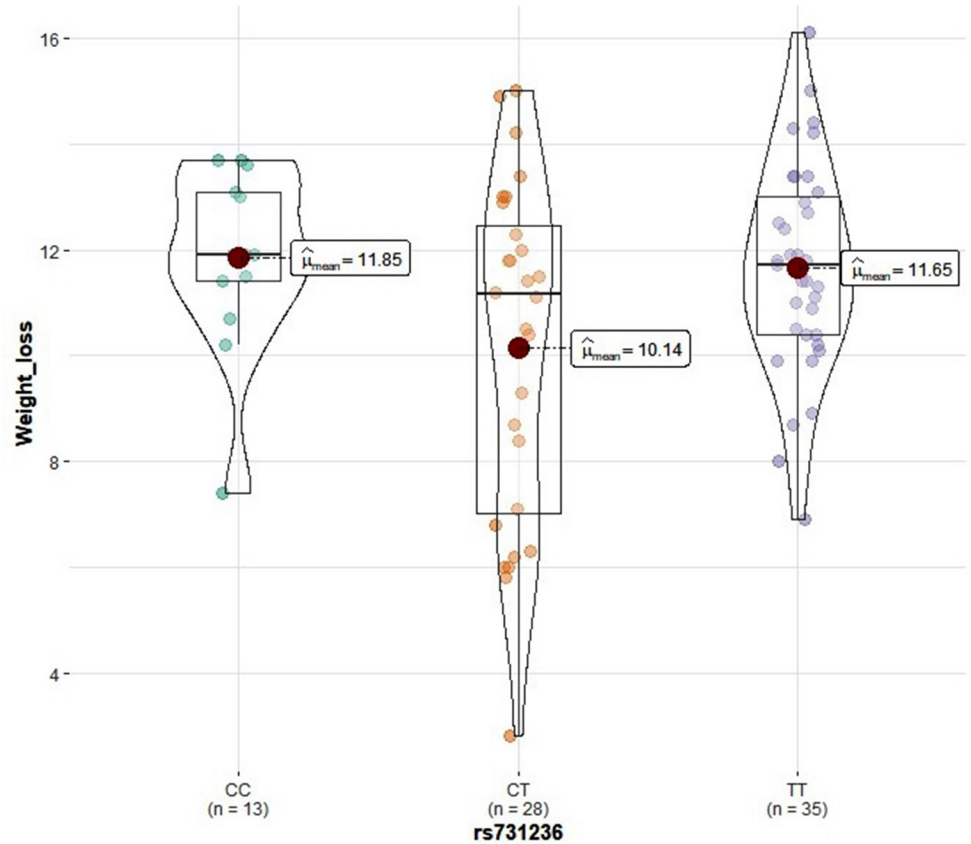

FIGURE 3 | Violin plots displaying the mean values of weight loss in the vitamin D group according to rs731236 genotype profile.

greater vitamin D level improvement compared with the homozygous C allele ($p = 0.067$) (**Figure 2**). Furthermore, heterozygous (CT) for the rs731236 tended to have lesser weight loss ($p = 0.068$) (**Figure 3**) and for the rs1042713, a lower decline in fat percentage was observed for homozygous AA carriers compared with the heterozygous ($p = 0.051$) (**Figure 4**). In the control group, differences in weight loss ($p = 0.055$) (**Figure 5**) and BMI ($p = 0.045$) (**Figure 6**) were observed between rs1544410 AA and GG homozygous. In particular, homozygous for the G allele tended to exhibit better weight loss and statistically significant more reduction in BMI level compared with the homozygous for the rs1544410 A allele.

DISCUSSION

The results of the current double-blind placebo-controlled study in overweight and obese (BMI>25 kg/m^2) Southeastern European Caucasians with vitamin D deficiency (serum 25(OH)D_3 <20 ng/ml) or insufficiency (serum 25(OH)D_3 = 20–30ng/ml), aged between 18 and 59 years, demonstrated that 12 weeks supplementation with vitamin D_3 3000 IU/d oral spray along with personalized calorie-restriction diet reduced significantly the mean of BMI and fat percentage while it significantly increased the level of serum 25(OH)D_3compared with the control group. Nonetheless, no significant impact of the vitamin D oral spray supplementation was observed on REE.

According to the findings of this study, vitamin D_3 3000 IU/d supplementation of obese and overweight individuals with inadequate vitamin D status in combination with weight-loss program can potentially improve weight loss and reduce fat percentage. The results of the current study are in agreement with the study of Salehpour *et al.* which indicates that 12 weeks supplementation with vitamin D_3 1000 IU/d without a weight loss program in healthy overweight and obese women ($n = 77$) with mean serum 25(OH)D level 16.4 ± 12.56 ng/ml (41.8 ± 31.4 nmol/L), significantly decreased body fat mass (19). Similarly, Khosravi *et al.* found a significant reduction in weight, BMI and waist and hip circumference, while vitamin D level was improved in 50 overweight and obese women aged 20–40, in a 6-week 50000 IU/week vitamin D supplementation, following their usual diet (20). In addition, Lotfi-Dizaji *et al.* concluded that a 12 week 50,000 IU/week vitamin D supplementation along with calorie restriction diet in44 obese

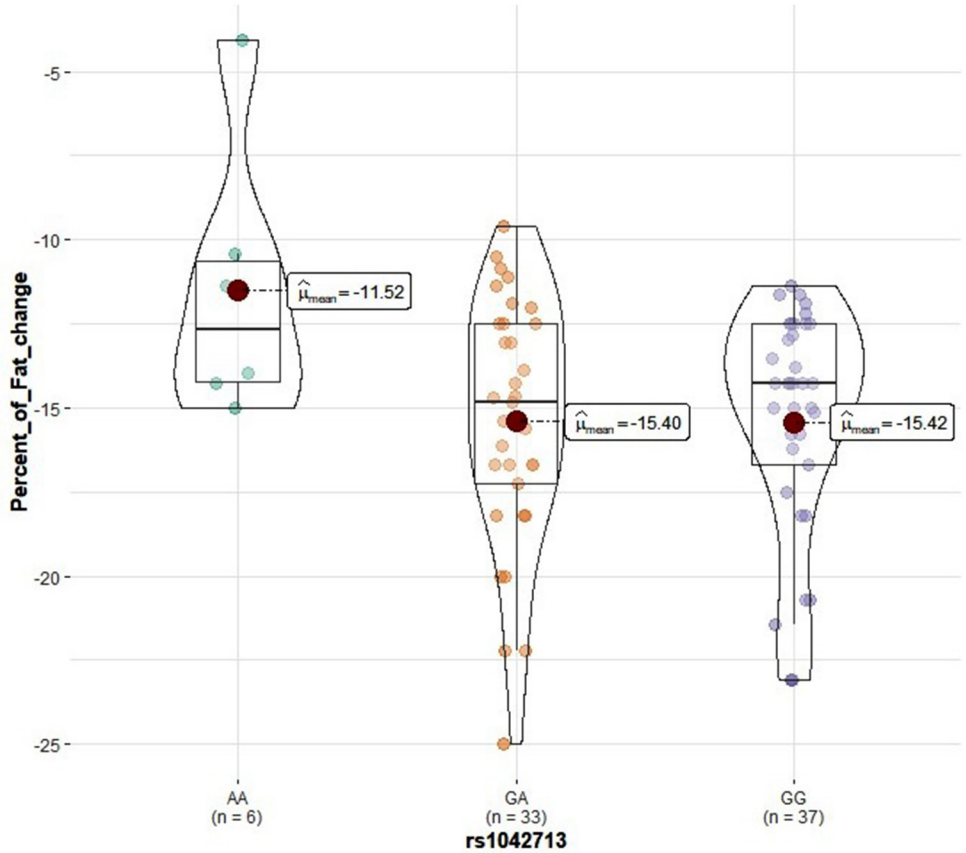

FIGURE 4 | Violin plots displaying the mean values of fat percentage change before and after the intervention in the vitamin D group according to rs1042713 genotype profile.

volunteers with vitamin D deficiency (25(OH)D < 20ng/ml) diminished significantly weight and fat mass and improved serum 25(OH)D level (28).

Contrary to the previous findings, Sneve *et al.* reported that 20000 IU vitamin D supplementation once or twice a week for 12 months in 334 overweight and obese men and women without vitamin D deficiency, did not have any effect on weight changes, waist-to-hip ratio and body fat percentage (29). Zittermann *et al.*, in a 12 months double-blind placebo controlled trialwith165 vitamin D deficient overweight or obese volunteers concluded that 3332 IU/d (83.3 μg) vitamin D_3 supplementation in combination with a weight-reduction program did not adversely affect weight loss (30). In addition, Mason *et al.* in a 12-months randomized double-blind placebo-controlled trial found that 2000 IU/d vitamin D supplementation during a weight-loss intervention in overweight and obese postmenopausal women with vitamin D insufficiency had no effect on weight or fat loss (31). The main difference between these studies from those mentioned above is the longer period duration, which may affect the compliance of the volunteers. A meta-analysis of randomized controlled trials on the effect of vitamin D and calcium supplements on obesity concluded that taking vitamin D supplements had no effect on obesity markers (32).

According to a recent review article, obesity may represent an underlying confounding factor modifying the association between vitamin D deficiency and cardiovascular disease due to its increasing prevalence and strong correlation with cardiovascular disease and vitamin D status (33). Although the causal relation between vitamin D deficiency and obesity remains to be determined, the findings of the current study indicate that obesity may play a critical role in vitamin D deficiency. In particular, a statistically significant increase in vitamin D status was observed after the calorie-restriction diet intervention in the placebo group (14.16 ± 5.01 vs. 14.62 ± 4.78, $p = 0.031$). In agreement with this finding, Onal et al. found a negative relationship between >10% weight loss and vitamin D level, i.e. higher rise in vitamin D level as BMI decreased, although not statistically significant, due to the low number of samples (34). Also, the current findings indicate that an increase in serum 25(OH)D level from around 14 ng/mL to 34 ng/mL in combination with calorie-restriction program decreased weight, BMI, and fat mass, compared to the control group.

Both vitamin D status and obesity are under remarkable genetic influence, as reported by twin and family-based studies (35–37). Studies indicate that *VDR* gene polymorphisms are related to vitamin D levels, as *VDR* gene regulates vitamin

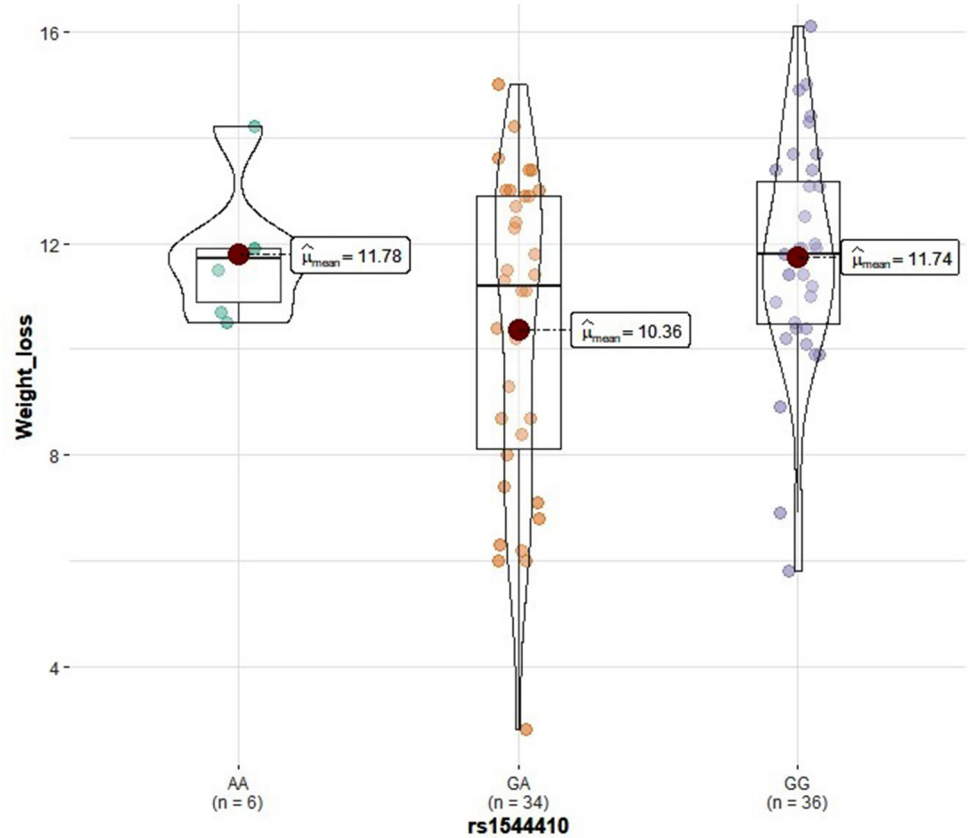

FIGURE 5 | Violin plots displaying the mean values of weight loss in the control group according to rs1544410 genotype profile.

D signaling pathways and vitamin D responsive genes, and may exert influence on adiposity and body composition (38–45). In the current study, the impact of three SNPs in *VDR* gene (rs2228570 (FokI), rs1544410 (BsmI), rs731236 (TaqI)) on the vitamin D status and obesity marker changes after the 3-month intervention was investigated. rs2228570 genotype status seems to influence vitamin D level in the vitamin D group, whereas rs1544410 and rs731236 genotype status was correlated with weight loss measures in control and vitamin D group, respectively. The effect of adrenergic receptor gene polymorphisms [rs1800544 (*ADRA2A*), rs1801252 (*ADRB1*), rs1042713 (*ADRB2*), rs4994 (*ADRB3*)] on obesity marker alterations after the intervention was also investigated, based on the major role of these receptor subtypes in the regulation of lipid mobilization and the correlation of these SNPs with obesity and metabolic syndromes (46–56). In the vitamin D group, rs1042713 genotype status seems to affect fat percentage changes, whereas no association was detected for *ADRA2A* rs1800544, *ADRB1* rs1801252, and *ADRB3* rs4994 polymorphisms.

VDR and *ADRs* genetic polymorphisms seems to influence weight-loss intervention outcomes. Consequently, identification of the most relevant genetic polymorphisms influencing weight-loss intervention outcomes could potentially improve dietary recommendations, advice and even drug therapy based on individual genetic susceptibility.

Strengths of the present study are the double-blind placebo-controlled design, the sufficient sample size, and the ethnic homogeneity of the study population. In addition, both genders were included in the study population, thus the results could be generalized in Southeastern European Caucasian population regardless gender and the occurrence of specific disease conditions. To our knowledge, the present study is the first to investigate the impact of genetic polymorphisms on obesity-related marker changes after weight loss diet and vitamin D supplementation.

The study has some limitation. Firstly, only one dose of vitamin D (3000 IU/d) supplementation was examined. Also, the effect of vitamin D supplementation on obesity markers was not examined independently, without a weight-loss diet intervention. The degree to which these findings can be generalized to non-Caucasian populations is not known. Finally, a possible limitation of the present study is not examining the potential role of additional biomarkers in vitamin D and obesity relationship.

In conclusion, 3-month vitamin D_3 3000 IU/d oral spray supplementation in overweight and obese Caucasian

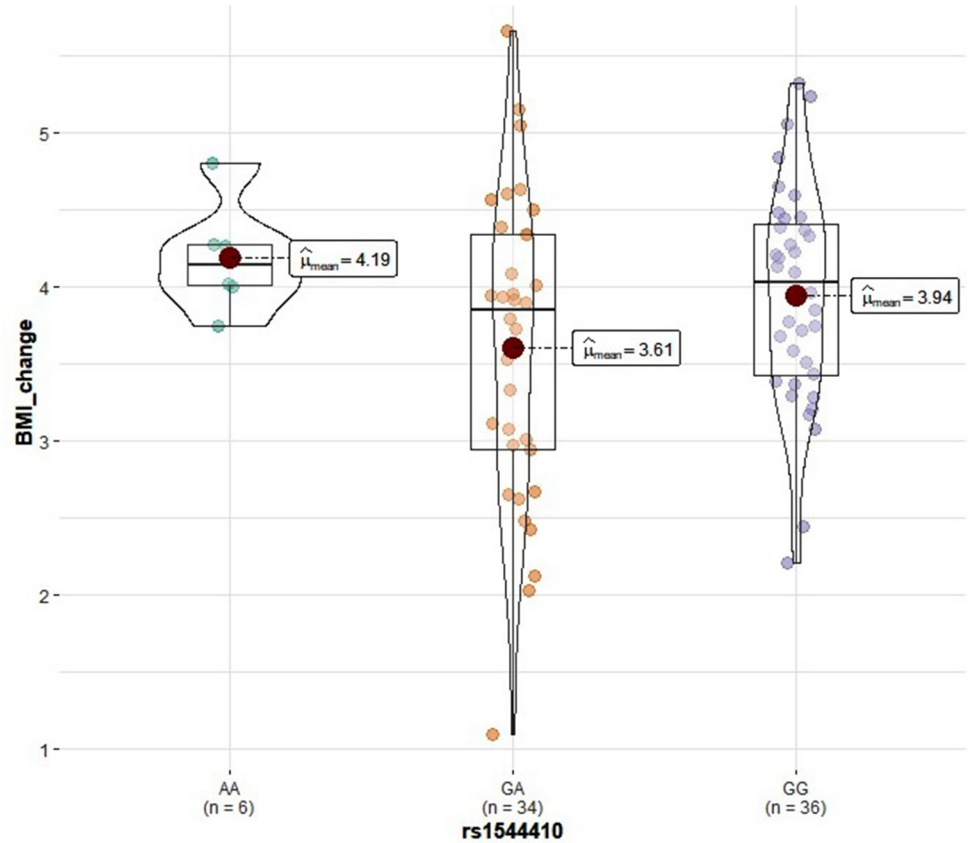

FIGURE 6 | Violin plots displaying the mean values of BMI change before and after the intervention in the control group according to rs1544410 genotype profile.

individuals with inadequate vitamin D status along with calorie-restricted diet program, improved 25(OH)D levels and contributed to a greater reduction in body weight, BMI, and fat percentage. Also, genetic polymorphisms seem to influence vitamin D supplementation response and obesity markers. Further, larger scale studies in Caucasian and non-Caucasian populations are required to validate the results of the present study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Athens Euroclinic Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KX and ND: conceptualized and designed the study. KX: performed the study and reviewed and edited the manuscript. MP: drafted the manuscript. AR: performed the statistical analysis. M-SK: analyzed the samples. All authors read, reviewed, and approved the final version of the manuscript.

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Association between chronic disease multimorbidity and leisure-time physical activity: Evidence from the China Multiethnic Cohort study

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Objective: To reveal the associations between multimorbidity and leisure-time physical activity (LTPA) by ethnicities in China.

Materials and methods: Self-reported information on a range of occupational, household, transport, and LTPA was collected by interviewer-administered questionnaire. A total of 17 chronic diseases were assessed based on self-reported lifetime diagnoses or medical examinations. Multivariable logistic regression models were used to assess the associations between multimorbidity and the risks of low LTPA.

Results: The mean age of all participants was 51.2 years old. Of all, 61.4% were women and 57.9% were from the Han population. A significantly negative association (OR = 0.92, 95% CI = 0.89–0.95) was found between multimorbidity and low LTPA, with a stronger association among minority populations (OR = 0.86, 95% CI = 0.82–0.91) than among the Han population (OR = 0.96, 95% CI = 0.92–1.01). For both the minority population and the Han population, digestive system multimorbidity and digestive-metabolic system multimorbidity had a significantly negative association with low LTPA. For the Han population, the association of intersystem multimorbidity for the circulatory-respiratory system (OR = 1.17, 95% CI = 1.04–1.31) with low LTPA was stronger than that of intrasystem multimorbidity for the circulatory (OR = 1.12, 95% CI = 1.01–1.25) and respiratory systems (OR = 1.14, 95% CI = 1.04–1.25).

Conclusion: There are significant associations between multimorbidity and low LTPA based on this large multiethnic population. Our findings suggest that LTPA-tailored interventions should be designed for specific ethnic groups according to different types of multimorbidity.

KEYWORDS

chronic diseases, multimorbidity, leisure-time physical activity, ethnic differences, system

Introduction

Multimorbidity, a term commonly used to describe the presence of two or more chronic physical conditions (1), has been widely acknowledged as a public health challenge. Despite the inconsistency in measurement, the prevalence of multimorbidity is ranging from 30% to 95% across age groups and countries (2, 3). People with multimorbidity suffer more pain, mobility limitations, and higher mortality (4, 5), and secondary prevention of a wide range of chronic diseases deserves attention in this population. To slow chronic disease progression and reduce mortality, regular physical activity (PA) has been recommended by the WHO in the multifaceted care of individuals with chronic disease or multimorbidity (6, 7). At the macro level, PA helps to reduce the disease burden and loss of economic output associated with the treatment of chronic disease complications, especially in low- and middle-income countries (LMICs) (8).

Evidence exists about the different levels of PA in people living with multimorbidity. For example, a study conducted on 46 LMICs revealed that people with chronic conditions and multimorbidity were significantly less physically active (9). Another study conducted on 96,706 United Kingdom Biobank participants (aged 40 years old or older) suggested that participants with chronic disease undertook 9% or 61 min less moderate activity and 11% or 3 min less vigorous activity per week than individuals without chronic disease (10). However, limited studies clarify whether and to what extent multimorbidity is associated with leisure-time physical activity (LTPA), the most important domain of PA (11). Moreover, multimorbidity of chronic disease may be present in multiple system groups (e.g., cardiorespiratory system (12) and psychiatric system (13)), and therefore usually refers to systematic multimorbidity (i.e., intersystem multimorbidity and intrasystem multimorbidity). Understanding the level of LTPA in people living with intersystem or intrasystem multimorbidity can help clinicians for better guidance on rehabilitation exercises to reduce the risk of complications, disability, and death, but such evidence is currently lacking.

Numerous studies have explored racial and ethnic disparities in PA worldwide (14, 15) and found racial/ethnic minorities are especially unlikely to engage in PA and tend to have poorer health outcomes (16). As a systematic review indicated, ethnic differences should also be considered in the relationship between chronic disease and PA (17). However, such studies are scarce, especially for LTPA.

To help fill these gaps, this study aimed to estimate the association between intersystem and intrasystem chronic disease multimorbidity and LTPA by ethnicities based on baseline data from the China Multi-Ethnic Cohort (CMEC) (18). Our study can help clinicians to better understand the levels of LTPA among different multimorbidity groups within Han and ethnic minorities groups, for better management of chronic disease and multimorbidity.

Materials and methods

Study design

This study was a cross-sectional study based on the baseline survey of a community population-based prospective observational study established in five provinces (Sichuan, Guizhou, Yunnan, Chongqing, and Tibet) of southwestern China (the CMEC). Seven ethnic groups were identified based on their census register, namely, the Han, Bouyei, Tibetan, Miao, Bai, Yi, and Dong ethnic groups. In the baseline survey, a total of 99,556 participants aged 30–79 years old were recruited from May 2018 to September 2019 by a multistage, stratified cluster sampling method. The details of the CMEC study design, survey methods, and inclusion criteria for participants have been reported previously (18).

For the current analyzes, we excluded (1) those with severe diseases or conditions that might affect participants' PA; (2) those with extreme body mass index (BMI), namely, a calculated value > 40 or < 15; (3) those who had both PA and sedentary leisure time equal to zero; and (4) those with missing information on any outcome, exposure, or adjusted covariates. Ultimately, a total of 76,084 participants remained in the analyzes (**Supplementary Figure 1**).

Exposures

Studies have shown that the relationship between low PA and multimorbidity is bidirectional (10, 19, 20). Also, a few studies examining multimorbidity have treated chronic diseases as exposure (9, 21). Thus, multimorbidity, including 17 chronic diseases, was treated as exposure in our studies, representing the most common chronic disease among the Chinese population (22). Among these chronic diseases, definitions of hypertension, chronic bronchitis, emphysema, gallstone, cholecystitis, diabetes, and hyperlipidemia were based on both the questionnaires and medical examinations; definitions of pulmonary heart disease, rheumatic heart disease, coronary heart disease, rheumatoid arthritis, asthma, cirrhosis, gastroenteritis, and peptic ulcer were only based on the questionnaire; and definitions of obesity and osteoporosis were only based on medical examinations.

Specific definitions for some chronic diseases were as follows. (1) Hypertension. Participants' blood pressure was measured three times using the OMROM HEM-8771 monitor (Omron [China] Co., Ltd., Shanghai, China). Hypertension was defined as a mean systolic blood pressure $\geq$ 140 mm Hg and/or mean diastolic blood pressure $\geq$ 90 mm Hg and/or a diagnosis of hypertension by doctors (23). (2) Diabetes. Fasting plasma glucose (FPG) and glycosylated hemoglobin (HbA1c) were measured in plasma enzymatically with a validated autoanalyzer (AU5800 Automated Chemistry Analyzer, Beckman Colter Commercial Enterprise, Shanghai, China). Diabetes was defined as FPG $\geq$ 126 mg/dl and/or HbA1c $\geq$ 6.5% and/or a diagnosis of diabetes by doctors (24). (3) Hyperlipidemia. Hyperlipidemia was defined based on the presence of one or more of the following components according to the Joint Committee for Developing Chinese Guidelines on Prevention and Treatment of Dyslipidemia in Adults: triglyceride $\geq$ 2.3 mmol/L, total cholesterol $\geq$ 6.2 mmol/L, high-density lipoprotein cholesterol $<$ 1.0 mmol/L, low-density lipoprotein cholesterol $\geq$ 4.1 mmol/L, and/or diagnosis of hyperlipidemia by doctors. (4) Obesity. Height and weight were measured in participants wearing light clothing and barefoot using a weight scale, and BMI was calculated as the body weight (kg) divided by the height squared (m^2). Obesity was defined as having a BMI $\geq$ 28.00, according to China's BMI criterion (25). (5) Osteoporosis. The mineral density of the anklebone was measured using an OSTEOKJ3000 ultrasonic bone densitometer, and a T-score of ≤ -2.5 was defined as osteoporosis (26).

Chronic disease multimorbidity was defined as having at least two of the defined chronic diseases (9). According to the International Classification of Diseases, Tenth Revisions (ICD-10), 17 chronic diseases in this study were organized into four chronic disease subgroups (i.e., diseases of the circulatory system, digestive system, respiratory system, and metabolic system) (**Supplementary Table 1**). According to the subgroups, multimorbidity was divided into two categories: (1) intrasystem multimorbidity, referred to as multimorbidity within the same system, including circulatory, respiratory, digestive, and metabolic system multimorbidity; and (2) intersystem multimorbidity, referred to as multimorbidity between two systems, including circulatory-respiratory, circulatory-digestive, circulatory-metabolic, respiratory-digestive, respiratory-metabolic, and digestive-metabolic system multimorbidity. This division helps to comprehensively capture the associations of LTPA with either chronic disease multimorbidity or special chronic disease multimorbidity subgroups. In the current study, each type of multimorbidity was treated as a separate exposure.

Outcomes

The questions on PA and LTPA were adapted from validated questionnaires used in several other studies, namely, the European Prospective Investigation into Cancer and Nutrition (27) and the China Kadoorie Biobank study (28). PA considered participants' occupational, household, transport, and leisure time (28). All of the participants were asked to report their usual type of LTPA (e.g., walking and jogging) and the average duration spent on LTPA every week over the past year. For a specific LTPA, the second update of metabolic equivalent tasks (METs) from the 2011 Compendium of Physical Activities was used to estimate how many calories are burned (29). The MET values were assigned to each type of LTPA: 3.5 METs for light LTPA (e.g., Taichi, Qigong, and walking), 4.5 METs for moderate LTPA (e.g., jogging and aerobic dancing), 6.0 METs for medium vigorous LTPA (e.g., ballgames and equipment sports), and 7.0 METs for high vigorous LTPA (e.g., swimming). The volume of LTPA (MET-h/week) was calculated by multiplying the intensity (METs) by duration (h/week) (30). According to the minimum level of LTPA recommended by the WHO, those with a volume of LTPA $<$ 7.5 MET-hours/week were defined as low LTPA. In addition, those with a volume of PA less than the median were defined as having low PA (31).

Statistical analyzes

Descriptive statistics were used for sample characteristics (i.e., demographics and behavior characteristics) under a specific LTPA and ethnicity in **Table 1**. Continuous and categorical characteristic variables between subjects engaged in low and adequate LTPA were presented as mean $\pm$ SD and numbers (percentages) and were compared using Student's *t*-test and chi-squared test. An intensity matrix was used to represent the linkages between different types of multimorbidity.

Multivariate logistic regression models were used to assess the associations among chronic diseases, multimorbidity, intrasystem multimorbidity, intersystem multimorbidity, and

TABLE 1 Sample characteristics by low or high leisure-time physical activity (LTPA) and ethnicity.

Variables	Han population (n = 44,025)			Minority population (n = 32,059)		
	High LTPA (n = 20,139)	Low LTPA (n = 23,886)	P -value	High LTPA (n = 8,105)	Low LTPA (n = 23,954)	P -value
Sex (%)			<0.001			0.045
Male	8,346 (41.4)	10,479 (43.9)		2,745 (33.9)	7,822 (32.7)	
Female	11,793 (58.6)	13,407 (56.1)		5,360 (66.1)	16,132 (67.3)	
Age (years) (SD)	52.65 (11.81)	49.46 (11.11)	<0.001	53.58 (11.45)	50.79 (10.92)	<0.001
Marital status (%)			0.001			<0.001
Married/cohabiting	17,951 (89.1)	21,528 (90.1)		7,037 (86.8)	21,396 (89.3)	
Unmarried/divorced/widowed	2,188 (10.9)	2,358 (9.9)		1,068 (13.2)	2,558 (10.7)	
Annual family income, yuan/year (%)			<0.001			<0.001
<20,000	4,315 (21.4)	7,192 (30.1)		3,076 (38.0)	12,435 (51.9)	
20,000–59,999	7,321 (36.4)	9,104 (38.1)		2,937 (36.2)	8,263 (34.5)	
≥ 60,000	8,503 (42.2)	7,590 (31.8)		2,092 (25.8)	3,256 (13.6)	
Education level (%)			<0.001			<0.001
Illiteracy	2,142 (10.6)	3,800 (15.9)		2,946 (36.3)	11,127 (46.5)	
Primary school	3,959 (19.7)	6,666 (27.9)		2,052 (25.3)	6,531 (27.3)	
Junior high school	6,505 (32.3)	7,173 (30.0)		1,595 (19.7)	4,279 (17.9)	
High school or more	7,533 (37.4)	6,247 (26.2)		1,512 (18.7)	2,017 (8.4)	
Smoking status (%)			<0.001			<0.001
Never	14,876 (73.9)	16,876 (70.7)		6,630 (81.8)	19,252 (80.4)	
Former	1,379 (6.8)	1,020 (4.3)		376 (4.6)	715 (3.0)	
Current	3,884 (19.3)	5,990 (25.1)		1,099 (13.6)	3,987 (16.6)	
Alcohol drinking status (%)			<0.001			0.025
Never	10,140 (50.4)	12,620 (52.8)		5,413 (66.8)	15,722 (65.6)	
Occasionally	7,091 (35.2)	7,444 (31.2)		2,021 (24.9)	6,021 (25.1)	
Often	2,908 (14.4)	3,822 (16.0)		671 (8.3)	2,211 (9.2)	
Non- LTPA (MET/day) (SD)	17.01 (14.37)	28.14 (18.81)	<0.001	19.53 (16.78)	29.31 (19.48)	<0.001
Sleep duration (h/day)			<0.001			<0.001
<6	2,190 (10.9)	2,423 (10.1)		976 (12.0)	2,884 (12.0)	
6–8	15,671 (77.8)	18,195 (76.2)		5,427 (67.0)	15,370 (64.2)	
>8	2,278 (11.3)	3,268 (13.7)		1,702 (21.0)	5,700 (23.8)	
Region (%)			<0.001			<0.001
Han ethnicity in Basin	17,697 (87.9)	17,274 (72.3)		–	–	
Han ethnicity in Yunnan	2,442 (12.1)	6,612 (27.7)		–	–	
Yi ethnicity in Yunnan	–	–		877 (10.8)	4,048 (16.9)	
Bai ethnicity in Yunnan	–	–		1,734 (21.4)	3,544 (14.8)	
Tibetans in Aba	–	–		972 (12.0)	2,389 (10.0)	
Tibetans in Lhasa	–	–		1,002 (12.4)	2,502 (10.4)	
Dong ethnicity in Guizhou	–	–		1,301 (16.1)	4,423 (18.5)	
Bouyei ethnicity in Guizhou	–	–		1,269 (15.7)	3,653 (15.3)	
Miao ethnicity in Guizhou	–	–		950 (11.7)	3,395 (14.2)	

SD, standard deviation; LTPA, leisure-time physical activity. The differences in dichotomous (i.e., sex) and polytomous (i.e., region) qualitative variables between subjects engaged in low and high LTPA were tested by the chi-squared test. The differences between quantitative variables were compared by Student's t-test.

LTPA among the Han and minority populations. The stratified analyzes were used to explore the associations between multimorbidity and LTPA by ethnic group, and the likelihood ratio test was used to examine the significance. The analyzes were adjusted for sex (male or female), age (continuous), marital status (married/cohabiting and unmarried/divorced/widowed), annual family income (3 categories: < 20,000 yuan, 20,000–59,999 yuan, and ≥ 60,000 yuan), educational level (4 categories: illiteracy, primary school, junior high school, and high school or more), smoking (3 categories: never, former,

and current), alcohol drinking (3 categories: never, occasionally, and often), sleep duration (3 categories: < 6 hours, 6–8 hours, and > 8 hours), region (9 categories: Han ethnicity in Basin, Han ethnicity in Yunnan, Yi ethnicity in Yunnan, Bai ethnicity in Yunnan, Tibetans in Aba, Tibetans in Lhasa, Dong ethnicity in Guizhou, Bouyei ethnicity in Guizhou, and Miao ethnicity in Guizhou), and non-LTPA (continuous). The number of hours spent per week on each activity was multiplied by the MET score for the activity, and the weekly amount of non-LTPA was obtained by totaling the MET-hours for activities related to occupation, household, and transport except for LTPA. We also detected the overall interaction by including an interaction term reflecting multimorbidity × ethnic group.

In the sensitivity analysis, we adjusted the exposure criteria to the self-reported chronic diseases aforementioned. Although both osteoporosis and obesity were diagnosed by medical examinations, we did not exclude obesity because it was easily perceived by the participants; osteoporosis was excluded since it was diagnosed by medical examination without self-reported data. Finally, we conducted the sensitivity analysis only on multimorbidity for 16 self-reported chronic diseases. The effect estimates were expressed as odds ratios (ORs) and 95% CIs. All of the statistical analyzes were performed using R software, version 4.0.2 (R Foundation for Statistical Computing). A two-tailed P-value < 0.05 was declared statistically significant.

Results

The analytic sample consisted of 76,084 participants aged 30–79 years old. Overall, the mean (SD) age was 51.2 (11.4) years old, 29,392 (38.6%) were men, and 32,059 (42.1%) were minorities. In brief, more than half (62.9%) of the participants engaged in a low level of LTPA, and the minority population (74.7%) was significantly larger than the Han ethnic population (54.3%). For minorities, participants engaged in low LTPA were more likely to be women who were younger, with low annual family income, low educational levels, current smoking, cohabiting, often drinking, high levels of non-LTPA, and long sleep durations. However, for the Han ethnic population, low LTPA tended to appear in men with the aforementioned characteristics (**Table 1**).

The prevalence of low LTPA in participants with each type of multimorbidity by ethnic group is shown in **Figure 1**. In brief, the prevalence of low LTPA among minorities with multimorbidity was significantly higher than that of the Han population. The ORs and 95% CIs for the associations of low LTPA with 17 chronic diseases are shown in **Supplementary Table 2**.

The results of the multivariable logistic regression analysis assessing the associations of multimorbidity, intrasystem multimorbidity, and intersystem multimorbidity with low LTPA are presented in **Figure 2**. In the overall sample, we observed that respiratory system multimorbidity (OR = 1.10, 95% CI = 1.03–1.18) and respiratory-metabolic system multimorbidity (OR = 1.14, 95% CI = 1.05–1.24) were associated with an increased risk of low LTPA in the adjusted models, while significantly negative associations were observed for multimorbidity (OR = 0.92, 95% CI = 0.89–0.95), digestive system multimorbidity (OR = 0.86, 95% CI = 0.82–0.91), circulatory-digestive system multimorbidity (OR = 0.87, 95% CI = 0.83–0.92), circulatory-metabolic system multimorbidity (OR = 0.92, 95% CI = 0.88–0.96), and digestive-metabolic system multimorbidity (OR = 0.91, 95% CI = 0.87–0.95). Notably, when we considered total PA (**Supplementary Table 3**), we found that all types of multimorbidity were positively associated with low PA. The intensity matrix suggested some types of multimorbidity (i.e., respiratory and respiratory-digestive) were closely associated (**Supplementary Figure 2**). The overall interaction between multimorbidity and ethnic group was not significant (OR = 0.95, 95%CI = 0.89–1.02).

When the analysis was stratified by the ethnic group, we found some interesting results (**Figure 2**). Minority population suffered from multimorbidity tended to have adequate LTPA (OR = 0.86, 95% CI = 0.82–0.91), while a significant association was not found among the Han population (OR = 0.96, 95% CI = 0.92–1.01). In addition, for intrasystem multimorbidity and intersystem multimorbidity, all significant associations with low LTPA were negative in the minority population but were mainly positive in the Han population. For both the minority and Han populations, digestive system multimorbidity and digestive-metabolic system multimorbidity had negative, significant associations with low LTPA. For the Han population, the association of circulatory-respiratory system multimorbidity (OR = 1.17, 95% CI = 1.04–1.31) with low LTPA was stronger than those of circulatory system multimorbidity (OR = 1.12, 95% CI = 1.01–1.25) and respiratory system multimorbidity (OR = 1.14, 95% CI = 1.04–1.25).

The sensitivity analyzes suggested similar effect estimates for low LTPA (**Supplementary Table 4**). Compared to the corresponding OR in **Figure 2**, for the Han population, the OR in the association between low LTPA and intrasystem multimorbidity of circulatory, respiratory, and circulatory-respiratory system multimorbidity increased; for minorities, the OR in the associations of low LTPA with circulatory system multimorbidity, circulatory-metabolic system multimorbidity, and digestive-metabolic system multimorbidity decreased.

Discussion

This study examined the associations of multimorbidity, intrasystem multimorbidity, and intersystem multimorbidity

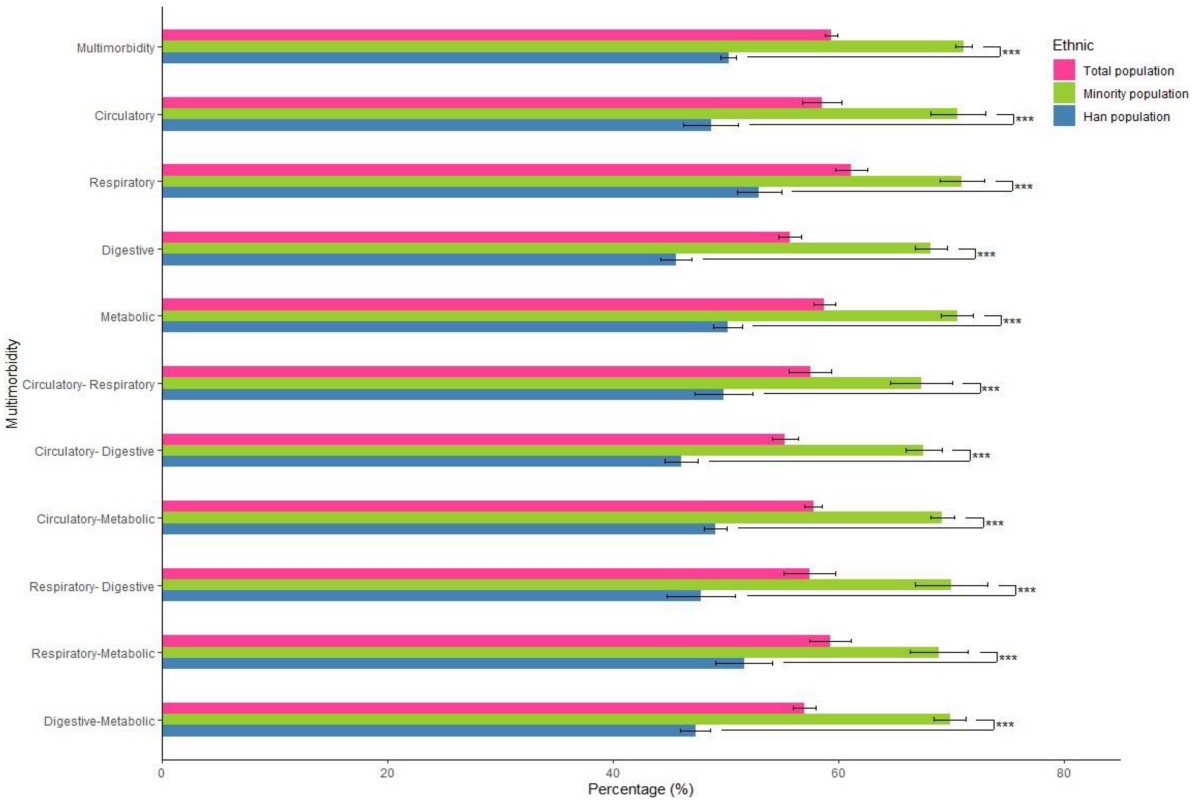

FIGURE 1
Prevalence of low leisure-time physical activity in participants with each type of multimorbidity by ethnic group. ***$p < 0.001$.

with low LTPA among Han and minority populations based on a large-scale, multiethnic cohort in China. Our findings revealed people with chronic disease multimorbidity were inclined to engage in more LTPA in the overall sample, and the association in minority populations was stronger than that in the Han population. In addition, the ethnic differences in the associations between multimorbidity and low LTPA were not found as the interaction between multimorbidity and ethnic group was not significant. These findings could enable clinicians and policymakers to carry out precise PA interventions and treatments for those with particular chronic disease multimorbidity.

To date, many studies have demonstrated that people with chronic diseases or multimorbidity tend to engage in less PA (21, 32–34). However, evidence about the association of chronic disease multimorbidity with LTPA has been relatively scarce. Considering that LTPA is largely modifiable, our study focused on LTPA and observed people with multimorbidity tend to engage in more LTPA, which could provide important evidence for chronic disease multimorbidity control. Notably, our finding is inconsistent with those of the studies aforementioned (21, 32–34). The most likely reason could be that our outcome variable is not the same as theirs, and we only focused on one domain of PA. As shown in **Supplementary Table 3**, we found that every multimorbidity was positively associated with low PA when considering PA related to occupation, household, transport, and leisure time as the outcome variable. In addition, a person living with multimorbidity may be recommended by clinicians to intentionally engage in more LTPA. Similar to our results, a negative association between chronic diseases and the different domains of PA was found in a study in Austria involving 8,251 participants (35). Our findings proved again that it is necessary to explore the relationships between different domains of PA and multimorbidity.

There are some interesting findings in the association between multimorbidity and low LTPA by the ethnic group. Among minority populations with multimorbidity, digestive system multimorbidity, digestive-metabolic, circulatory-metabolic, or circulatory-digestive system multimorbidity took part in more LTPA. As shown in **Supplementary Table 4**, the ORs for most multimorbidity decreased compared with **Figure 2** among minorities, suggesting that minorities with multimorbidity were likely to engage in adequate LTPA

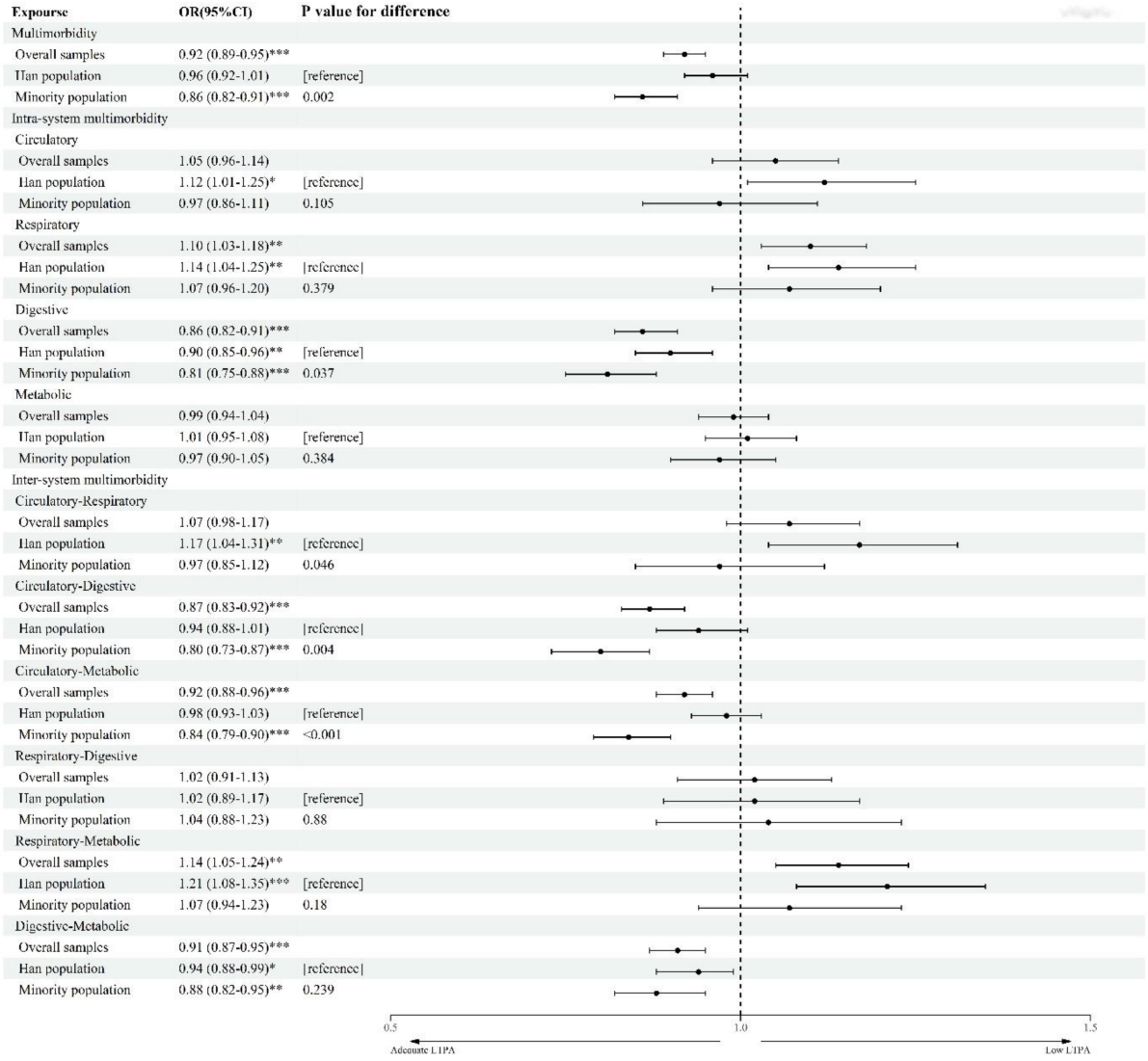

FIGURE 2
ORs for low leisure-time physical activity associated with chronic disease multimorbidity. Low leisure-time physical activity (LTPA) (<7.5 MET-h/week). Each type of multimorbidity was treated as a separate exposure. All models were adjusted for sex, age, marital status, annual family income, educational level, smoking, alcohol drinking, sleep duration, region, and non-LTPA. Multimorbidity refers to the coexistence of two or more chronic diseases. Intrasystem multimorbidity refers to multimorbidity within the same system. Intersystem multimorbidity refers to multimorbidity between two systems. The likelihood ratio test was used to examine the significance. OR, odds ratio; CI, confidence interval. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

when they have learned about their body status. In addition, people might not expect digestive-metabolic system disorders to limit activity (10). Conversely, LTPA can contribute to the therapy of chronic diseases, leading to a higher level of LTPA in the population with chronic diseases (36). For the Han population with respiratory system multimorbidity or circulatory system multimorbidity, their LTPA levels were lower. As shown in **Supplementary Table 4**, the ORs for all types of multimorbidity increased compared with **Figure 2** except for respiratory-metabolic system multimorbidity among the Han population. One reason could be that the disease might be directly responsible for lower activity levels due to a reduced exercise capacity by influencing cardiopulmonary function (37). A UK Biobank study of 52,556 participants reported that people with the worst cardiometabolic diseases performed approximately half of moderate to vigorous PA on a daily basis compared to healthy individuals (38). Alternatively, physical inactivity leads to an increased risk of these diseases, and individuals might have been habitually less active than their healthy

counterparts for some time to develop the condition (10). Participants with lower LTPA had a greater likelihood of developing chronic diseases, such as metabolic syndrome (39). Moreover, a cohort study conducted for 3 years found that the number of patients with metabolic syndrome having sufficient PA did not significantly increase despite advice to increase PA (40). Multimorbidity can contribute to dementia and cognitive decline (41), which could influence LTPA. For digestive system multimorbidity and digestive-metabolic system multimorbidity among the Han population, their associations with LTPA could be attributed to the same reasons aforementioned in the minority population. The association between chronic conditions or multimorbidity and LTPA might differ in ethnicities due to different disease profiles (42), suboptimal treatment of chronic conditions (43, 44), differences in knowledge regarding the benefits of PA (45), or other environmental factors, such as work conditions (46). Han populations with multimorbidity tended to report low LTPA in this study, contradicting our current knowledge that ethnic minorities engage in less healthy behaviors than the Han people. More future studies are warranted to verify such findings.

We observed that intersystem multimorbidity has a possible synergistic effect on LTPA. For example, among the overall sample, the ORs for low LTPA with respiratory system multimorbidity and metabolic system multimorbidity were 1.10 and 0.99, respectively. However, the effect values increased to 1.14 in the association between low LTPA and respiratory-metabolic system multimorbidity. Consistent synergistic effects among circulatory-respiratory system multimorbidity, respiratory-metabolic system, and low LTPA were also observed among the Han population. The effect has some biological plausibility, with the consideration of several pathways. First, shortness of breath caused by chronic respiratory disease might be worse due to the additional influence of chronic circulatory disease. It seems to be difficult for patients to engage in sufficient LTPA owing to breathing problems (47). Second, the connectedness of several chronic diseases might increase symptom severity, especially pain perception, which could in turn explain why patients with chronic respiratory disease and other chronic diseases avoid LTPA (48). Finally, there is evidence that diseases belonging to common patterns of multimorbidity can interact, curtailing compensatory mechanisms and resulting in more severe physical inactivity (37).

As is known to all, limitations in physical and cognitive function due to multimorbidity decisively reduce people's levels of PA, which can in turn increase multimorbidity (19, 20). However, our study found people suffering from multimorbidity may engage in adequate LTPA, which suggested that clinicians should not blindly advise patients with multimorbidity to do physical exercise in their spare time.

Limitations and strengths

Several limitations to this study should be considered. First, eight chronic diseases were self-reported based on the questionnaire, and the results could have been affected by information bias. Second, PA could have been influenced by many factors. Although it was impossible to adjust for all of the potential confounding factors, variables such as green space, the built environment, and cognitive function (41, 49, 50) should have been considered. Third, our research only focused on seventeen chronic diseases with a high prevalence in southwest China and excluded people with special health statuses (e.g., infectious diseases, pregnancy, and injury) that might severely affect PA; other diseases affecting PA were not considered either. Fourth, since this study was a cross-sectional study, caution should be taken in making causal interpretations between LTPA and multimorbidity. Nonetheless, our study has notable strengths. Our findings clarified multimorbidity into intrasystem multimorbidity and intersystem multimorbidity, which helps us comprehensively understand the associations of LTPA with specific multimorbidity subgroups.

Conclusion

This large-scale epidemiologic study provides an improved understanding of the impact of multimorbidity on LTPA in Han and minority populations in southwest China. Our findings suggest that LTPA-tailored interventions should be designed for specific ethnic groups according to a different type of multimorbidity. Future large, prospective studies are required to further determine the temporality of the associations observed in this investigation and better explain whether changes in the nature of the dependent variable produce different results.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Sichuan University (K2016038). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YL, QN, and JZ: conceptualization. JL, RH, and QN: data curation. YL, XL, and BY: formal analysis and writing—original draft. YL, QN, JL, and RH: investigation. YL, BY, XL, and JZ: methodology. QN: project administration. JZ, YL, XL, and BY: writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.874456/full#supplementary-material

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Prevalence and associated risk factors of chronic kidney disease: A case study within SIME clinics in Quito, Ecuador 2019–2021

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Background: Ecuador has been experiencing an epidemiological transition due to its demographic and lifestyle changes, where non-communicable diseases are the leading cause of death, including chronic kidney disease (CKD). Quito, Ecuador's capital city, is one of the cities burdened by CKD, yet it is unknown the factors that contribute to the rising incidence of this disease. The purpose of this study was to estimate the prevalence of CKD among non-institutionalized adults in Quito between 2019 and 2021, and to examine its associations with various risk factors.

Methods: For the analysis of prevalence, the *Kidney Disease: Improving Global Outcomes* guidelines were used, where an estimated glomerular filtration rate (eGFR) of < 60 ml/min/1.73 m^2 was counted as a presumed case of CKD. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used to calculate eGFR. Multiple linear regression models were used to determined associations between blood pressure, blood glucose, sex, and zone with eGFR. A *t*-test of independence was used to determine difference in means between sex and zone and eGFR.

Results: A prevalence of 7.2% was found, in which almost 45% of the participants were classified within stages 2–4 of this disease. The risk factors that were significantly associated with eGFR were systolic blood pressure ($\beta = -0.43$, $p < 0.001$), sex, and zone ($p < 0.001$).

Conclusions: Overall a high prevalence of CKD was found among adults who visited SIME clinics in Quito. Associations between main risk factors and eGFR were found, yet further research is needed to explore CKD in Ecuador and its main cities.

KEYWORDS

chronic kidney disease, prevalence, blood pressure, blood glucose, eGFR

Introduction

Chronic kidney disease (CKD) is a major public health concern with its rising prevalence and higher mortality rates (1). Globally, the prevalence of CKD is 9.1% or about 700 million people, in which females have a higher prevalence (9.5%) compared to males (7.8%), and a mortality rate of 4.6% (2). CKD has five different stages based on the estimated Glomerular Filtration Rate (eGFR), where CKD is presumed with an eGFR of <60 ml/min/1.73 m^2 (3). eGFR is considered the best overall indicator of kidney function (4) and is determined through the utilization of serum creatinine and characteristics of the patient such as sex, race and age (5). The Modification of Diet in Renal Disease (MDRD) and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) are used globally to calculate the eGFR. A meta-analysis compared eGFR from adults with CKD based on the CKD-EPI equation to the MDRD equation. Results showed that the CKD-EPI equation had less bias when classifying different populations, greater precision and accuracy considering the demographic profiles of participants (e.g., age, sex, race), in comparison to the MDRD equation (6). Furthermore, Levey et.al discovered that the MDRD equation led to higher prevalence estimates due to its imprecision when detecting higher values of GFR (4). Meanwhile, the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines state that in the absence of specific modifications such as race, ethnicity, or regional differences, it is acceptable to use the CKD-EPI equation for determining eGFR (3).

The progression of this disease can be attributed to different risk factors such as elevated blood glucose and high blood pressure (2). Studies demonstrated that blood pressure levels below 130/90 mmHg reduces the risk for CKD, cardiovascular disease, and others whereas above 130/90 mmHg increases that risk (7–10). Nonetheless, it is worth noting that the systolic and diastolic blood pressure, may be equally and individually important to predict the risk for CKD. A few studies have shown that systolic blood pressure (SBP) is highly associated with adverse kidney outcomes, but not diastolic blood pressure (DBP) (11, 12). Moreover, elevated blood glucose levels (i.e., hyperglycemia) have been known to disrupt kidney's function and lead to impairment of glucose homeostasis (13). When hyperglycemia is present, there are several consequences that progressively damage the structure of the kidney, such as a mesangial expansion of the matrix and thickening of the glomerular basement membrane, which are known to cause an increase of the systemic pressure and elevated excretion of protein in urine (i.e., proteinuria), resulting in a reduction of glomerular filtration (14). Furthermore, when diabetic nephropathy is present, hemodynamic modifications occurs, which changes normal renal blood flow and causes the kidney to increase glomerular filtration, triggering not only an alteration of kidney's functions but also affecting the body homeostasis overall (14).

CKD is greatly affecting Latin American countries, especially Ecuador (15) in which CKD has increased by more than 50% of the disability-adjusted life years (DALYs) rate from 1990 to 2017 (2). Few studies, though, have focused on risk factors and prevalence of certain regions within Ecuador. One study performed in Cuenca, Ecuador, revealed the prevalence of CKD was at 10.6% with the highest prevalence observed in individuals younger than 65 years of age, and a higher prevalence of CKD detected in urban zones (16). Even though this is one of the few prevalence studies performed in Ecuador, the results are based on the MDRD equation, thus caution must be considered when comparing to similar studies using the CKD-EPI equation.

One specific region in Ecuador where CKD is the leading cause of mortality is Quito. Quito, the capital of Ecuador, is located within the province Pichincha. Compared to other cities in Pichincha, it is a densely populated area with 2.6 million people, who are predominately female (60.5%), are between the ages of 20–39 years (34%) and considered Mestizos (American India and White, 82.8%) (17). Even though mortality rates from CKD are high in this region (18), limited information is available with the prevalence and risk factors associated with this disease in Quito. Therefore, this study aimed to explore CKD prevalence and the impact that different risk factors such as blood glucose and blood pressure, and demographics have on estimated glomerular filtration rate (eGFR) among adults residing in Quito.

Materials and methods

Study design

A retrospective cross-sectional case study was conducted among non-institutionalized adults who visited Sistemas Médicos (SIME) clinics in Quito, for routine physical exams during the years of 2019–2021. Sistemas Médicos (SIME), is a group of clinics that offers primary health care attention. SIME clinics are found within the main parishes of Quito. Two of these clinics are in rural zones: Carapungo-Calderón and Los Chillos, while the other two are in urban zones: Cumbayá and La Carolina. SIME clinics have a commitment to the community to reduce and prevent the prevalence of non-communicable diseases through research and medical advancements (19). This study was approved by the Ethics and Research Committee of Human subjects of CEISH-USFQ (IE02-E158-2021-CEISH-USFQ) and by the University of Florida Institute of Review Board (IRB202101202) as exempt.

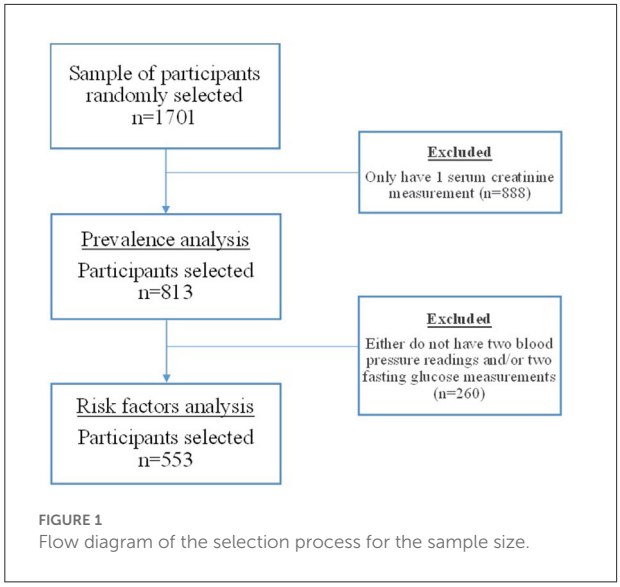

FIGURE 1
Flow diagram of the selection process for the sample size.

Study population

For this study, the inclusion criteria comprised: (1) adults over 18 years of age, (2) demographic data (sex and age), (3) at least two readings of blood pressure, (4) at least two measures of blood glucose, and (5) at least two measures of serum creatinine. The criteria followed World Health Organization (WHO) (20, 21), and KDIGO guidelines, for detection of presumed hypertension, diabetes and CKD cases, respectively, in which the blood pressure readings, blood glucose and serum creatinine measurements were mean averaged for analysis. These readings and measurements may have occurred at different time points for each participant (e.g., collection of all markers within the same year or every other year). Participants were excluded if they did not meet the above criteria and/or if they were pregnant/lactating women. The de-identified data was sent to one of the researchers (L.E.-J.), who collected and organized the information in a matrix previously created by the researchers (L.E.-J., J.M.A). The dataset included 17,000 participants from which 1,701 participants (10%) were randomly selected following the methodology of another population-based study (22). The primary outcome in this study was eGFR. Based on the sample of 1,701 participants, 813 met the study inclusion criteria to analyze CKD prevalence, while 553 met the criteria for all the variables of interest. Assuming the current prevalence of 11.3% among the entire Ecuadorian population (2) and assuming an incidence of 5% within the study group, this would be a 95% power with a precision of 6.3–16.3% to detect incidence of CKD. See Figure 1 for a flow diagram of the selection process for the sample size of this study.

CKD-EPI equation

The eGFR was determined based on the updated 2022 CKD-EPI equation to determine CKD prevalence. The CKD-EPI equation can be seen below:

$$eGFR = 142 * \left(minstandardized\frac{Scr}{K}, 1\right)^{\alpha} * \left(maxstandardized\frac{Scr}{K}, 1\right)^{-1.200} * 0.9938^{Age} * 1.012 \left[if\ female\right]$$

where Scr is serum creatinine, K is 0.7 for females and 0.9 for males, α is−0.241 for females and - 0.302 for males, min indicates the minimum of Scr/K or 1, and max indicates the maximum of Scr/K or 1.

Statistical analysis

Statistical analyses were carried out using the statistical program R version 4.1.2 (23, 24). Descriptive analyses were used to represent the distribution of the sample by different variables such as sex, rural or urban zones, while age, blood glucose, and blood pressure, and serum creatinine were presented as mean ± standard deviation (SD). To identify the prevalence of CKD, a dichotomous eGFR variable (<60 vs. ≥60 ml/min/1.73 m^2) was used to determine the presence of CKD according to the definition of KDIGO guidelines (3) where an eGFR < 60 ml/min/1.73 m^2 was counted as a CKD case. Additionally, based on the eGFR, participants were categorized into the five different stages of CKD to determine frequencies. To examine the impact of blood glucose and blood pressure on eGFR values, a multiple linear regression model was used. A t-test was used to determine differences between sex and zone on eGFR. Statistical significance was determined at $p < 0.05$.

Results

Characteristics of the samples

For the analysis of CKD prevalence, the majority of participants were females (61.1%), all identified as Mestizo race, had an average age range between 18 and 44 years, and more participants visited urban zone clinics (85.6%) compared to those who visited the clinics located at rural zones (14.4%). Similarly, for the analysis of CKD risk factors, most of the participants were females (59.5%), the majority were less than 65 years of age, and more participants visited urban zones clinics (84.4%). Furthermore, 11.9% of participants had a mean systolic blood pressure above 140 mmHg, and 4.2% had mean values higher than 90 mmHg in diastolic blood pressure. The percentage of participants that presented mean values of fasting

TABLE 1 Demographic characteristics.

Group	N	Percentage (%)
Sex ($n = 813$*)		
Females	497	61.1
Males	316	38.9
Zone ($n = 813$)		
Urbana	696	85.6
Rural	117	14.4
Age ($n = 813$)		
18–44	334	41.1
45–64	287	35.3
> 64	192	23.6
CKD ($n = 813$)		
eGFR < 60 ml/min/1.73m^2	58	7.2
eGFR ≥ 60 ml/min/1.73m^2	755	92.8
Systolic blood pressure ($n = 553$**)		
<140 mmHg	487	88.1
≥140 mmHg	66	11.9
Diastolic blood pressure ($n = 553$**)		
<90 mmHg	530	95.8
≥90 mmHg	23	4.2
Blood Glucose ($n = 553$**)		
<126 mg/dl	532	96.2
≥126 mg/dl	21	3.8

*Participants that met inclusion criteria for: age, sex, zone, and 2 serum creatinine measurements.
**Participants that met inclusion criteria for: age, sex, zone, 2 serum creatinine, 2 blood pressure and 2 blood glucose measurements.

TABLE 2 Prevalence of CKD by stage.

Stage (eGFR ml/min/1.73m^2)	CKD-EPI (N)	Prevalence Percentage (CKD-EPI) [%]
Stage 1 (≥90)	455	55.9
Stage 2 (60–89.9)	300	36.9
Stage 3 (30–59.9)	56	6.9
Stage 4 (15–29.9)	2	0.3
Stage 5 (<15)	0	0

blood glucose above 126 mg/dl, was 3.8%, while presumed cases of CKD (<60 ml/min/1.73 m2) accounted for 7.2% of the sample (Table 1).

CKD prevalence

The prevalence of CKD was 7.2% and almost 45% of the participants were classified within stages 2–4 (Table 2). Furthermore, the prevalence of CKD among those who visited an urban zone clinic compared to a rural zone clinic was determined at 6.62 and 1.02%, respectively. Prevalence of CKD among males was at 7.9% and among females was at 6.6%. Chi-square analysis revealed no significant correlations between rural and urban zones and eGFR, X^2, $(1, N = 813) = 1.49$, $p = 0.221$; and between sex and eGFR, X^2, $(1, N = 813) = 0.30$, $p = 0.584$.

Risk factors associated with eGFR

A multiple linear regression analysis showed that systolic blood pressure ($\beta = -0.43$, $p < 0.001$) and blood glucose ($\beta = -0.09$, $p = 0.024$) was negatively associated with eGFR (model 1) (Table 3). A second multiple regression model adding sex and zone as covariates showed that there was a negative association between sex ($\beta = -3.87$, $p = 0.027$), zone ($\beta = -11.89$, $p < 0.001$) and eGFR. However, the blood glucose variable was not significant in model 2 ($\beta = -0.06511$, $p = 0.107$) (Table 4). Furthermore by performing a t-test for independence, it was found that males had a lower eGFR than females, $t_{(731.2)} = 4.71$, $p < 0.001$, and adults who had visited rural zone clinics had lower eGFR compared to those who visited urban zone clinics, $t_{(174.1)} = -6.56$, $p < 0.001$.

Discussion

In this study, a prevalence of 7.2% was found among adults who visited SIME clinics. The majority of participants were classified within stages 1 and 2 of this disease following the KDIGO guideline criteria. The sample was best characterized as participants below 65 years of age, most of them women, and who visited more urban zone clinics than rural zone clinics. The risk factors that were significantly associated to eGFR, based on multiple regressions, were systolic blood pressure, blood glucose, sex, and zone.

CKD is a progressive disease in which detection can be a challenge since symptoms do not start until later stages of this disease, 3–5 (25), hence obtaining accurate information about the prevalence of this disease is fundamental to reduce the rates. In 2017, the Global, regional, and national burden of chronic kidney disease study showed that Ecuador's prevalence was 11.3% (2), which differs from this study where the prevalence was 7.2%. This report indicated to have limitations, one of them, was the use of predictive statistics to obtain prevalence estimates for the countries that lacked information about the incidence and prevalence of CKD, like Ecuador (2). Furthermore, classification of the eGFR through the different stages of CKD has been also considered an important barrier to determine accurate prevalence in different countries, especially considering the use of different equations such as CKD-EPI and

TABLE 3 Factors associated with eGFR (Model 1).

Coefficient	Estimate (95% CI)	SE	t. value	Pr(>|t|)
(Intercept)	145.51 (129.853, 161.161)	7.97	18.26	<0.01**
Systolic blood pressure	−0.426 (-0.584,−0.267)	0.08	−5.27	<0.01**
Diastolic blood pressure	0.082 (-0.171, 0.335)	0.13	0.64	0.53
Blood Glucose	−0.092 (-0.173,−0.012)	0.04	−2.25	0.03

Multiple linear regression (Model 1): R2 = 0.09, R2 adjusted = 0.09, $p < 0.001$; **p-value < 0.01.

TABLE 4 Factors associated with eGFR (Model 2).

Coefficient	Estimate (95% CI)	SE	t.value	Pr(>|t|)
(Intercept)	156.41 (140.68, 172.14)	8.01	19.53	<0.01**
Systolic blood pressure	−0.43 (-0.58,−0.27)	0.08	−5.43	<0.01**
Diastolic blood pressure	0.16 (-0.09, 0.42)	0.13	1.24	0.22
Blood Glucose	−0.07 (-0.14, 0.01)	−0.04	−1.61	0.11
Sex	−3.87 (-7.30,−0.44)	1.75	−2.22	0.03*
Zone	−11.89 (-16.29,−7.49)	2.24	−5.30	<0.01*

Multiple linear regression (Model 2): R2 = 0.14, R2 adjusted = 0.14, $p < 0.001$; **p-value < 0.01; *p-value < 0.05.

MDRD to calculate eGFR (1). In congruence with this study, another study (26) showed that the prevalence of CKD stages 3-5 in low to middle income countries ranged from 7.4 to 13.1% with a median of 10.7%. Furthermore, a study conducted in Nicaragua among 1,242 participants showed that the prevalence of CKD was 5.3% and was most prevalent in those who were older, self-reported diagnosis of hypertension or diabetes (27). In a similar study done in another major city of Ecuador (16), the percentage of prevalence was 10.6%, using the MDRD equation, thus to truly compare prevalence and identify the severity of CKD, it is necessary for further studies to use one equation.

While detecting CKD is fundamental, understanding its main risk factors is equally important due to their impact on the progression of the disease. Hypertension is one of the leading causes of CKD, and in developing countries, it is attributed to be the cause of 21% of CKD cases (28). Yet, in Ecuador, and its main cities, accurate information elucidating the association between these chronic diseases is scarce. In this study, blood pressure components [Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP)] were analyzed individually, and the results revealed that SBP was negatively associated with eGFR, while DBP showed no significance. Similar to these results, the main findings of an observational retrospective study that included 1,323 individuals, indicated that time updated SBP was associated with CKD progression. Time-updated in that study referred to an average SBP at different time points. Furthermore, that study revealed that hazard ratios were higher among adults with SBP between 130 and 139 mmHg and above 140 mmHg (HR: 1.48 and 2.53 respectively), compared to the reference group (e.g., < 120 mmHg). Indicating that the higher SBP contributed to CKD. Meanwhile, DBP did not show a significant association with CKD progression (29). A possible explanation for this is the stiffness of arteries caused by high blood pressure, in which there is a return of blood pressure flowing back to the heart that increases the pressure of the systole compared to the diastole (30). Moreover, high blood pressure, in general, will cause a progressive loss of kidney function by injuring the afferent arteriole of the kidney, in which its ability to constrict and dilate is compromised, thus affecting the kidney's ability to remove waste and reduce GFR (31).

Although blood glucose was not significant after the addition of sex and zone as covariates in the second regression model, there remained a negative trend, in which a higher blood glucose will lower eGFR levels. Moreover, blood glucose is one of the main contributors of CKD and the prevalence of diabetes is on the rise in the Ecuadorian population (32) and one of the main causes of mortality in Quito (18). In the initial regression model, blood glucose had a statistically significant negative association with eGFR. Similar results were found in a descriptive study of 40 diabetic patients, in which they observed a decrease in GFR when glucose levels were reduced. In that study GFR was calculated through the creatinine clearance formula, which used creatinine from blood and urine samples. Through this equation, a normal range of GFR is between 80 and 130 ml/min, thus a decrease in GFR was considered a positive result. Furthermore, that study also demonstrated a reduction in hyperfiltration when glucose levels were reduced, which indicates a possible improvement of kidneys' function (33). These results could be explained by the kidneys' function to re-absorb glucose when needed, thus high

glucose levels will enhance the kidney's absorption and filtration, causing microvascular complications, in which the kidney starts filtrating glucose and/or other large molecules, thus contributing to kidney disease (34).

In addition to the findings with blood glucose and SBP, sex and zone also were associated with eGFR. These findings are comparable to another population-based CKD prevalence study that was completed in Nicaragua, where males had higher odds (POR = 3.47) of CKD compared to females and those living in a rural zone was associated with an increased odds (POR = 2.10) of CKD. Even though this Nicaragua study did not compare risk factors with eGFR values directly, but with presumed cases of CKD, they followed the criteria for counting a CKD case at an eGFR < 60 ml/min/1.73 m^3, which means that zone and sex were associated with decreased eGFR values (22). Another study conducted among agricultural workers in El Salvador, obtained similar results, in which two rural communities had decreased eGFR (<60 ml/min/1.73 m^3) compared to urban communities. Furthermore, males had a significantly higher prevalence of decreased eGFR than females. The authors suggested that a decreased eGFR in males was due to their occupation. Regarding the difference between rural and urban zones, the authors explained that the studied communities were exposed to different climates and altitudes. In those residing in warmer climates, there may have been slight dehydration, contributing to the reduction in eGFR (35). In this study, even though the climates and altitudes were similar between rural and urban areas, individuals residing in the rural areas had lower mean eGFR compared to those residing in the urban areas. This difference might be explained by reduced access to health care centers, lack of nephrologists, and dialysis centers located in rural areas compared to urban zones (36). Concerning a lower mean eGFR in males compared to females, a probable justification might be that in Ecuador the majority of registered works are classified within the group of agriculture, livestock, forestry and fishing (37), where all of them are consider hard labor work, which might be related to dehydration issues and could possibly lead to decrease of kidney function as well.

Limitations and strengths

To the best of the authors' knowledge, this is the first retrospective study that explored CKD prevalence and the associations between different risk factors and eGFR among adults who visited different clinic centers in Quito. Moreover, the aims of this study aligned with different goals set by a group of researchers as an action plan for determining CKD, which provided specific activities that can improve CKD monitoring and detection, highlighting the importance of surveillance systems within a country (1). However, this study does have limitations. First, more than half of the participants had to be excluded due to lacking at least two measurements of serum creatinine to calculate eGFR values. This indicates that serum creatinine is not part of basic blood work, even though the prevalence of CKD was high. Also, the lack of other laboratory markers that could help in the identification of proteinuria, is an important limitation. Second, for the analysis of risk factors, more participants were excluded due to lacking either blood glucose measurements and/or blood pressure readings, which could impact the results, especially considering the change in significance of blood glucose on eGFR. Also, the procedures used for the collection of serum creatinine, blood pressure and blood glucose, is unknown to the authors. Furthermore, no time series or method to assess progression of CKD prevalence in the population could be conducted as the time for collection of these measurements varied. Third, results cannot be comparable to other studies performed in Ecuador or Ecuador's main cities that can further contribute to elucidate the associations found in this study.

Conclusions

Overall, the main findings of this study showed a high prevalence (7.2%) of CKD among adults who visited SIME clinics, which is comparable to the worldwide prevalence (9.1%) of CKD. Moreover, systolic blood pressure, sex and zone are the risk factors found to be associated with eGFR, yet further research needs to elucidate these associations and other risk factors that might be contributors of CKD in Ecuador. Although this study had its limitations, still can be considered as an incentive for Ecuador's main authorities to execute high quality population-based studies that accurately describe CKD in Ecuador and its provinces.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Due to HIPPA compliance records are not available to the public. Requests to access these datasets should be directed to leguiguren@ufl.edu.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics and Research Committee of Human subjects of Universidad San Francisco de Quito CEISH-USFQ (IE02-E158-2021-CEISH-USFQ) and by the University of Florida Institute of Review Board (IRB202101202). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

LE-J and JA: conceptualization, methodology, and writing—original draft preparation. LE-J and JM: data analysis, formal analysis, and data curation. LE-J, JM, JO, and JA: writing—review and editing. JA: supervision and project administration. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Effect of electroacupuncture on cyclic adenosine monophosphate-protein kinase A-vanillic acid receptor subtype 1 of the transient receptor potential/PLK-protein kinase C-vanillic acid receptor subtype 1 of the transient receptor potential pathway based on RNA-seq analysis in prostate tissue in rats with chronic prostatitis/chronic pelvic pain syndrome

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Objective: To investigate the analgesic mechanism of electroacupuncture (EA) in rats with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS).

Methods: Thirty male SD rats were randomly divided into sham group, model group and EA group, with ten rats in each group. The CP/CPPS model was prepared by injecting 50 μL of complete Freund's adjuvant (CFA) into the ventral lobes of the prostate tissue, and the sham group was injected with the same dose of saline. After 14 days of modeling, EA was applied to Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) in the EA group. After four courses, H&E staining was performed to observe the prostate tissue

morphology, transcriptome sequencing (RNA-Seq) was performed for each group, and the selected signaling pathways were verified by qRT-PCR.

Results: The RNA-Seq analysis results suggested that the analgesic effect of EA on CP/CPPS may be achieved by regulating prostate gene expression, which may be related to multiple biological processes and signaling pathways. qRT-PCR results showed that the vanillic acid receptor subtype 1 of the transient receptor potential (TRPV1), phospholipase C (PLC), protein kinase C (PKC), cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA) were all upregulated in the model group compared to the sham group ($p < 0.01$). Compared with the model group, TRPV1, PLC, PKC, cAMP, and PKA were all downregulated in the EA group ($p < 0.05$, $p < 0.01$).

Conclusion: The analgesic mechanism of EA on CP/CPPS may be achieved through modulation of cAMP-PKA-TRPV1/PLC-PKC-TRPV1 signaling pathway.

KEYWORDS

chronic prostatitis/chronic pelvic pain syndrome, electroacupuncture, RNA-seq technology, analgesic mechanism, animal experiment

Introduction

Prostatitis is one of the most common urinary diseases in adult men. Based on the National Institutes of Health Prostatitis Collaborative Network classification, chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is referred to as category III prostatitis, which is the most common chronic prostatitis, accounting for about 90∼95% of prostatitis (Chen et al., 2021). Its main characteristics are persistent pelvic pain, urinary tract irritation and sexual dysfunction. Among them, pain is the main symptom of most CP/CPPS patients. Long-term and repeated symptom of pain will bring negative psychological effects, substantial health care costs and seriously affect their daily life (McNaughton and Pontari, 2001).

At present, the clinical treatments for CP/CPPS mainly include alpha-blockers, antibiotics and non-steroidal anti-inflammatory drugs (Cohen et al., 2012). However, these drugs' treatment has obvious negative effects, such as dizziness, nausea, and postural hypotension and gastrointestinal side effects, which reduce patients' compliance with treatments (Nickel et al., 2013). Despite recent advances in novel treatment schemes development, its clinical effectiveness needs further appraisal (Engeler et al., 2013). Previous studies indicated that EA may be a potential therapy for CP/CPPS (Qin et al., 2016a,b).

EA, a special modern type of acupuncture, originates from the combination of traditional acupuncture and modern electrical stimulation (Cao et al., 2021). With the advantages of inexpensive, safe and fewer side effects, in recent years, EA has been widely used in the prevention and rehabilitation of CP/CPPS (Franco et al., 2018). A multicenter, randomized, sham-controlled trial showed that EA can alleviate the symptoms of pain, micturition dysfunction, anxiety and depression in patients with CP/CPPS, and its efficacy may last for 24 weeks after treatment (Sun et al., 2021). While the efficacy of EA on CP/CPPS has been proven in clinical practice, little is studied and elucidated, however, about the analgesic mechanism. Most animal experiments related to the mechanism of EA on CP/CPPS are without in-depth and systematic exploration of possible signal pathways and targets (Wu et al., 2021).

Transcriptome sequencing (RNA-Seq) is a novel method for gene expression profiling by next-generation sequencing of transcripts (Shi et al., 2018). With the advantages of high sensitivity, high throughput and low cost, it has been widely used to explain disease gene expression signatures. For example, Zhao (2018) used RNA-Seq technology to preliminarily explore the possible mechanism of CP/CPPS. However, studies related to the analgesic mechanism of EA on CP/CPPS using RNA-Seq technology have been not reported. In this study, we tried to comprehensively elucidate the potential molecular mechanisms underlying the analgesic effects of EA on CP/CPPS rats using

Abbreviations: CP/CPPS, chronic prostatitis/chronic pelvic pain syndrome; EA, electroacupuncture; RNA-Seq, transcriptome sequencing; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2;β-EP, β-endorphin; DEG, differential expression gene; CFA, complete Freund's adjuvant; CV4, "Guanyuan"; CV3, "Zhongji"; SP6, "Sanyinjiao"; BL35, "Huiyang"; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cell composition; MF, molecular function; TRP, transient receptor potential ion channel protein; TRPV1, vanillic acid receptor subtype 1 of the transient receptor potential; PLC, phospholipase C; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; SD, Sprague–Dawley.

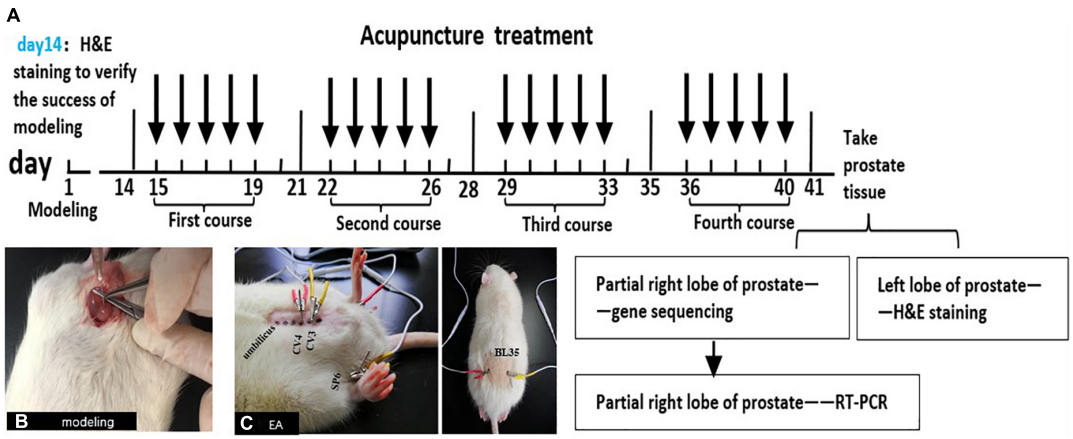

FIGURE 1
The schematic diagram of this experiment. **(A)** The schematic diagram for methodologies. Male SD rats were injected with CFA into the ventral lobes of the prostate on both sides on the first day, and H&E staining was used to judge whether the model was successful or not on the 14th day. All groups were treated on the 15th day after modeling. Treatment once a day for 40 min, 5 days as a course of treatment, with 2 days of rest between the two courses, a total of 4 courses. At the end of the last course of treatment, the rats were anesthetized and sacrificed on the next day and take the prostates. The left lobe of the prostate was stained with H&E, and partial right lobes were analyzed with RNA-Seq technology. Then, combined with the results of RNA-Seq and modern research progress on the pain-related mechanism of CP/CPPS, the DEG and pain-related signal pathways were screened out. Finally, the key genes in the pathways were verified by qRT-PCR. **(B)** Injecting CFA into the prostate of rats to make CP/CPPS model. **(C)** Acupoint location and EA process in CP/CPPS rats. CV3(zhongji), CV4(guanyuan), SP6(sanyinjiao), and BL35(huiyang).

RNA-Seq technology and provide a scientific basis in clinics (Figure 1).

Results

The results of H&E staining of the prostate tissue

In the sham group, the cavity was filled with evenly distributed pink secretion, without inflammatory cell infiltration in the glandular cavity and stroma. In the model group, the prostatic epithelium was papillary hyperplasia, with a narrow glandular cavity, and the pink secretion in the cavity was reduced, with a large number of inflammatory cells in the glandular cavity and stroma. In the EA group, the pathological changes were significantly improved, with the basically complete structure of the glandular cavity, and the inflammatory cell infiltration was rare in the glandular cavity and stroma, with more pink secretions in the cavity (Figure 2).

Bioinformatics analysis of rat prostate tissue

Analysis results of quality control

To ensure the quality of the original sequencing data, it is necessary to evaluate the quality of the original data before analysis. The results showed that 670 million reads were obtained, with an average of about 44.49 million reads per sample; Q20 of all samples ranged from 98.3 to 98.63%, Q30 ranged from 95.13 to 95.71%, and 92.78–96.26% of all samples could map to the reference genome, indicating that the sequencing results were reliable (Table 1).

Results of correlation heat map

Through the correlation heat map, it could be seen that the correlation coefficient between samples in each group was high, which revealed the high similarities in expression patterns between samples in each group, and further indicated good experiment repeatability, as well as a reasonable selection of samples in each group. The correlation coefficient between the model group and the sham group was relatively low, which revealed that there were some differences in the expression pattern between the model group and the sham group. After EA intervention, compared with the correlation coefficient between the model group and the sham group, the correlation coefficient between the EA group and the sham group was relatively high, which indicated that after EA intervention, the expression pattern of rats in the EA group tended to that of rats in the sham group (Figure 3).

Analysis results of differential expression gene

The differential expression gene (DEG) in the prostate tissue was analyzed after four courses of EA intervention. The two-dimensional hierarchical cluster heat map showed that

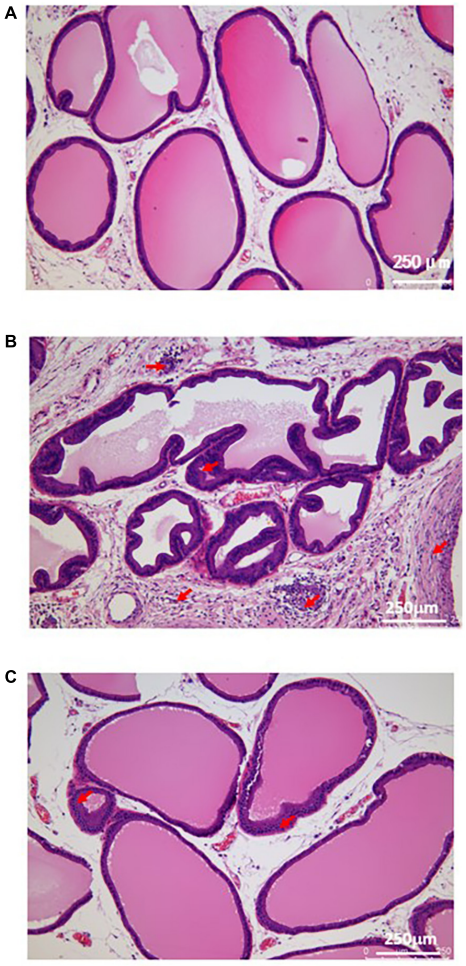

FIGURE 2
Histological morphology of prostatic tissue in each group. **(A)** The result of H&E staining in sham group; **(B)** The result of H&E staining in model group; **(C)** The result of H&E staining in EA group. Red arrows show the infiltration of inflammatory cells.

samples expression in each group was relatively consistent. Compared with the EA group and sham group, the color of the model group was significantly different, suggesting that the degree of gene expression separation in the model group was significantly different from that in EA group and sham group. The color of the EA group was close to that of sham group, suggesting that the gene expression pattern of EA group was close to that of sham group (Figure 4). The volcano plot showed that there were 1994 intersecting DEGs between model group and sham group ($p < 0.05$, Pmodel/sham), of which 1,794 were upregulated and 200 were downregulated; these DEGs may be related to the modeling of rats. There were 499 intersecting DEGs between EA group and model group ($p < 0.05$, PEA/model), of which 262 were upregulated and 237 were downregulated (Figure 5). The intersection DEG was further analyzed. The result showed that there were 147 intersecting DEGs between Pmodel/sham and Pmodel/EA ($p < 0.05$, Pmodel/sham∩PEA/model), of which 103 intersecting DEG between the upregulated genes in Pmodel/sham and the upregulated genes in Pmodel/EA and 37 intersecting DEG between the downregulated genes in Pmodel/sham and the downregulated genes in Pmodel/EA suggested that EA could reverse the up-/downregulation of these genes in CP/CPPS model rats (Figure 6). These results suggested that the analgesic effect of EA on CP/CPPS rats may be associated with gene expression in prostate tissue.

Analysis results of Gene Ontology and Kyoto Encyclopedia of Genes And Genomes

Analysis results of Gene Ontology function

Gene ontology (GO) function mainly includes three parts: biological process (BP), cell composition (CC), and molecular function (MF). To illustrate the DEG function of rats after modeling, the GO function of Pmodel/sham was analyzed. The results showed that the BP mainly included leukocyte activation regulation, T cell activation regulation, lymphocyte activation regulation, adaptive immune response, leukocyte differentiation, monocyte proliferation, inflammatory response, etc. The CC mainly included the outer side of the plasma membrane, the side of the mold, synapse, plasma membrane receptor complex, the inflammatory body complex, lysosome, etc. The MF mainly included chemokine activity, chemokine receptor binding, cytokine activity, cytokine receptor binding, G protein-coupled receptor binding, actin binding, etc. (Figure 7A).

To illustrate the DEG function of CP/CPPS rats after EA intervention, the GO function of PEA/model was carried out. The results showed that BP mainly included myeloid leukocyte activation, cell response to biological stimulation, cell response to lipopolysaccharide, response to bacterial-derived molecules, pain response, regulation of body fluid level, regulation of cytokine secretion, cell respiration, etc. The CC mainly included outer plasma membrane, motor cartilage membrane, organelle membrane, mitochondrial protein inner membrane complex, mitochondrial respiratory chain complex, respiratory enzyme complex, respiratory chain complex, etc. The MF mainly included cytokine receptor activity, cytokine binding, chemokine activity, oxidoreductase activity, G protein-coupled receptor binding, dehydrogenase activity, etc. (Figure 7B).

What was worth noting was that Pmodel/sham and PEA/model have similar intersecting GO functions, of which the BP mainly included immune response and inflammatory response; the CC mainly included plasma membrane, organelle membrane and other membrane surfaces; the MF is mainly involved in the functions of inflammation and immunity, such as cytokine activity and chemokine activity. The results suggested that the analgesic mechanism of EA on CP/CPPS may

TABLE 1 Quality control and sequencing information for samples.

NO	Sample	Raw reads	Clean reads	Q20 (%)	Q30 (%)	Total mapped
1	Sham1	41,225,988	40,457,396	98.63	95.71	38,413,513 (94.95%)
2	Sham2	42,578,460	40,854,460	98.37	95.23	39,026,425 (95.53%)
3	Sham3	44,344,402	42,449,774	98.33	95.14	40,252,894 (94.82%)
4	Sham4	45,633,734	43,329,820	98.37	95.15	41,165,825 (95.01%)
5	Sham5	44,166,684	41,622,168	98.32	95.19	39,459,030 (94.8%)
6	Mode1	45,693,778	43,839,792	98.3	95.13	41,732,871 (95.19%)
7	Mode2	45,900,210	43,959,880	98.42	95.4	41,801,767 (95.09%)
8	Mode3	47,510,362	45,683,786	98.49	95.46	43,685,288 (95.63%)
9	Mode4	45,538,110	44,545,936	98.41	95.37	42,239,898 (94.82%)
10	Mode5	46,465,170	44,539,906	98.58	95.68	42,775,386 (96.04%)
11	EA1	42,013,976	40,135,674	98.37	95.26	38,633,546 (96.26%)
12	EA2	42,207,288	40,110,152	98.43	95.39	38,229,295 (95.31%)
13	EA3	45,244,586	42,868,484	98.5	95.4	40,910,076 (95.43%)
14	EA4	42,968,022	41,204,250	98.37	95.17	39,316,288 (95.42%)
15	EA5	45,988,586	43,599,800	98.42	95.28	40,449,826 (92.78%)

(1) Sample, sample name; 15 cDNA libraries are sham group (sham1, 2, 3, 4, and 5), model group (model1, 2, 3, 4, and 5), EA group (EA1, 2, 3, 4, and 5); **(2)** raw reads, counting the number of original sequence data; **(3)** clean reads, counting the number of sequencing data after filtering; **(4)** Q20, Q30, counting Phred values, respectively. **(5)** Total mapped, the number of clean reads that can be located on the genome.

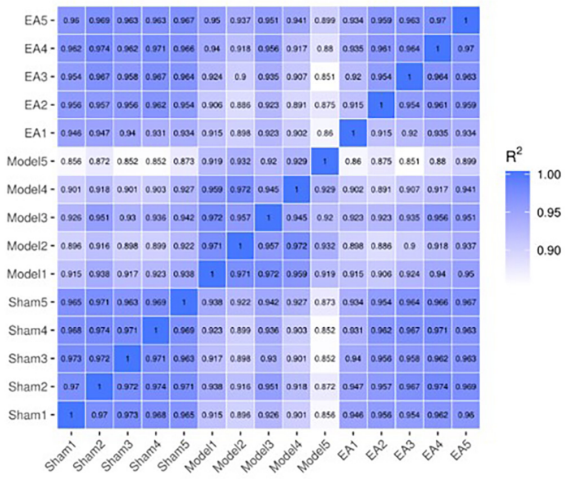

FIGURE 3
Correlation heat map between samples. The correlation heat map between samples is often used to evaluate the sample difference between groups and the sample repetition within groups. The *X*-axis and *Y*-axis in the above figure are sham group (sham1, 2, 3, 4, and 5), model group (model1, 2, 3, 4, and 5) and EA group (EA1, 2, 3, 4, and 5). The values between the *X*-axis and *Y*-axis are the square of the correlation coefficient between the corresponding samples. The darker the color indicates the higher the correlation coefficient between samples, and the lighter the color indicates the lower the correlation coefficient between samples.

be related to the process and function of immune inflammation and the composition of the membrane (Table 2).

Analysis results of Kyoto Encyclopedia of Genes and Genomes enrichment

To illustrate the signaling pathways of rats after modeling, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed on the Pmodel/sham. The results showed that the pathways mainly included chemokine signal pathway, B-cell receptor signal pathway, NOD-like receptor signal pathway, NF-κB signal pathway, phosphatidylinositol signal pathway, cAMP signal pathway, cytokine–cytokine receptor interaction, rheumatoid arthritis, primary immunodeficiency disease, platelet activation, etc. (Figure 8A).

To illustrate the pathways of CP/CPPS rats after EA intervention, KEGG enrichment analysis was carried out on PEA/model. The results showed that the pathways mainly

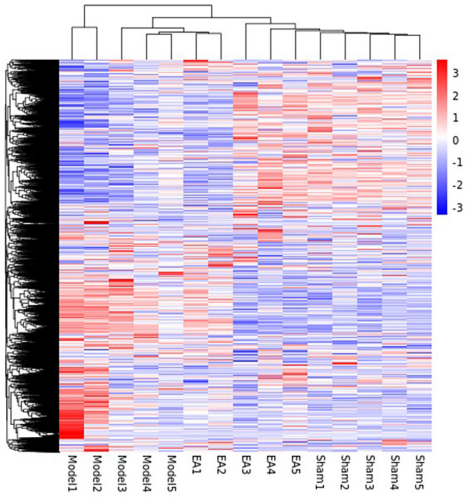

FIGURE 4
Hierarchical cluster heat map of DEG in each group. This heat map can intuitively compare the homogeneity and differences between groups. Each column in the figure represents a sample, and each row represents a gene. The color in the figure represents the expression amount of the gene in the sample, red represents the high expression amount, and blue represents the low expression amount. The number label next to the color bar at the top left is the specific change trend of the expression amount. On the left is the dendrogram of gene clustering and the module diagram of sub clustering, the closer the two gene branches are, the closer their expression is. The upper part is the dendrogram of sample clustering, and the lower part is the name of the sample. The closer the two sample branches are, the closer the expression pattern of all genes in the two samples is.

included Toll receptor signal pathway, NF-κB signal pathway, cAMP signal pathway, phosphatidylinositol signal pathway, complement coagulation cascade, cytokine receptor interaction, staphylococcus aureus infection, non-alcoholic fatty liver disease, platelet activation, Parkinson's disease, Alzheimer's disease, osteoclast differentiation, etc. (**Figure 8B**).

Both of Pmodel/sham and PEA/model were involved in NF-κB signaling pathway, cAMP signaling pathway, phosphatidylinositol signaling pathway, B-cell receptor signaling pathway, chemokine signaling pathway, cytokine receptor interaction, and other immune-related pathways, as well as some immune and inflammatory-related diseases, such as rheumatoid arthritis, primary immune deficiency diseases, staphylococcus aureus infection, and non-alcoholic fatty liver disease (**Table 3**). The results suggested that the analgesic mechanism of EA on CP/CPPS may be related to the key genes in these intersecting signaling pathways.

Results of qRT-PCR

To verify the RNA-Seq results and further scientifically study the related signal pathways involved in the analgesic effect of EA on CP/CPPS. Based on the results of RNA-Seq analysis in prostate tissue of rats in each group, and combined with the modern research progress on the pain-related mechanism of CP/CPPS, our team screened out the pain-related DEG and the key genes in pain-related pathways, vanillic acid receptor subtype 1 of the transient receptor potential (TRPV1), phospholipase C (PLC), protein kinase C (PKC), cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA). qRT-PCR results showed that compared with sham group, TRPV1, PLC, PKC, cAMP, and PKA in model group were increased significantly ($p < 0.01$). Compared with the model group, TRPV1, PLC, PKC, cAMP, and PKA in EA group were decreased ($p < 0.05$, $p < 0.01$) (**Figure 9**). qRT-PCR results suggested that the analgesic effect of EA on CP/CPPS may be achieved by regulating the expression of TRPVI, PLC, PKC, cAMP, and PKA (**Figures 10A,B**).

Discussion

Pain is a major symptom in patients with CP/CPPS (Lin et al., 2016). Most clinical studies have shown that EA is an effective means of improving pain symptoms in CP/CPPS patients, because it is inexpensive, safe, has few side effects and has a long-lasting efficacy (Sun et al., 2021). Combined with medication, it can significantly improve the clinical efficacy of the medication, including improving various symptoms and indicators in CP/CPPS patients (Liang et al., 2021). Our previous studies (Wu et al., 2022; Xu et al., 2022) also assessed the effects of EA on the behavioral pain profile of CP/CPPS rats. The result showed that EA was effective in reducing mechanical and thermal pain in CP/CPPS rats; it also revealed a decrease in serum substance P (SP) and prostaglandin E2 (PGE2) and a decrease in cyclooxygenase-2 (COX-2) and PGE2 in prostate tissue, suggesting that the analgesic mechanism of EA in CP/CPPS rats may be related to the modulation of the body's inflammatory response. This study has investigated the analgesic mechanism of EA therapy in CP/CPPS rats from the prostate transcriptome level. As a peripheral injury receptor in CP/CPPS rats, the prostate tissue is a key site for sensing and transmitting nociceptive stimuli. Various causative factors can cause sensitization of pain throughout the pelvis by stimulating sensory nerves in the prostate tissue and generating pain signals (Jiang et al., 2021). In the H&E staining results of this study, we found that EA significantly improved the histopathological changes of prostate tissue in CP/CPPS rats, suggesting that the effect of EA on CP/CPPS may be related to the protective effect on prostate tissue. This is one of the reasons why we investigated the analgesic mechanism of EA on CP/CPPS from the transcriptome level of the prostate tissue.

The acupoints used in this study included CV4, CV3, SP6 and BL35 (**Figure 11**). A meta-analysis showed that these acupoints are the most frequently used acupoints in the clinical treatment of patients with CP/CPPS (Zhang et al., 2021).

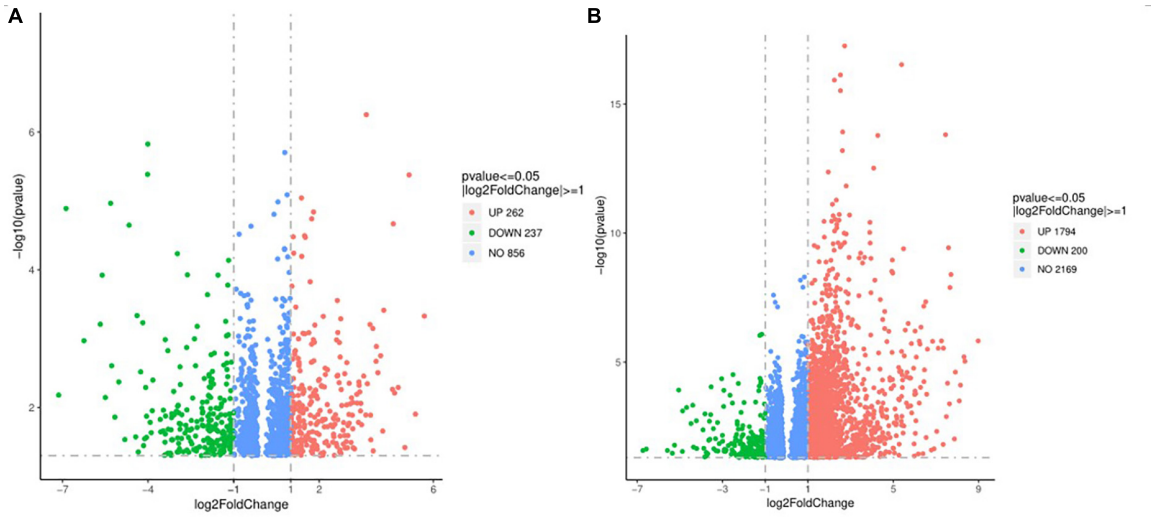

FIGURE 5
Volcano plot of DEG in each group. **(A)** Volcano plot of DEG between model group and sham group. **(B)** Volcano plot of DEG between model group and EA group. In the figure, the *X*-axis is log2FoldChange, and the *Y*-axis is -log10 (*p*-value). The gray dotted line is the threshold line representing the screening criteria of DEG. Red represents the up-regulated DEG, green represents the down-regulated DEG.

In terms of Traditional Chinese Medicine (TCM) theory, CV3 and CV4 are acupoints on the Ren Vessel, which have a function of warming Yang and dredging collaterals (Zheng and Hu, 2017; Ying H. Z. et al., 2021). SP6 is the intersection acupoint on Three Yin Meridian of Foot, which has the functions of soothing liver and regulating Qi, fortifying the spleen and disinhibiting dampness, warming kidney and tonifying Qi (Ying Z. H. et al., 2021). BL35 is on the Bladder Meridian of Foot Taiyang, which has the function of promoting Qi and water (Liu et al., 2016). The combination of the above acupoints can relieve CP/CPPS pain by warming Yang, promoting Qi and disinhibiting dampness. In addition, from modern neuroanatomy, the prostate is innervated by the parasympathetic nerves, and the painful areas of CP/CPPS patients are mainly distributed by the pubic nerve, perineal nerve and pelvic nerve, and these nerve fibers mainly originate from the lumbar and sacral plexuses of the spinal cord segments (Wang and Shang, 2010). The sympathetic and parasympathetic nerves are distributed under the CV3 and CV4, and some studies (Chang et al., 2009) have shown that by stimulating CV3 and CV4, it can relieve pelvic and prostate pain. This can relieve the muscle tension around the pelvis and prostate, promote local blood circulation and lower PGE2 levels, thereby relieving the stimulation of parasympathetic and sympathetic afferent fibers by these substances and reducing the transmission of undesirable impulses to the center, thus reducing the painful symptoms of chronic prostatitis. The tibial nerve is located under the SP6, and some studies have shown (Abdel-Karim et al., 2005) that electrical stimulation of SP6 allows impulses to reach the posterior roots of the spinal cord through the tibial nerve, which regulates the function of the corresponding organs through central feedback. Stimulation of SP6 may regulate prostate activity through tibial nerve conduction. BL35 is in the projection area of the prostate, which has caudal nerves in the superficial part and pudendal nerve trunks in the deep part; acupuncture at BL35 can regulate the excitability of the pudendal and pelvic nerve (Li et al., 2009). From the above, it can be seen that the nerves under CV4, CV3, SP6, and BL35 overlap with those of the CP/CPPS pain site. The analgesic effect of EA on CP/CPPS at CV3, CV4, SP6, and BL35 may be related to the corresponding relationship between the nerves distributed at these acupoints and the nerves distributed at the pain site of CP/CPPS.

RNA-Seq allows the study of gene function and gene structure at a holistic level, revealing specific biological processes and molecular mechanisms in the development of disease (Lv et al., 2020). In this study, there were 147 intersecting DEG between Pmodel/sham and Pmodel/EA, of which 103 were upregulated and 37 were downregulated (**Supplementary Table 1**), indicating that EA can reverse the up-/downregulated genes in prostate tissue of CP/CPPS rats. These intersecting DEGs may be closely related to the analgesic mechanism of EA on CP/CPPS rats.

Comprehensively comparing the GO analysis of Pmodel/sham and PEA/model, there is a crossover of GO functions between Pmodel/sham and PEA/model (**Table 2**). The results of GO analysis suggest that the analgesic effect of EA on CP/CPPS may be related to inflammation-related ion channels in the cell membrane of prostate tissue. In the process of inflammatory pain, the inflammatory reaction will make the nociceptor sensitive, resulting in the decrease of activation threshold, and the pain hypersensitivity at the

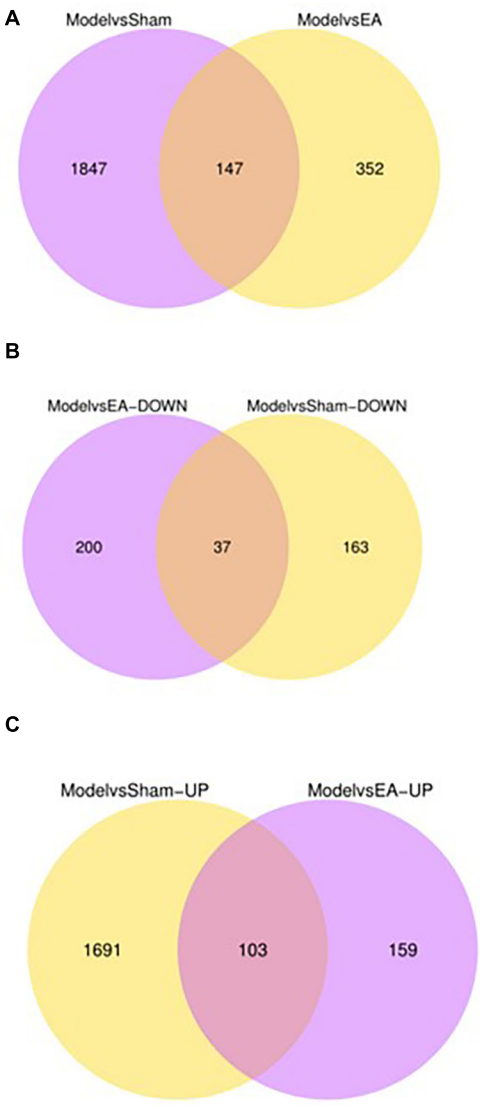

FIGURE 6
Number of DEG and intersecting genes in each group. **(A)** The purple circle represents the amount of DEG between the model group and the sham group. The yellow circle represents the amount of DEG between the model group and the EA group. **(B)** The purple circle represents the amount of down-regulated DEG between the model group and the EA group. The yellow circle represents the amount of down-regulated DEG between the model group and the sham group. **(C)** The purple circle represents the amount of up-regulated DEG between the model group and the EA group. The yellow circle represents the amount of up-regulated DEG between the model group and the sham group. Overlapping regions represent the intersecting gene between two comparable groups.

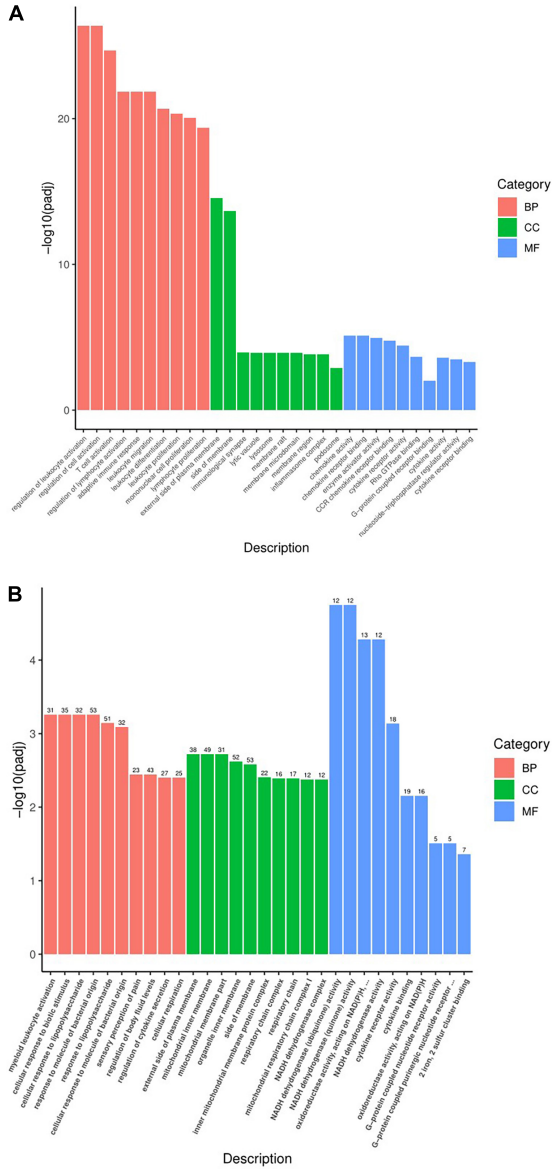

FIGURE 7
Statistical chart of GO function analysis. **(A)** Statistical chart of GO function analysis of Pmodel/sham. **(B)** Statistical chart of GO function of Pmodel/EA. The *X*-axis represents the secondary classification term of GO, and the *Y*-axis represents the percentage of genes included in the secondary classification. Three colors represent three categories: Red refers to BP (biological process), green refers to CC (cell composition), and blue refers to MF (molecular function).

inflammatory site (Liu and Zhang, 2009). The largest receptor that acts as noxious stimulus detectors in injury receptors are the transient receptor potential ion channel protein (TRP) family. TRP channels are expressed on cell membranes and mediate non-selective cation inward flow by integrating intra- and extracellular information (Szallasi et al., 2007; Darré and Domene, 2015). TRPV1 is a subtype of TRP channels whose physiological function primarily responds to inflammatory pain stimuli and is involved in nociceptive signaling (Julius, 2013). The binding of inflammatory mediators to TRPV1 in peripheral tissues enhances cell membrane excitability, decreases inflammatory pain thresholds and causes nociceptive

TABLE 2 Similar GO function analysis between Pmodel/sham and PEA/model.

GO function	Pmodel/sham ∩ PEA/model
BP	Immune cell activation, other immune and inflammatory reactions
CC	Cell membrane, cell organelles membrane
MF	Cytokine activity, chemokine activity, other functions related to immunity and inflammation

Pmodel/sham, GO function analysis results of DEG between model group and sham group. PEA/model, GO function analysis results of DEG between EA group and model group. Pmodel/sham∩PEA/model, similar GO function analysis results between Pmodel/sham and PEA/model.

sensitization (Zhang and Wang, 2017). In modern medical research, several studies (Caterina et al., 2000; Davis et al., 2000) found that TRPV1 gene-deficient mice can reduce inflammatory mediator-induced nociceptive sensitization. Several studies (Lu et al., 2016) have also shown that effective relief of inflammatory pain by EA is associated with the modulation of TRPV1. In the present RNA-Seq results of CP/CPPS rat prostate tissue, we also found TRPV1 to be an upregulated gene in Pmodel/sham and a downregulated gene in PEA/model (**Supplementary Table 1**). qRT-PCR results also found that TRPV1 was significantly upregulated in the model group and significantly downregulated after EA intervention, in line with the trend of RNA-Seq results, suggesting that the analgesic effect of EA on CP/CPPS may be related to the regulation of TPPV1.

In the results of KEGG enrichment analysis, Pmodel/sham intersected with the pathways mainly involved in PEA/model (**Table 3**), and the results suggest that the effect of electrodes on CP/CPPS rats may be related to the regulation of phosphatidylinositol signaling pathway and cAMP signaling pathway. In the phosphatidylinositol signal pathway, extracellular signaling molecules can bind to G protein-coupled receptors to activate PLC, which then indirectly activate PKC, and regulate downstream signaling molecules (Yi et al., 2021). Recent studies (Bai, 2018; Xue et al., 2021) have shown that PLC-PKC pathway plays a significant role in chronic pain generation and maintenance. In the cAMP signaling pathway, recent studies (Smith et al., 2000; Joseph and Levine, 2003) have shown that pain hypersensitivity in rats, an inflammation model, is closely associated with the cAMP-PKA pathway. In this qRT-PCR results, PLC, PKC, cAMP, and PKC increased significantly in the model group and significantly decreased after EA intervention. The results were consistent with the KEGG enrichment analysis, suggesting that the analgesic effect of EA on CP/CPPS may be related to the modulation of the cAMP-PKA and PLC-PKC signaling pathways.

Recent studies (Bhave et al., 2002; Mohapatra and Nau, 2005) also have suggested that the TRPV1 phosphorylation mediated by cAMP-PKA and PLC-PKC signaling pathways

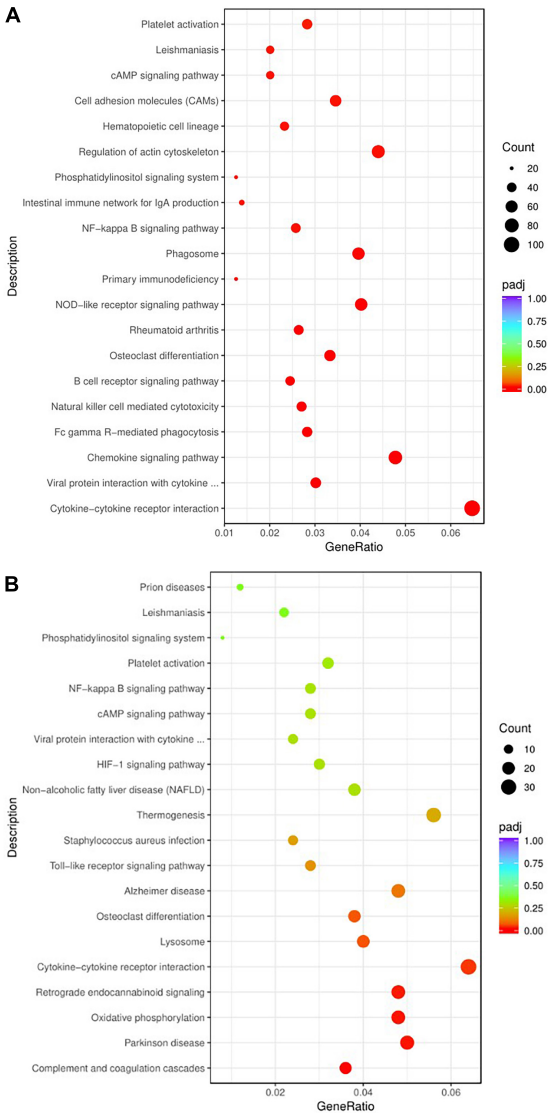

FIGURE 8
The bubble chart of KEGG enrichment analysis. **(A)** KEGG enrichment analysis bubble chart of Pmodel/sham. **(B)** KEGG enrichment analysis bubble chart of Pmodel/EA. The *Y*-axis represents the pathway name, and the *X*-axis represents the enrichment factor, that is the ratio of the amount of DEG enriched in the KEGG term to the total amount of differential genes. The greater the ratio, the greater the degree of enrichment. The size of the bubble indicates the number of genes in this pathway, and the color of the bubble from red to purple represents the significance of enrichment.

may be the mechanism of inflammatory pain. In the present qRT-PCR results, PLC, PKC, cAMP, PKA, and TRPV1 in the model group were significantly increased and significantly decreased after EA intervention, suggesting that the analgesic effect of EA on CP/CPPS may be achieved by reducing the TRPV1 phosphorylation mediated by cAMP-PKA and PLA-PKC signaling pathways.

TABLE 3 Similar KEGG enrichment results between Pmodel/sham and PEA/model.

KEGG enrichment analysis	Pmodel/sham ∩ PEA/model
Signal pathway	NF-kB signaling pathway, cAMP signaling pathway, phosphatidylinositol signaling pathway, B-cell receptor signaling pathway, chemokine signaling pathway, cytokine receptor interaction and other immune-related pathways
Disease	Rheumatoid arthritis, primary immunodeficiency disease, Staphylococcus aureus infection, non-alcoholic fatty liver and other diseases related to immunity and inflammation

Pmodel/sham, KEGG enrichment analysis results of DEG between model group and sham group. PEA/model, KEGG enrichment analysis results of DEG between EA group and model group. Pmodel/sham∩PEA/model: similar KEGG enrichment analysis results between Pmodel/sham and PEA/model.

In conclusion, the present study is the first to apply RNA-Seq technology to the study of the analgesic effect of EA on CP/CPPS. The results indicate that the analgesic effect of EA on CP/CPPS involves a variety of GO biological functions and multiple signaling pathways. However, some questions still need to be addressed in this study. The study did not investigate how EA affects cAMP, PKA, PLC, PKC, TRPV1 and other important genes in the cAMP signaling pathway and PKC signaling pathway, and the specific targets of EA intervention on CP/CPPS need to be further investigated. In the KEGG enrichment analysis results, the DEGs were also enriched in the NF-κB pathway and other immune-related pathways in the model rats after EA intervention. Whether the analgesic effects of EA at CV3, CV4, SP6, and BL35 on CP/CPPS are related to these pathways and their extensiveness also need to be further investigated in the future.

Materials and methods

Animals

Thirty male Sprague–Dawley (SD) rats of 7–8 weeks' weight (210 ± 10 g) were fed in the laboratory of Beijing University of Chinese Medicine (SPF level), with food and water freely. All rats were provided by *Beijing Vitong Lihua Laboratory Animal Technology Co., Ltd*. The feeding temperature was 23 ± 2°C, the humidity was 45%, and the light and dark periods were 12 h (turning on the light at 8 a.m.). Adaptive feeding occurred for 1 week. Rats were randomly divided into sham group (n = 10), model group (n = 10) and EA group (n = 10). Ethical approval was granted by the Beijing University of Chinese Medicine (BUCM-3-20151202-4001); therefore, experiments were performed in accordance with the ethical standards laid out by the IACUC.

Modeling methods

Referring to the modeling method of Tang (2007), fasting and water deprivation occurred 24 h before modeling. Rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (350 mg/kg body weight). All rats were in the supine position, cut off the hair on the right lower abdomen, and a longitudinal incision (1 cm beside the anterior midline) was made to expose the bilateral ventral lobes of the prostate. Stabilizing the prostate with hemostatic forceps, the rats in model group and EA group were injected with 0.05 mL of complete Freund's adjuvant (CFA) solution (Beijing Benovir Biotechnology Co., Ltd.) into the ventral lobes of the prostate on both sides, while the rats in sham group were injected with the same dose of saline. Then layer by layer disinfection, suturing, and disinfection. All rats were kept in a warm place until awake. All of the rats fasted for 24 h postoperatively, but free water was allowed. Pathological changes of prostate tissues were observed by H&E staining to evaluate the successful model. Under the microscope, a large number of inflammatory cell infiltration in the prostate gland was to prove the successful establishment of the model. All experiment procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Intervention methods

In sham and model groups, the rats were bounded and fixed every day without other treatment. In EA group, firstly, stainless steel acupuncture needles (0.18 × 13 mm, *Beijing Zhongyan Taihe Medical Instrument Co., Ltd.*) were inserted at a depth of 3 mm into the CV4 (located at points 3/5 down the ventral midline connecting the umbilicus to the pubic tubercle (Huang, 2020)), CV3 (located at points 4/5 down the ventral midline connecting the umbilicus to the pubic tubercle (Huang, 2020)) and bilateral SP6 (located at 10 mm proximal to the highest prominence of the medial malleolus, on the posterior border of the medial crest of the tibia (Wu et al., 2019)) in the supine position, 20 min for each time; then, bilateral BL35 (located at anteromedial of the transverse process of the 6th lumbar spine (Liu and Wang, 2013)) was inserted at a depth of 6 mm inward toward the spine in the prone position, 20 min for each time. The two ipsilateral needles were connected to the output terminals of the HANS-200E EA instrument with 2 Hz/100 Hz alternating frequencies. The output current was set as 2–3 A, with the tail of the rats swinging slightly without obvious struggle. All groups were treated on the 15th day after modeling. The treatment was once a day for 40 min, 5 days as a course, with 2 days

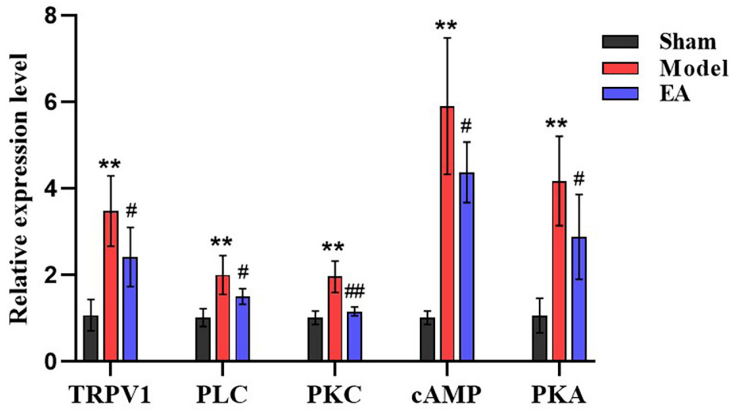

FIGURE 9
Results of qRT-PCR in each group (n = 6, $\bar{x} \pm s$). The X-axis is the gene name and the Y-axis is the relative expression of the gene. Compared with the sham group, $^{**}P < 0.01$. Compared with the model group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

of rest between the two courses, 4 courses in total. Before the formal intervention, the animals were stroked for 5 min every day, and all rats were loosely fixed throughout the treatment (Figures 1, 11).

Sample collection

The day after the last treatment, all rats were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (350 mg/kg body weight). The lower abdominal skin of rats was cut to fully expose the prostate. After carefully stripping and removing the prostate, the left lobe of the prostate was fixed in 4% paraformaldehyde, and the right lobe of the prostate was stored in a refrigerator at 80°C.

Histological examination

The prostate tissue fixed in 4% paraformaldehyde was dehydrated in a fully automatic dehydrator and then embedded in paraffin and sectioned at a thickness of 4 μm. Paraffin sections were dewaxed with xylene, hydrated with alcohol, stained with hematoxylin and eosin, dehydrated with alcohol and made transparent with xylene. The morphology of the prostate tissue was observed under an optical microscope.

RNA extraction and construction of sequencing library

Five frozen right lobes of the prostate tissues were taken from each group for processing and sequencing (n = 5), Trizol (Invitrogen, United States) method was used to extract total RNA, and the quality inspection of the obtained total RNA was performed using Agilent 2100 bioanalyzer to observe its purity, concentration and integrity. For library establishment, the library was constructed of qualified samples with a starting quantity of 1 μg total RNA $\geq$ 50 ng/μL. PCR amplification was performed to obtain the final cDNA library. The Agilent 2100 bioanalyzer was used to check the library quality. The transcriptome sequencing was completed based on the HiSeq sequencing platform. The illumine PE library was constructed by Illumina TruSeq TM RNA Sample Prep Kit method for 2 × 150 bp sequencing. The quality control of the sequencing data was carried out, and then the transcriptome data were analyzed by bioinformatics. Fragments per kilobase per million mapped reads (FPKM) value was used to determine gene expression level. DEG was analyzed with the full transcriptomic data of each sample.

Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses

GO terms are used to describe and classify the functions of genes and proteins. When the corrected p-value < 0.05, it is considered that the GO function is significantly enriched. KEGG is a large knowledge base for systematic analysis of gene function and association of genomic information. Corrected p-value < 0.05 was used to identify which pathway is significantly enriched in DEG compared with the whole genome background.

qRT-PCR

Total RNA was extracted using HiPure Total RNA Mini Kit (Guangzhou Magen Biotechnology Co., Ltd.). RNA was

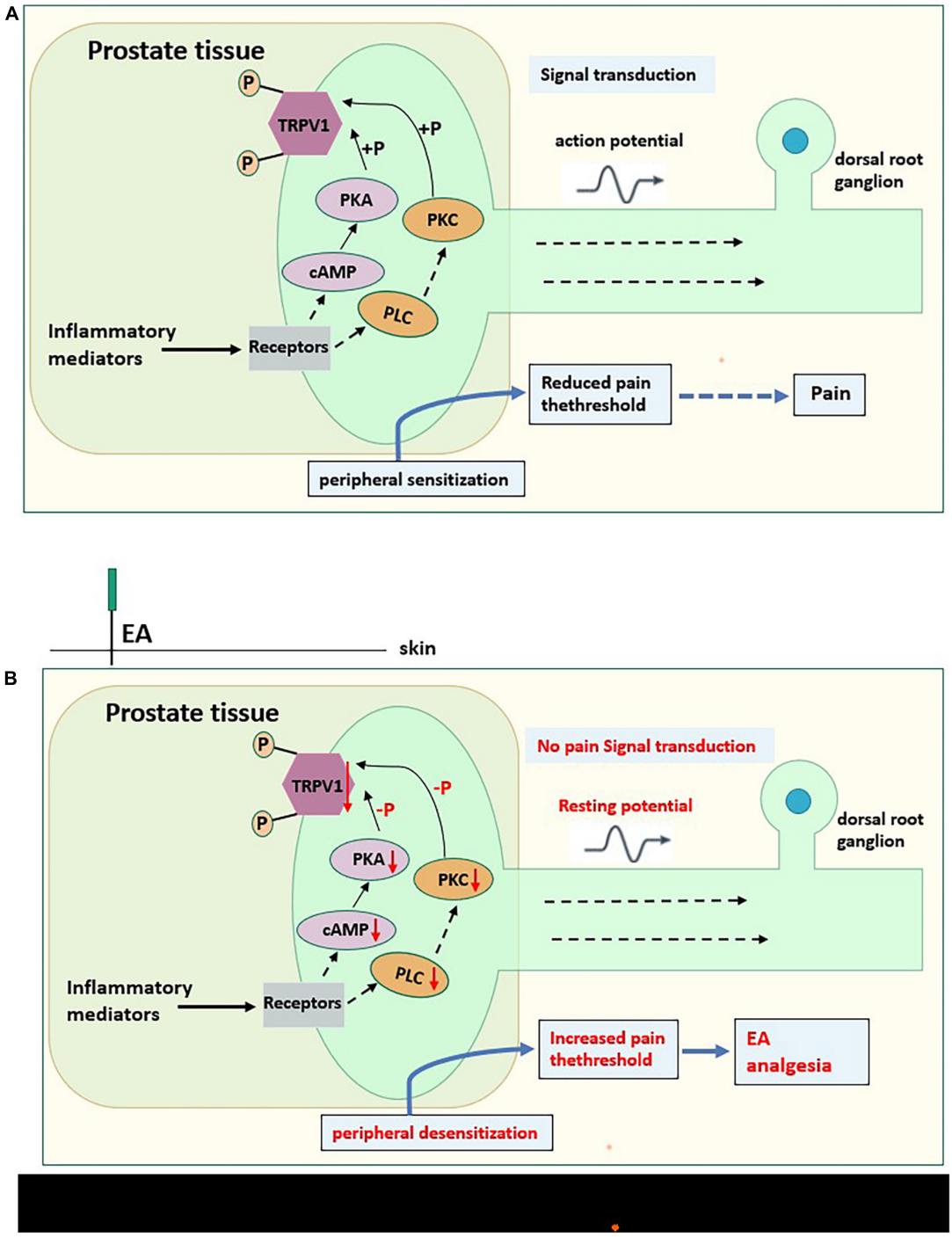

FIGURE 10
(A) The possible Pain-related mechanism diagram of CP/CPPS. Prostate tissue of CP/CPPS model rat produces a variety of inflammatory mediators (sensitizers), which act on homologous receptors expressed by nociceptors to activate the cAMP-PKA pathway and PLC-PKC pathway of intracellular signal transduction. These pathways can phosphorylate TRPV1, causing peripheral sensitization, converting chemical signals into electrical signals, then transmitting to the central system, and reducing the pain threshold. **(B)** The possible analgesic mechanism diagram of EA on CP/CPPS rats. EA on acupoints may interfere with the activation of the cAMP-PKA pathway and PLC-PKC pathway, hindering intracellular signal transduction, reducing the phosphorylation of TRPV1, then causing peripheral desensitization, increasing the pain threshold, and do not transmit noxious stimulation to the central system, to effectively for pain relief. "⇢"Directly acting on downstream substances,"→" Indirect action on downstream substances.

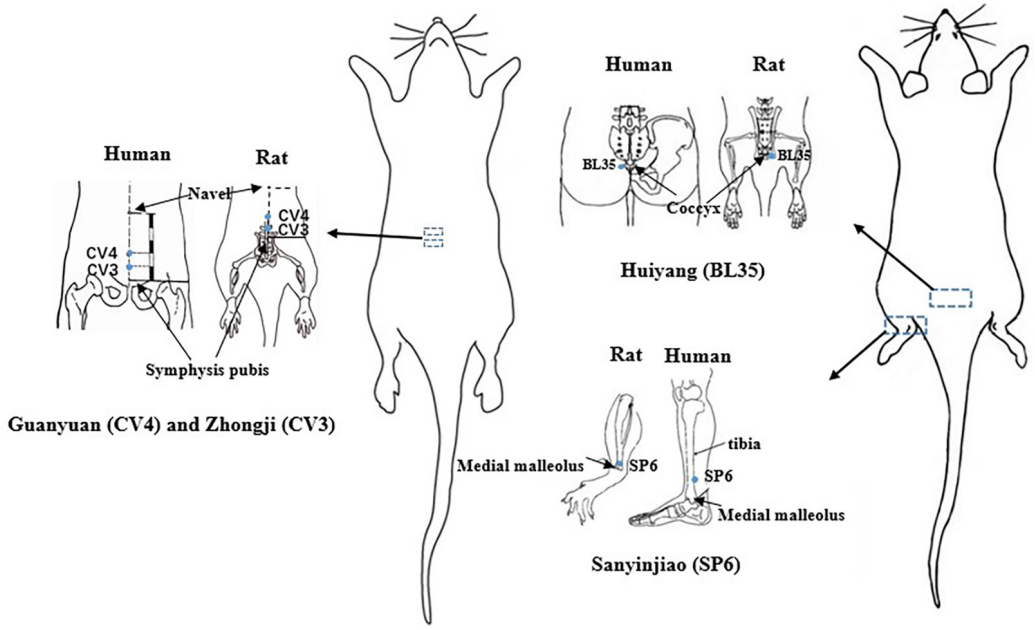

FIGURE 11
Schematic representation of the points in the EA group. Anatomical localization of the acupoints are shown on the rat and human bodies. Points are indicated by blue dots.

quantified by spectrophotometry. The First-Strand Synthesis Master Mix (Beijing LABLEAD Biotech Co., Ltd.) was used for reverse transcription to obtain cDNA. Premier 5.0 software (Premier, Canada) was used to design specific oligonucleotide primers for rat TRPV1, PLC, PKC, cAMP, PKA and GAPDH (as an internal reference gene). The primers listed were amplified (Table 4). The reaction mixture (total volume 10 μL) was prepared using cDNA (1 μg/μL), forward and reverse primers (0.5 μL), PowerUpTMSYBRTM Green Master Mix (5 μL) (Life Technologies) and Nuclease-Free Water (3 μL). The amplification was carried out with an initial denaturation step at 95°C for 3 min, followed by 44 repeated thermal cycles (95°C for 30 s, 60°C for 30 s, 72°C for 30 s, 95°C for 2 min). The relative quantification of TRPV1, PLC, PKC, cAMP, PKA normalized to GAPDH and relative to a calibrator was measured by $2^{-\Delta\Delta\ Ct}$.

TABLE 4 qRT-PCR primers.

Gene	Primer name	Sequence (5′–3′)
1	TRPV1-F	AGAAGGGGAACCAGGGCAAAG
	TRPV1-R	TCAACGAGGACCCAGGCAACT
2	PLC-F	AAGCCTTTGACCCCTTTGAT
	PLC-R	CCAGCCACTTCAATCTCCAC
3	PKC-F	TCTGGAAGCAGCAATAGAGTT
	PKC-R	TCATCAAGGTGTTAGGCAAAG
4	cAMP-F	CCCTGAACTCAACTGTGAAATAGCA
	cAMP-R	CCCAAGTCAAGGGCTTGGAA
5	PKA-F	ACCTTGGGAACGGGTTCCTTCG
	PKA-R	TACACCCAATGCCCACCAGTCC
GAPDH	GAPDH-F	ACCACAGTCCATGCCATCAC
	GAPDH-R	TCCACCACCCTGTTGCTGTA

Statistical analysis

IBM SPSS 20.0 software was used for statistical analysis of the data. The data in the normal distribution were represented as the mean ± standard deviation (−x ± s), and the one-way analysis of variance least significant difference method was used for comparison between groups with a homogeneous variance, while Dunnett's T3 method was used for those with a heterogeneous variance. The data that did not follow a normal distribution were represented by the median and quartile [median (P25, P75)], and non-parametric tests were used for comparison between the groups. The test level was set as $\alpha = 0.05$, with $p < 0.05$ considered statistically significant.

Conclusion

After modeling, the expression of some genes in prostate tissue increased or decreased. EA acting on CP/CPPS rats

could reduce the upregulated genes expression and increase the downregulated genes expression, so that the genes expression tends toward normal and therefore could relieve pain symptoms of CP/CPPS. The RNA-Seq data obtained in this study have been proven to be verified with qRT-PCR. The analgesic effect of EA on CP/CPPS may be related to the cAMP-PKA-TRPV1/PLC-PKC-TRPV1 signal pathway. This study, for the first time, applied RNA-Seq to analyze the gene expression profiling in prostate tissue after EA intervention and provides theoretical support and scientific basis for the analgesic effect of EA on CP/CPPS in clinics.

Data availability statement

The data is publicly available at the following link: https://dataview.ncbi.nlm.nih.gov/?archive=bioproject.

Ethics statement

The animal study was reviewed and approved by the Beijing University of Chinese Medicine (BUCM-3-20151202-4001).

Author contributions

NL contributed to the conception and design of the study. X-LW wrote the first draft of the manuscript. X-LW, CX, T-HY, Z-WY, Q-HS, and X-HQ performed the experiments. KC, Y-MC, and YT organized the database. WC and J-NZ performed the statistical analysis. X-YY wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.938200/full#supplementary-material

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Potential lipid-lowering effects of *Ulmus macrocarpa* Hance extract in adults with untreated high low-density lipoprotein cholesterol concentrations: A randomized double-blind placebo-controlled trial

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Introduction: *Ulmus macrocarpa* Hance extract (UME) has demonstrated an antilipidemic effect *via* upregulation of the adenosine monophosphate-activated protein kinase pathway and regulation of lipid metabolism in both laboratory and animal studies. Therefore, we examined the effects and safety of UME on plasma lipids in adults with untreated high, low-density lipoprotein cholesterol (LDL-C) concentrations.

Materials and methods: In the current double-blind placebo-controlled randomized clinical trial, 80 patients with untreated high LDL-C concentrations (130–190 mg/dl) were randomly allocated to either the "UME group" (received 500 mg UME as two capsules per day) or the "Placebo group" (received placebo containing cornstarch as two capsules per day) for 12 weeks. The primary outcome was the change in LDL-C concentration within the 12-week treatment period; secondary outcomes included changes in total cholesterol (TC), triglyceride, high-density lipoprotein cholesterol, apolipoprotein A1, and apolipoprotein B (ApoB) concentrations.

Results: UME over 12 weeks led to a greater decrease in LDL-C, TC, and ApoB concentrations than did the placebo as follows: by 18.1 mg/dl ($P < 0.001$); 23.3 mg/dl ($P < 0.001$); 9.3 mg/dl ($P = 0.018$), respectively. When LDL-C, TC, and ApoB concentrations were expressed as a lsmeans percentage of the baseline concentration, they after 12 weeks of UME had greater % differences

compared to the placebo as follows: by 11.9% ($P < 0.001$); 10.0% ($P < 0.001$); 8.6% ($P < 0.05$), respectively. However, no significant inter- and intra-group changes in liver enzyme, free fatty acid, anti-inflammatory marker, and fasting glucose concentrations were observed. None of the participants experienced notable adverse events.

Discussion: UME causes a significant improvement in lipid profiles in adults with untreated high LDL-C concentrations.

Clinical trial registration: [www.clinicaltrials.gov/], identifier [NCT03773315].

KEYWORDS

dietary supplements, dyslipidemia, *Ulmus macrocarpa* Hance, lipids, lipoprotein, randomized controlled trial

Introduction

Dyslipidemia is recognized as one of the most common modifiable risk factors for developing atherosclerosis and subsequent ischemic heart disease (IHD) (1). The global burden of dyslipidemia has steadily increased over the past 30 years (2, 3). The World Health Organization (WHO) reported a global prevalence of hypercholesterolemia in adults aged ≥18 of 39% in 2008 (4). High LDL-cholesterol rapidly increased from the 15th leading risk for death in 1990 to the 8th in 2019 (2). The prevalence of dyslipidemia in the young population also is increasing (5). Compared to those without dyslipidemia, adults with dyslipidemia are at approximately twice the risk of developing cardiovascular disease (CVD), among the leading causes of mortality worldwide (1, 6). Further, hypercholesterolemia is estimated to cause 56% of IHDs and 18% of strokes worldwide (6).

The initial management of dyslipidemia involves optimizing lifestyle changes and correcting secondary exacerbating factors before beginning antilipemic drug use (7). Weight loss, changes in dietary macronutrient composition such as a Mediterranean-style diet, and physical activity, or the combination of them, contribute to triglyceride reduction (8). They remain important even when using medications (9). Lipid-lowering medications must also be administered to patients with higher CVD risk who do not respond to non-pharmacological therapy. Currently, statins are the most used therapeutic option for treating dyslipidemia as they reduce the risk of cardio-cerebrovascular events and mortality (10). Previous studies have reported on various statin drugs, such as lovastatin, simvastatin, atorvastatin, and rosuvastatin, which induce hypolipidemia *via* inhibiting β-hydroxy β-methylglutaryl-CoA reductase (HMGCR), a rate-limiting enzyme of the cholesterol biosynthetic pathway (11). Although statins are effective for lowering cholesterol and protecting against cardiovascular and cerebrovascular events, they may elicit side effects in some patients, including muscle- and skeletal-related adverse events (AEs) (pain, weakness, myopathy, and rhabdomyolysis), liver damage, increased risk of developing type 2 diabetes, memory loss, and confusion (12).

A focus-group study in Germany revealed that people use herbal medicine primarily to treat mild to moderate illnesses for all age groups and prevent illnesses or promote health, especially for the elderly. Also, they were aware of the limits of herbal medicine for severe illnesses (13). Although these standard lipid-lowering treatments should be used in patients with high or very high CVD risk, functional foods may be recommended for individuals with borderline lipid profile levels or drug intolerance (14). In recent years, lipid-lowering nutraceuticals and functional foods identified through clinical studies have included phytosterols, oat β-glucan, chitosan, and probiotic lactobacillus as inhibitors of intestinal cholesterol absorption; monacolin K as an inhibitor of liver cholesterol synthesis; green tea catechin extract and milk polar lipids as inducers of low-density lipoprotein cholesterol (LDL-C) excretion; and spirulina supplementation, krill oil, turmeric, and curcuminoids as nutraceuticals with mixed mechanisms of action (14).

Recently, *Ulmus macrocarpa* Hance extract (UME) exhibited potential as supporting therapy for lowering plasma total cholesterol (TC), triglyceride (TG), and LDL-C concentrations in hypercholesterolemic conditions by regulating the adenosine monophosphate-activated protein kinase (AMPK) pathway and lipid metabolism *in vitro* and *in vivo* using oleic acid (OA)-treated HepG2 cells and high-cholesterol diet (HCD)-induced hyperlipidemia rats (15). However, no randomized, placebo-controlled trial in humans has explored the effects and safety of UME in hyperlipidemia. We hypothesized that UME has a lipid profile-improving effect in adults based on previous studies. Thus, this randomized, double-blinded, placebo-controlled trial aimed to investigate the impact of UME administration for 12 weeks on lipid profiles in adults with untreated high LDL-C concentrations and to test its safety.

Materials and methods

Study participants and ethical aspects

This study was approved by the Institutional Review Board at Pusan National University Yangsan Hospital (IRB 02-2018-029, 8 October 2018). It was conducted in accordance with the principles of the Declaration of Helsinki and the Korean Good Clinical Products guidelines. Written informed consent was obtained from all study participants recruited through advertisements at a tertiary hospital in Yangsan, South Korea. The trial was conducted between April 2019 and October 2019. The trial was registered in the Registry Clinical Trial.[1]

According to the clinical practice guideline of the Korean Society of Lipid and Atherosclerosis for the Korean population, statins are recommended for patients with LDL-C concentration ≥190 mg/dl, irrespective of the level of risk. Also, statins are considered when LDL-C concentration ≥130 mg/dl persists even after weeks or months of lifestyle modification for moderate-risk and low-risk groups (16). Therefore, participants ≥20 years of age and with LDL-C concentrations ranging from 130 to 190 mg/dl were eligible for the study. Participants using of lipid-lowering drugs within the previous 3 months; with a history of cerebrovascular diseases (such as cerebral infarction, cerebral hemorrhage, etc.) or heart disease (such as unstable angina, myocardial infarction, heart failure, etc.) for which lesser than 6 months had passed since hospital discharge; with abnormal liver or renal function (aspartate aminotransferase or alanine aminotransferase concentration more than two times the upper limit of normal; creatinine concentration more than two times the upper limit of normal; or proteinuria, defined as a urinalysis dipstick reading of ≥2+); with hyperthyroidism or hypothyroidism; with diabetes (diagnosed clinically or with a fasting glucose concentration >126 mg/dl); with uncontrolled hypertension [blood pressure (BP) ≥160/100 mmHg); with any cancer; with use of any medication or supplements within the preceding 1 month, which could have caused a change in body weight, including anti-absorptive agents, appetite suppressors, and any other hormonal products; with psychiatric disorder; alcohol abuser; who had quit smoking within 3 months of enrollment; with severe gastrointestinal symptoms; or any allergic reaction to the involved ingredients; or pregnant or lactating women were excluded.

Study design

The study was a randomized, placebo-controlled, double-blinded controlled trial. Simple randomization of the two study groups was performed using a random number table. The table of random numbers was generated using the Excel® random number macro (Microsoft Corp., Redmond, WA, USA). Participants were assigned sequentially randomized numbers, and these randomization codes were held by the company that manufactured the UME and the dummy placebo (**Supplementary Table 1**). The authors who selected the study participants and those who performed the measurements were blinded to the randomization assignments.

After the baseline assessment, participants were randomly allocated to either the UME-supplemented group or the placebo-supplemented group. Participants were requested to log when they took the supplement in a diary, which was turned in along with the bottle to the researcher at every visit. Compliance was assessed by pill counting of the supplements that participants brought with them at each visit; if more than 20% were unused, the participant was considered to have dropped out of the study. Adherence rates of ≥80% were required for optimal therapeutic efficacy. This cut-off is widely used as a conventional threshold for good adherence (17). Each participant was instructed to visit the clinic at 6 weeks (±7 days) and 12 weeks (±7 days) after the initiation of treatment. BP and blood tests, including the lipid profile, were performed at each visit. BP was measured three times in the sitting position after a 10-min rest using a model BP-203 RV II device (Colin Corp., Aichi, Japan), and the average was used. Physical activity and nutrition assessments were performed at baseline and 12 weeks (±7 days) after treatment. Participants were counseled to maintain their usual lifestyle and diet during the 12 weeks of the study.

Intervention

Participants were randomly assigned to the UME group (supplied by Naturetech Co., Ltd., Seoul, South Korea) or the placebo group. The UME group was administered 500 mg UME/day orally, that is, one 250 mg capsule 30 min after breakfast and dinner, for 12 weeks. The UME contained a mean 5.08 mg of total catechin/g, obtained through hydrothermal extraction, as determined by HPLC analysis. The proportions of each catechin were (−)-epigallocatechin (EGC, 37.19%), (−)-epigallocatechin-gallate (EGCG, 3.58%), (−)-epicatechin (EC, 38.04%), and (−)-epicatechin-gallate (ECG, 21.19%). The placebo group was administered the same quantity of the placebo identically. The placebo was identical in appearance to the UME capsule but was filled with corn starch. Based on the results of a previous animal study, which showed that the efficacious dose of UME for lowering lipids was 100 mg/kg (15), the dose used in the animal subjects was converted to a human equivalent dose based on the person's body surface area, that is, 480 mg for individuals weighing 60 kg. Thus, 500 mg/60 kg was selected as the final dose. In the preclinical toxicity test, this dose of UME satisfied all standards for hazardous substances

1 https://clinicaltrials.gov/ct2/show/NCT03773315

such as heavy metals, microorganisms, safe pesticides, and residual sulfur dioxide. Furthermore, it reduced hepatotoxicity in experimental animals (15).

Measurements of efficacy

The primary study outcome measure was the change in LDL-C concentration within the 12-week treatment period. Secondary outcome measures were changes in TC, TG, high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), free fatty acid, and high-sensitivity C-reactive protein (hs-CRP) concentrations.

Biochemical measurements

All laboratory analyses were performed in a central laboratory. After a 12-h overnight fast, blood samples were collected at the baseline and at 6 and 12 weeks after the randomization to evaluate the antilipidemic effect of UME and monitor any potential adverse effects. Plasma hs-CRP was measured by latex particle-enhanced immunoturbidimetric assay on the AU5800 chemistry analyzer (Beckman Coulter, Brea, CA, USA). Free fatty acids were determined by an enzymatic colorimetric method assay (NEFA-HR2, ACS-ACOD; Wako Chemicals, Neuss, Germany) on the Cobas 8000 c502 analyzer (Roche Diagnostics, Mannheim, Germany). Plasma TC, TG, HDL-C, and LDL-C concentrations were measured using an enzymatic colorimetric assay on the AU5800 chemistry analyzer (Beckman Coulter, Brea, CA, USA). ApoA1 and ApoB concentrations were measured using an immunoturbidimetric method (Tina-quant, Roche Diagnostics, Mannheim, Germany) on the Cobas 8000 c502 analyzer (Roche Diagnostics, Mannheim, Germany). Serum liver enzyme, glucose, and creatinine concentrations were measured using the TBA200FR biochemical analyzer (Toshiba Co. Ltd., Tokyo, Japan).

Dietary intake and physical activities assessments

At the baseline and after 12 weeks of the trial, participants were asked to answer a questionnaire on dietary intake and physical activities that may influence changes in lipid profiles. Information on the nutritional intake of participants was collected using the 24-h dietary recall method. The CAN-Pro version 4.0 (Computer Aided Nutritional Analysis Program for Professionals 4.0; Korean Nutrition Society) was used for nutrient analysis of the surveyed dietary intake. The frequency, intensity, and type of physical activities performed by participants during the preceding 7 days were reported using the International Physical Activity Questionnaires (IPAQ) (18). The number of physical activities was represented as the metabolic equivalent of task (METs).

Safety and tolerability assessments

All randomized participants exposed to at least one dose of the study intervention were included in the safety analysis. All randomized participants exposed to at least one dose of the study intervention were included in the safety analysis. Per protocol, safety was assessed at each study visit based on AEs, vital signs, physical examination, and laboratory test results (complete blood counts, liver enzymes, glucose, and creatinine). Reports of any other AEs or unpredicted allergic reactions were collected throughout the study. All AEs were coded using version 21.0 of the Medical Dictionary for Regulatory Activities.

Statistical analyses

Data were presented as either mean ± SD, median [IQR], or mean (95% CI) for continuous variables and number (%) for categorical variables. We used MedCalc version 19.4.1 (MedCalc Software Ltd., Ostend, Belgium) to calculate the sample size based on a previous similar study (19). The estimated sample size was determined to be 32 subjects per group for 80% power to detect a difference of 14.4 mg/dl in the LDL-C concentrations, assuming an SD of 20.3 mg/dl in the primary outcome and an α error of 5% (19). By considering the changes in ox-LDL level as a main outcome, a 1/4 0.05, power of 80%, and anticipating a probable dropout rate of 20% during the intervention course, 40 patients were recruited in each group. Eighty participants (40 per group) were recruited, with an assumed dropout rate of 20%. Intention-to-treat (ITT) was the primary analysis for comparisons of outcomes between the UME and placebo groups, with multiple imputation of missing data (n = 80). Because the percentage of missing values at the 12-week follow-up was 11.3% for all variables, 5 imputed data sets were created, and the results of the analyses from the different imputed data sets were pooled according to Rubin's rules using R software version 3.6.2 (R Foundation for Statistical Computing). Multivariate imputation by the chained equations algorithm was used with the predictive mean matching method. A per-protocol (PP) analysis was also performed (n = 71) to assess the effectiveness of the supplementation. Shapiro–Wilk's test was used to test the normality assumption for all variables. Intergroup comparisons of baseline characteristics were performed using the two-sample t-test for continuous variables (or Mann–Whitney's U test for non-parametric continuous variables) and the Chi-square test for categorical variables (or Fisher's exact test for non-parametric categorical variables). ANCOVA or rank ANCOVA was used for the main analysis, with adjustment for each baseline variable and baseline dietary fat intake percentage as covariates. Model assumptions were checked by histograms, normal probability plots, and residual scatter plots. The change from baseline to week 12 in outcomes was expressed as a lsmean

percentage of the baseline levels using an ANCOVA model. *P*-values < 0.05 were considered statistically significant. Data were analyzed using SPSS Statistics 25.0 software (IBM Corp., Armonk, NY, USA) and R software version 4.1.2.[2]

Results

Consolidated standards of reporting trials flow diagram and baseline characteristics of the subjects

The flow of participants through the controlled interventional trial is depicted in a consolidated standards of reporting trials (CONSORT) conform diagram (**Figure 1**). A total of 131 participants were screened. Of them, 80 (mean age 50.6 ± 9.8 years) were included in this study and randomly allocated to the UME or placebo group. The median LDL-C concentration was 147.0 mg/dl [interquartile range, IQR 137.0–162.5]. Four participants in the UME group and one in the placebo group withdrew from the study for personal reasons; this was not associated with any adverse effects. Two participants in each group were excluded due to protocol violations of non-compliance. Overall, 71 subjects (88.8%) completed the trial. Two (5%) subjects had comorbid disorders (one osteopenia and one irritable bowel syndrome) in the UME group, and three (7.5%) had comorbid disorders (one osteopenia and two hypertension) in the placebo group. Randomization was successful, as most variables were comparable between the two groups, and no significant differences were observed in the baseline demographic or anthropometric characteristics between the groups except daily fat intake (**Table 1**). There were no significant changes in the total calorie intake, macronutrient (carbohydrate, fat, and protein) intake, and physical activities checked at the baseline and 12 weeks of the trial among the participants, reflecting no additional effects that might have influenced the lipid profile, aside from the intervention (**Table 2**). During the entire study period, the double-blind requirement was maintained.

Primary outcome

Table 3 shows that the LDL-C concentration of the UME group was significantly lower than in the placebo group after 6 and 12 weeks. In the ITT analysis, the concentrations of LDL-C were significantly decreased in the UME group compared to those in the placebo group at 6 weeks, by 8.02 mg/dl (95% CI: −15.37, −0.67; $P = 0.033$), and 12 weeks, by 18.05 mg/dl (95% CI: −25.00, −11.10; $P < 0.001$). The PP analysis also revealed that the LDL-C concentration in the UME group had decreased by 8.85 mg/dl (95% CI: −16.55, −1.15; $P = 0.025$) and 20.28 mg/dl (95% CI: −27.50, −13.07; $P < 0.001$) after 6 and 12 weeks of treatment, respectively, compared with that in the placebo group (**Table 4**). When LDL-C concentration was expressed as a lsmean percentage of the baseline concentration, LDL-C concentration of the UME group after 12 weeks demonstrated an 11.86% decrease compared to the placebo group. This intergroup difference in LDL-C concentrations was significant at the last visit, with an overall percentage change of −7.69 vs. 4.17% in the UME and placebo groups, respectively, from baseline (**Figure 2**, $P < 0.001$).

Secondary outcome

As shown in **Table 3**, based on ITT analysis, the UME group presented significantly decreased TC and ApoB concentrations, which were reduced by 23.29 and 9.31 mg/dl, respectively, compared with placebo group after 12 weeks of treatment (95% CI: −33.64, −12.94; $P < 0.001$ and 95% CI: −16.95, −1.66; $P = 0.018$, respectively). Also, based on PP analysis (**Table 4**), in UME group, TC and ApoB concentrations were significantly lower (decreased by 27.18 and 11.51 mg/dl, respectively) than those of the placebo group after 12 weeks of treatment (95% CI: −37.66, −16.69 mg/dl; $P < 0.001$ and 95% CI: −19.57, −3.46 mg/dl; $P = 0.006$, respectively). Moreover, in the UME group, the HDL-C concentration was significantly reduced, by 3.43 mg/dl (95% CI: −7.29, −0.24; $P = 0.037$), compared with the placebo group after 12 weeks of treatment, but only in the PP analysis. When TC and ApoB concentrations were expressed as a lsmean percentage of the baseline concentrations, TC and ApoB concentrations after 12 weeks of UME supplementation demonstrated a 10.02% ($P < 0.001$) and 8.56% ($P = 0.01$) decrease, respectively, compared to placebo (**Figure 2**). However, secondary outcomes, including TG, HDL-C, ApoA, and free fatty acids concentrations, did not differ between the two groups throughout the study period.

Safety

All subjects completed the protocol without any adverse or serious AEs. There were no subject complaints in either of the groups. After the 12-week trial, there were two cases of diastolic BP exceeding 100 mmHg in the placebo group but none in the UME group. No significant changes in liver enzymes, glucose, or creatinine concentrations were observed between the two groups during the 12-week trial. Comparatively, diastolic BP in the UME group was lower than in the placebo group after 12 weeks of the trial (mean difference: −3.44 mmHg, $P = 0.006$). However, there was no significant difference in the systolic BP (**Table 5**).

2 http://www.r-project.org/

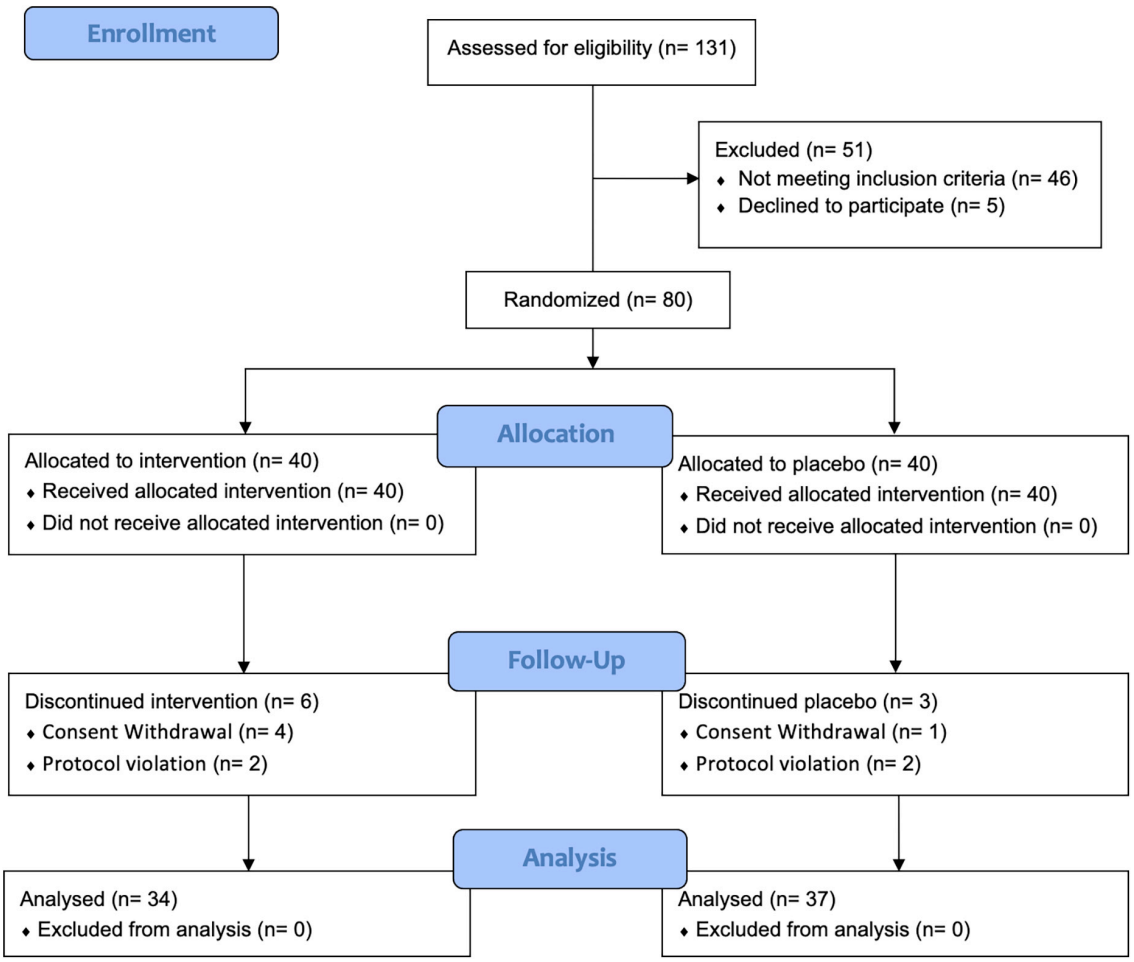

FIGURE 1
Consolidated standards of reporting trials (CONSORT) flow diagram.

Discussion

Ulmus macrocarpa Hance (UMH) is a large shrub endemic to the Far East. The stem and root bark of UMH have been used as traditional herbs to treat various conditions such as swelling, stomach disease, enteritis, dysuria, skin disease, mastitis, and arthritis (15, 20). Catechins, such as EGC, EGCG, EC, and EC, have several health benefits, such as antioxidant, antimicrobial, anti-inflammatory, and antiviral activities (21–23). Although catechins are the main components of green tea and UMC, EGCG is the most abundant catechin in green tea, and EGC and EC are the most abundant catechin in UMC. Green tea extract could suppress the mRNA level of HMGCR and increase the level of LDL receptors, leading to a lowered cholesterol level in mice fed with high-fat and high-sucrose diets. EGCG and EC could lower TC, LDL-C, and TG and increase HDL-C in hyperlipidemic rats (21–23). Green tea, containing catechin, was shown to remarkably reduce concentrations of LDL-cholesterol in humans (21–23). Previous studies have reported that UME has significant pharmacological potential, including antimicrobial, antioxidative, antiallergic, anti-inflammatory, antiplatelet, antihypertensive, and vasorelaxant effects (15, 20, 24). Recent studies have demonstrated that UME attenuates testosterone propionate-induced benign prostate hyperplasia *via* its pro-apoptotic and anti-proliferative activities (25); inhibits *Heliobacter pylori* colonization synergistically, especially when used in combination with *Rubus crataegifolius* (26); and prevents anti-photoaging of the skin by activating antioxidant enzymes and inhibiting the mitogen-activated protein kinase pathways (27). However, no study has assessed the effects of UME on lipid profiles in humans. Cardiovascular disease is still the major cause of morbidity and mortality. Despite the availability of different pharmacological drugs, new approaches are needed due to side effects and the general skepticism of many patients. Therefore, this study was designed as a primary prevention approach.

Our study evaluated the positive effect of UME on lipid profiles, which is another potential use of UME. To the best of our knowledge, this is the first randomized, double-blind, placebo-controlled trial to investigate the efficacy and safety of UME supplementation on lipid metabolism in adults with untreated high LDL-C concentrations. Our study showed that a 500-mg daily supplement of UME administered over 12 weeks positively affected the lipid profiles in adults aged with LDL-C concentrations ranging from 130 to 190 mg/dl. Supplementation of UME over 12 weeks led to a decrease in

TABLE 1 Baseline characteristics of the study group.

Variables	Intention-to-treat population			Per protocol population		
	UME group (n = 40)	Placebo group (n = 40)	P^1	UME group (n = 34)	Placebo group (n = 37)	P^1
Age, year	50.6 ± 10.1	50.7 ± 9.6	0.955	49.2 ± 9.9	50.1 ± 9.4	0.685
Male, %	15 (37.5)	14 (35.0)	0.816	13 (38.2)	14 (37.8)	0.973
BMI, kg/m^2	24.8 ± 2.9	24.8 ± 3.9	0.908	24.7 ± 2.8	25.0 ± 4.0	0.673
Systolic BP, mmHg	126.0 ± 13.9	127.2 ± 13.4	0.696	126.4 ± 14.1	128.3 ± 12.8	0.551
Diastolic BP, mmHg	82.6 ± 8.3	82.4 ± 10.7	0.935	82.4 ± 8.4	83.4 ± 10.2	0.676
Alcohol drinker, %	11 (27.5)	5 (12.5)	0.244	11 (32.3)	5 (13.5)	0.165
Moderate[2]	9 (22.5)	4 (10.8)		9 (26.5)	4 (10.8)	
Heavy[3]	2 (5.0)	1 (2.5)		2 (5.9)	1 (2.7)	
Current smoker, %	3 (7.5)	1 (2.5)	0.615	3 (8.8)	1 (2.7)	0.344
Energy intake, Kcal/day	1,754.4 ± 841.2	1,516.8 ± 396.9	0.110	1,812.5 ± 894.2	1,533.6 ± 404.5	0.090
Carbohydrate, %	58.7 ± 9.6	61.6 ± 9.6	0.173	57.9 ± 9.9	61.0 ± 9.2	0.166
Fat, %	25.4 ± 7.4	22.0 ± 7.4	0.048	25.9 ± 7.5	22.5 ± 6.6	0.044
Protein, %	15.8 ± 2.6	15.7 ± 3.3	0.807	15.8 ± 2.7	15.8 ± 3.3	0.954
IPAQ, METs	982.5 [535.5–2,283.5]	1,428.0 [714.0–2,171.0]	0.870	949.5 [495.0–2,274.0]	1,470.0 [724.5–2,245.5]	0.696
Adherence, %	93.7 ± 5.8	94.1 ± 4.4	0.718	94.1 ± 5.7	94.3 ± 4.1	0.889

Values are mean ± SD, median [IQR] or n (%). UME, *Ulmus macrocarpa* Hance extract; BMI, body mass index; BP, blood pressure; IPAQ, international physical activity questionnaires; MET, metabolic equivalent task.
[1] P-value by two-sample t-test for parametric variables, Mann–Whitney's U test for non-parametric variables, and Chi-square test, or Fishers exact test for categorical variables.
[2] Moderate, 2 drinks or less in a day for men or 1 drink or less in a day for women, on days when alcohol is consumed.
[3] Heavy, more than moderate.

TABLE 2 Energy intake and physical activity between the two groups for 12 weeks.

	UME group		Placebo group		Adjusted difference of UME vs. placebo over 12 weeks	P^1
	Baseline	12 weeks	Baseline	12 weeks		
Intention to treat (n = 80)						
Energy intake, Kcal/day	1,754.4 ± 841.2	1,769.0 ± 601.2	1,516.8 ± 396.9	1,533.8 ± 467.2	135.02 (−75.20, 345.24)	0.205
Carbohydrate, %	58.7 ± 9.6	57.2 ± 10.9	61.6 ± 9.6	59.1 ± 12.9	−1.49 (−6.89, 3.91)	0.584
Fat, %	25.4 ± 7.4	25.9 ± 9.0	22.0 ± 7.4	24.4 ± 9.9	0.73 (−3.55, 5.01)	0.734
Protein, %	15.8 ± 2.6	16.0 ± 3.4	15.7 ± 3.3	17.4 ± 6.1	−1.49 (−3.63, 0.65)	0.169
IPAQ, METs	982.5 [515.3–2,288.3]	1,282.5 [459.0–1,750.1]	1,428.0 [703.5–2,182.5]	1,490.0 [681.8–2,398.0]	3.44 (−570.47, 577.35)	0.445
Per protocol (n = 71)						
Energy intake, Kcal/day	1,812.5 ± 894.2	1,783.1 ± 638.4	1,533.6 ± 404.5	1,563.7 ± 472.5	104.07 (−130.52, 338.67)	0.379
Carbohydrate, %	57.9 ± 9.9	57.1 ± 11.5	61.0 ± 9.2	58.3 ± 12.9	−1.00 (−6.95, 4.94)	0.738
Fat, %	25.9 ± 7.5	25.7 ± 9.6	22.5 ± 6.6	24.9 ± 10.0	0.15 (−4.62, 4.93)	0.949
Protein, %	15.8 ± 2.7	16.1 ± 3.4	15.8 ± 3.3	17.7 ± 6.2	−1.68 (−4.04, 0.67)	0.159
IPAQ, METs	949.5 [495.0–2,278.8]	1,282.5 [476.5–1,686.3]	1,470.0 [714.0–2,297.0]	1,584.0 [685.5–2,433.0]	−22.21 (−656.14, 611.73)	0.488

Values are mean ± SD or median [IQR] or mean (95% CI). UME, *Ulmus macrocarpa* Hance extract; IPAQ, international physical activity questionnaires; MET, metabolic equivalent task.
[1] ANCOVA or rank ANCOVA adjusted for each baseline value as covariates over the 12-week period.

TABLE 3 Primary and secondary outcome measures of the two groups (intention-to-treat population).

	UME group (n = 40)			Placebo group (n = 40)			Adjusted difference of UME vs. placebo			
	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks	Δ 6 weeks	P^1	Δ 12 weeks	P^1
LDL-C, mg/dl	152.5 ± 19.5	144.1 ± 21.0	139.0 ± 18.8	147.7 ± 17.1	145.6 ± 17.9	154.4 ± 19.9	−8.02 (−15.37, −0.67)	0.033	−18.05 (−25.00, −11.10)	<0.001
TC, mg/dl	238.2 ± 27.5	230.1 ± 30.7	225.3 ± 26.0	233.7 ± 27.0	231.7 ± 23.4	245.6 ± 28.0	−5.07 (−14.81, 4.67)	0.303	−23.29 (−33.64, −12.94)	<0.001
TG, mg/dl	104.5 [83.0–149.5]	123.0 [77.8–187.3]	104.0 [83.2–157.8]	120.0 [77.3–169.5]	119.0 [86.0–156.3]	117.0 [91.0–159.0]	13.38 (−10.13, 36.89)	0.423	10.42 (−11.53, 32.36)	0.657
HDL-C, mg/dl	58.4 ± 10.6	56.2 ± 12.7	56.7 ± 11.5	56.9 ± 14.3	55.9 ± 13.7	58.8 ± 13.9	−1.01 (−3.96, 1.94)	0.498	−3.23 (−6.64, 0.18)	0.063
ApoA1, mg/dl	148.0 ± 19.1	147.8 ± 23.6	146.0 ± 20.9	144.3 ± 26.5	144.8 ± 28.3	146.4 ± 25.1	−0.95 (−8.27, 6.37)	0.797	−3.61 (−11.16, 3.95)	0.345
ApoB, mg/dl	119.7 ± 15.9	116.8 ± 21.3	114.1 ± 20.9	123.8 ± 20.3	122.9 ± 18.6	126.7 ± 22.6	−3.69 (−11.21, 3.82)	0.331	−9.31 (−16.95, −1.66)	0.018
FFA, mg/dl	403.0 [277.3–551.0]		419.0 [242.0–615.0]	413.0 [312.0–551.8]		356.5 [259.5–520.8]	–	–	129.61 (−10.55, 248.67)	0.100
hs-CRP, mg/dl	0.1 [0.0–0.1]		0.1 [0.0–0.1]	0.1 [0.0–0.2]		0.1 [0.0–0.1]	–	–	−0.09 (−0.03, 0.20)	0.043

Values are mean ± SD, median [IQR] or mean (95% CI). UME, *Ulmus macrocarpa* Hance extract; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; FFA, free fatty acid; hs-CRP, high-sensitivity C-reactive protein. FFA and hs-CRP, not measured at 6 weeks.

[1]ANCOVA or rank ANCOVA adjusted for each baseline value and baseline dietary fat intake% as covariates over the 12-week period.

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TABLE 4 Primary and secondary outcome measures of the two groups (per protocol population).

	UME group (n = 34)			Placebo group (n = 37)			Adjusted difference of UME vs. placebo			
	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks	Δ 6 weeks	P^1	Δ 12 weeks	P^1
LDL-C, mg/dl	153.2 ± 20.8	142.5 ± 22.1	138.5 ± 19.3	147.1 ± 16.8	146.7 ± 17.7	155.3 ± 20.4	−8.85 (−16.55, −1.15)	0.025	−20.28 (−27.50, −13.07)	<0.001
TC, mg/dl	238.0 ± 28.9	230.0 ± 32.4	223.5 ± 26.4	233.0 ± 25.9	232.5 ± 23.4	247.5 ± 28.2	−6.50 (−16.80, 3.80)	0.212	−27.18 (−37.66, −16.69)	<0.001
TG, mg/dl	101.0 [81.8–142.8]	126.5 [79.3–181.8]	105.0 [87.0–157.3]	134.0 [78.5–174.0]	121.0 [88.0–162.5]	117.0 [94.0–162.5]	−11.94 (−10.05, 33.92)	0.517	3.55 (−14.35, 21.45)	0.991
HDL-C, mg/dl	58.7 ± 9.7	56.3 ± 12.6	56.4 ± 11.7	56.7 ± 14.4	55.8 ± 13.9	58.9 ± 14.4	−1.48 (−4.51, 1.54)	0.331	−3.43 (−7.29, −0.24)	0.037
ApoA1, mg/dl	148.2 ± 18.6	147.1 ± 22.0	145.8 ± 20.9	145.1 ± 26.1	145.1 ± 28.6	147.2 ± 25.5	−1.59 (−9.05, 5.88)	0.673	−3.43 (−10.92, 4.05)	0.363
ApoB, mg/dl	119.7 ± 16.4	116.2 ± 22.7	113.3 ± 21.3	123.6 ± 20.7	123.9 ± 18.2	127.9 ± 22.9	−5.76 (−13.83, 2.31)	0.159	−11.51 (−19.57, −3.46)	0.006
FFA, mg/dl	389.5 [293.8–545.0]		427.5 [285.5–669.5]	414.0 [307.0–551.5]		362.0 [261.0–554.0]	–	–	100.68 (−15.20, 216.56)	0.253
hs-CRP, mg/dl	0.1 [0.0–0.1]		0.1 [0.0–0.1]	0.1 [0.0–0.2]		0.1 [0.0–0.1]	–	–	0.07 (−0.03, 0.17)	0.064

Values are mean ± SD, median [IQR] or mean (95% CI). UME, *Ulmus macrocarpa* Hance extract; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; FFA, free fatty acid; hs-CRP, high-sensitivity C-reactive protein. FFA and hs-CRP, not measured at 6 weeks.

[1] ANCOVA or rank ANCOVA adjusted for each baseline value and baseline dietary fat intake% as covariates over the 12-week period.

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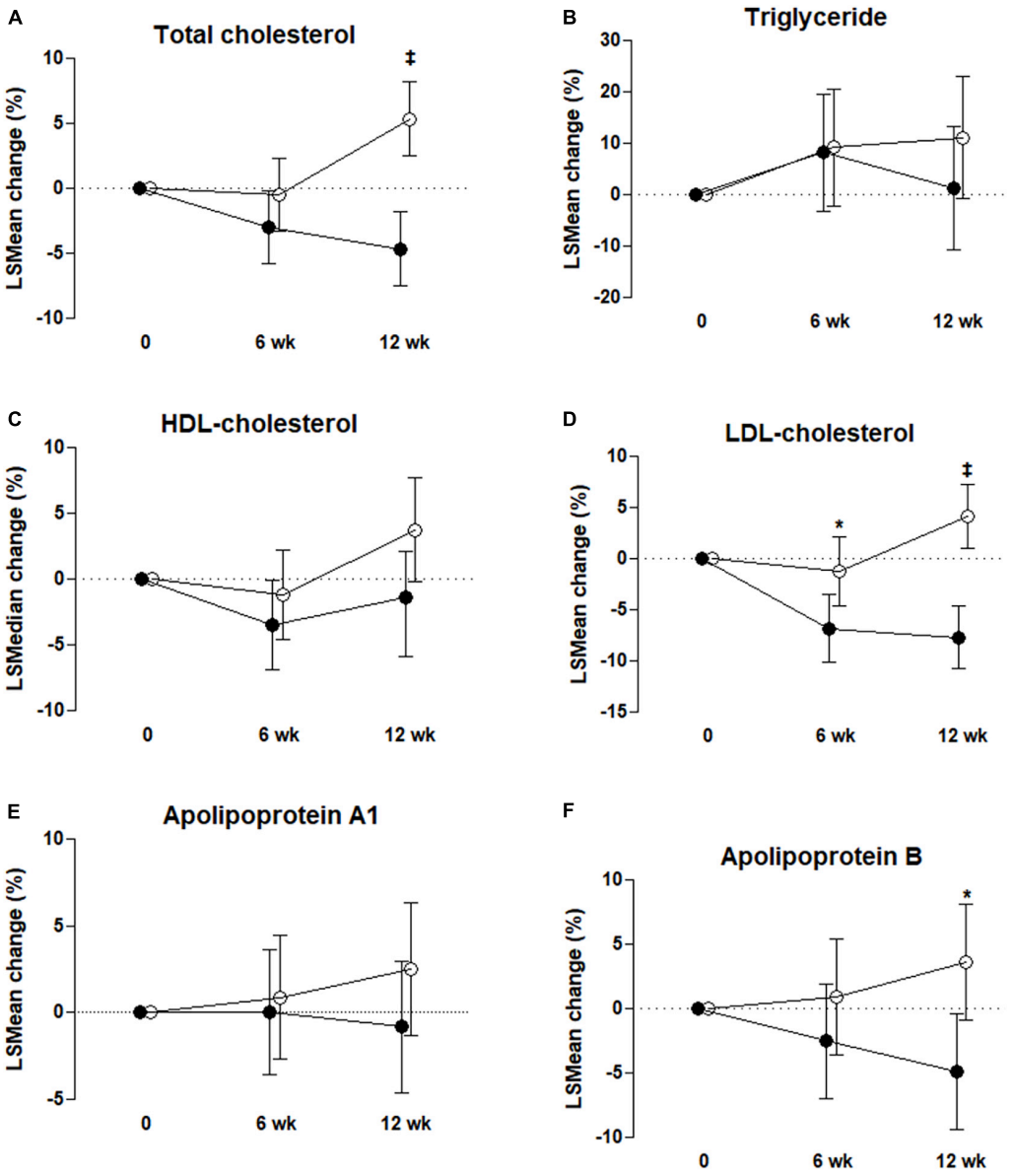

FIGURE 2
Percentage change from baseline to 6 and 12 weeks for total cholesterol **(A)**, triglyceride **(B)**, high-density lipoprotein (HDL)-cholesterol **(C)**, low-density lipoprotein (LDL)-cholesterol **(D)**, apolipoprotein A1 **(E)**, and apolipoprotein B **(F)** in the control group, ○, and UME group, ●. Values are mean ± SD except for triglyceride and HDL-cholesterol (median with IQR). $^{*}P < 0.05$, $^{‡}P < 0.001$, *P*-value by ANCOVA or rank ANCOVA with adjustment for each baseline value and baseline dietary fat intake % as covariates; intent-to-treat analysis.

LDL-C concentration by 17.71 mg/dl, TC concentration by 20.83 mg/dl, and ApoB concentration by 9.22 mg/dl, which was significant compared to the placebo group.

However, it had no favorable effects on the TG, HDL-C, and ApoA concentrations. No AEs were reported in this study. This is consistent with the results of our other previous study (20), and it can be said that the safety of UME has been proven. This may be because the optimal low dose was administered to minimize the possibilities of adverse effects and toxicity but to have lipid-lowering effects (28, 29). Interestingly, diastolic BP decreased at 12 weeks in the UME group compared to the placebo group. A study in spontaneously hypertensive rats reported that prolonged (42 days) administration with UME reduced systolic BP (24). Although it can be assumed that UME has vasorelaxant and antioxidant properties, it is necessary to reconfirm the effect of UME on BP in humans and to conduct further studies on the mechanism.

The mechanism underlying the effects of UME on lipid pathways and metabolism has been reported in a previous animal study (15). Han et al. (15) investigated the impact of

TABLE 5 Laboratory findings evaluating the adverse effects.

	UME group		Placebo group		Adjusted difference of UME vs. placebo over 12 weeks	P^1
	Baseline	12 weeks	Baseline	12 weeks		
Intention-to-treat (n = 80)						
Systolic BP, mmHg	126.0 ± 13.9	123.7 ± 14.3	127.2 ± 13.4	125.5 ± 12.1	−0.73 (−5.32, 3.86)	0.751
Diastolic BP, mmHg	82.6 ± 8.3	80.1 ± 11.2	82.4 ± 10.7	83.4 ± 9.9	−3.44 (−6.82, −0.06)	0.046
AST, IU/L	25.1 ± 6.9	24.1 ± 6.8	26.0 ± 7.2	26.7 ± 8.9	−2.28 (−5.71, 1.15)	0.190
ALT, IU/L	22.1 ± 11.2	23.2 ± 12.6	24.5 ± 13.3	26.0 ± 17.7	−1.49 (−7.76, 4.78)	0.637
Creatinine, mg/dl	0.75 ± 0.18	0.74 ± 0.18	0.73 ± 0.18	0.73 ± 0.20	−0.01 (−0.06, 0.04)	0.740
Glucose, mg/dl	94.6 ± 9.3	93.8 ± 8.1	93.4 ± 9.9	93.1 ± 11.6	0.18 (−3.82, 4.18)	0.929
Per protocol (n = 71)						
Systolic BP, mmHg	126.4 ± 14.1	122.9 ± 14.9	128.3 ± 12.8	125.2 ± 12.0	−1.00 (−5.90, 3.89)	0.684
Diastolic BP, mmHg	82.4 ± 8.4	79.7 ± 11.6	83.4 ± 10.2	83.8 ± 9.7	−3.33 (−6.96, −0.29)	0.071
AST, IU/L	25.7 ± 7.2	23.6 ± 6.5	25.4 ± 4.9	26.6 ± 9.1	3.20 (−0.40, 6.80)	0.081
ALT, IU/L	23.1 ± 11.7	22.5 ± 12.6	23.8 ± 10.8	26.1 ± 18.3	3.16 (−3.09, 9.42)	0.317
Creatinine, mg/dl	0.76 ± 0.18	0.75 ± 0.18	0.74 ± 0.18	0.74 ± 0.18	0.01 (−0.03, 0.04)	0.752
Glucose, mg/dl	95.0 ± 9.7	93.8 ± 8.2	93.4 ± 10.2	93.3 ± 11.9	0.24 (−4.15, 4.63)	0.913

Values are mean ± SD or mean (95% CI). UME, *Ulmus macrocarpa* Hance extract; BP, blood pressure; AST, aspartate transaminase; ALT, alanine transaminase.
[1]ANCOVA adjusted for each baseline value as covariates over the 12-week period.

UME administration on lipid accumulation in HepG2 cells and hyperlipidemia in HCD-induced Sprague Dawley rats. They observed that, at the treatment concentrations of 50 and 100 μg/ml, UME attenuated OA-induced lipid accumulation *via* activation of the AMPK pathway in a dose-dependent manner. The oral administration of UME decreased the concentrations of TC, TG, and LDL-C and increased the concentration of HDL-C in HCD-induced hyperlipidemia rats. In addition, UME supplementation increased the expression of phosphorylated AMPK and phosphorylated acetyl CoA carboxylase proteins and decreased the expression of the sterol regulatory element binding protein-1 (SREBP-1) and HMGCR proteins in the experimental rats. These results suggest that UME has a favorable ameliorating effect on lipid profiles *via* activation of the AMPK pathway and regulation of lipid metabolism.

Unlike the results of a previous experimental study, which indicated that UME supplementation did not improve all lipid profiles, this human study showed that UME had a positive effect in lowering the TC and LDL-C concentrations but no effect on reducing TG concentration and in raising the HDL-C concentration. Such differences compared to the previous study could be partly explained by a relatively normal range of TG and HDL-C concentrations in both groups at the start of the study. When the TG concentration is higher than 200 mg/dl or the HDL-C concentration is lower than 40 mg/dl, it is traditionally defined as dyslipidemia (30). However, since our study focused on patients with high LDL-C concentrations, TG and HDL-C concentrations were relatively normal at the beginning of the study. Thus, it is presumed that there was no further change when UME supplementation was administered. For this reason, more studies may be needed to verify the effect of UME on lipid profiles in subjects with higher TG or lower HDL-C concentrations.

Epidemiological studies have suggested that ApoB predicts atherosclerotic risk better than traditional TC or LDL-C (31). Among bioactive natural compounds, red yeast rice extract, berberine, and flaxseed have some roles in reducing ApoB concentrations in clinical trials (32). The potential reported mechanisms regarding the effects of nutraceuticals on ApoB are decreased ApoB mRNA expression and secretion, increased upregulation of ApoB receptors, and enhanced protection of ApoB against oxidation (32). In our study, ApoB concentration in the UME group reduced by 9.22 mg/dl (7.8%) compared to that in the placebo group after 12 weeks of treatment. This finding was consistent with that observed in another experimental study (33). In the previous study (33), as in a study investigating the effect of isoflavone on lipid metabolism (34), a decrease in SREBP-2 was also observed. Hwang et al. (33) presumed this as a mechanism of apoB reduction (35), but further studies are warranted to understand the mechanism clearly.

This study has some limitations, including the lack of biological confirmation to determine the mechanism of action of UME on ApoB reduction. Because this study focused on subjects with untreated high LDL-C concentrations (130–190 mg/dl), the effect of UME in patients with elevated TG or low HDL-C concentrations remains unknown. Also, there were hardly any smokers included. The smoking rates for men and women in Korea are 40–50 and 4–8%, respectively (36). Considering that the male-to-female ratio of the subjects of this study was 1:1.8, the overall smoking rate of 5% was very low. Furthermore, physical activity and nutrition intake in this study

were assessed by IPAQ and 24-h dietary recall, respectively; therefore, the information may not represent the usual state of participants. Although the lipid-lowering effect of UME decreased more at 12 weeks than at 6 weeks, there is no data for more than 12 weeks, so the impact of using it for more than 12 weeks is unknown. Also, this study did not evaluate whether major adverse cardiovascular events, the endpoint of anti-lipid therapy, could be avoided. Despite these limitations, this study is still considered valuable owing to several strengths. First, to our knowledge, this is the first well-designed clinical study to examine the efficacy and tolerability of UME supplementation in adults with untreated high LDL-C concentrations. Another strength of this study is the use of valid self-report instruments to evaluate participants' physical activity and dietary intake.

Conclusion

In conclusion, UME supplementation could improve lipid profiles in adults with high LDL-C concentrations without toxicity or severe adverse effects. However, unlike the results of previous experimental studies, there was no decrease in the concentrations of TG or HDL-C. Further clinical studies are needed to determine the effect of UME supplementation in adults with high TG or low HDL-C concentrations.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board at Pusan National University Yangsan Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SL contributed to the conceptualization of the study, carried out the formal analysis of the data, and coordinated and supervised the entire project. YL and SL designed the methodology of the work, had an active role in the process of participant recruitment and data acquisition, contributed to the validation of results, worked together for data curation, wrote the work's draft, and reviewed the final document. Both authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1000428/full#supplementary-material

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