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Molecular biomarkers of cardiometabolic disease

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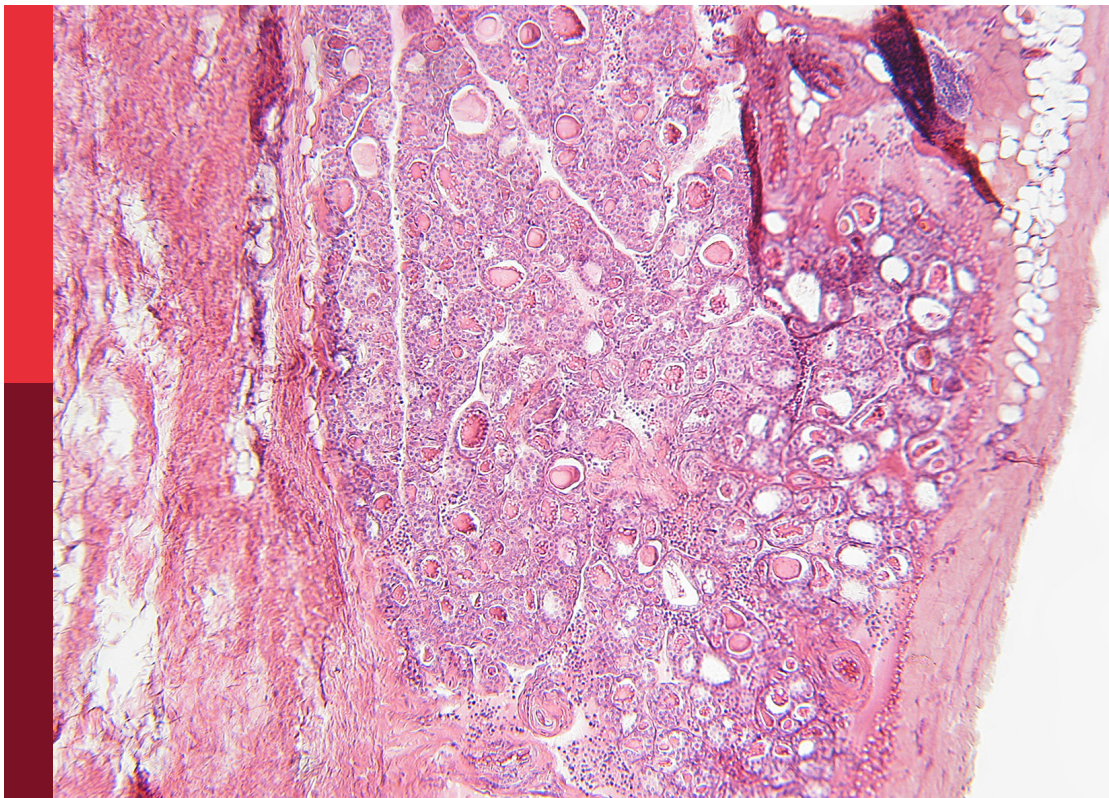

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Molecular biomarkers of cardiometabolic disease

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Editorial: Molecular biomarkers of cardiometabolic disease

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Editorial on the Research Topic
Molecular biomarkers of cardiometabolic disease

Cardiometabolic disease (CMD) is a leading global cause of mortality and refers to a complex sequence of pathophysiological processes resulting in both metabolic and cardiovascular disease (CVD). Central to its development is insulin resistance, which involves perturbed regulation of glucose levels, inflammation, and endothelial dysfunction. Timely detection, accompanied by appropriate intervention, is crucial in preventing CMD-associated complications such as type 2 diabetes mellitus (T2DM) and CVD. However, the major challenge of CMD management lies in its complex and diverse manifestations that often defy prediction based solely on traditional risk factors due to the absence of reliable and accurate molecular CMD biomarkers. The present Research Topic, "Molecular biomarkers of cardiometabolic disease" aims to showcase new developments in identifying, characterizing, and evaluating novel molecular biomarkers for CMD risk assessment, screening, and diagnosis/prognosis. By covering novel research advances and clinical trends in identifying and evaluating molecular CMD biomarkers, this Research Topic offers insights into novel potential diagnostic solutions that may support early interventions and improve outcomes.

This Research Topic has collected nine original research articles. The study by Garcia et al. reports that serum low-density lipoprotein receptor-related protein 1 (sLRP1) levels, which predict cardiovascular risk, are upregulated, whereas atrial natriuretic peptide (ANP) levels are downregulated in T2DM patients at disease onset. Increased sLRP1 and decreased ANP levels are normalized in the T2DM patients that reached optimal glycemic and metabolic control. The authors propose that the sLRP1/ANP ratio could be a reliable marker of cardiometabolic function and the cardiovascular benefits of glycemic control in T2DM patients.

Gou et al. combined experimental and bioinformatics approaches to investigate energy metabolism-related genes (EMRGs) in heart failure with preserved ejection fraction (HFpEF). Gene expression profiles in the HFpEF mouse dataset were compared to control groups to identify differentially expressed EMRGs (DE-EMRGs), and the potential biomarkers with diagnostic value were screened using machine learning

algorithms. The analysis revealed five potential diagnostic biomarkers for HFpEF as well as several promising therapeutic targets that deserve future investigation.

Chen et al. conducted a cross-sectional study to assess the relationship between serum testosterone (TT) and apoB in diverse populations exposed to different factors. The study reports a statistically significant, inverse correlation between serum TT concentration and apoB concentration. The authors suggest that an investigation of the correlation between serum TT and apoB may be used for screening individuals with CVD risk within the population that exhibits normal or low LDL-C levels.

A retrospective observational study by Bosco et al. investigated SLCO1B1 rs4149056 impact on LDL-C target achievement after lipid-lowering therapy optimization in men and women with familial hypercholesterolemia (FH). The authors found that the genotype effect of SLCO1B1 rs4149056 is more pronounced in FH women since the prevalence of subjects on the LDL-C target and high-intensity lipid-lowering therapy was significantly lower in FH women with SLCO1B1 rs4149056 than in other groups.

A study by Chen et al. reports that the serum level of fetuin-A, which is a glycoprotein that acts as an inhibitor of insulin secretion and arterial calcification progression (1), negatively correlates with the risk of TTAs and is accompanied by decreased descending thoracic aortic diameter. These findings suggest that monitoring of serum fetuin-A levels may be used in the early detection and diagnosis of TTA.

Su et al. performed a drug target Mendelian randomization (MR) analysis on seven genetic variants encoding lipid-lowering drug targets (LDLR, HMGCR, NPC1L1, PCSK9, APOB, APOC3, and LPL) to explore the impact of lipid-lowering drug targets on erectile dysfunction (ED). The results show that APOB inhibitors are associated with an increased risk of ED occurrence, whereas APOC3 inhibitors, LDLR, and LPL agonists are significantly associated with a reduced risk of ED occurrence. LDLR AND LPL agonists were also significantly associated with increased TT levels. The authors propose that APOB, APOC3, LDLR, and LPL may be new drug target candidates for ED treatment.

Xu et al. assessed clinical correlation and demographic characteristics of hyperhomocysteinemia (HHcy) within the Chinese urban population with hypertension, focusing on the identification of risk factors for HHcy in hypertensive patients. The authors report high HHcy prevalence in the Chinese urban population, as well as a significant association between homocysteine (Hcy) levels, gender, methylenetetrahydrofolate reductase (MTHFR) genotypes, and fatty acid (FA) levels. Male gender and the presence of the MTHFR genotype represent a significant risk factor for HHcy in the studied population. A study by Su et al. reports the absence of genetic evidence suggesting a causal association between plasma levels of Hcy, folate, vitamin B12, vitamin B6, and polycystic ovary syndrome (PCOS) in individuals of European ancestry.

Finally, the molecular mechanism responsible for the association between T2DM and atherosclerosis is investigated in a study by Qi et al. who combined identification of differentially expressed genes with bioinformatic enrichment analyses, protein-protein interaction network construction, and core genes identification. The authors also built a transcription factor-mRNA regulatory network and analyzed infiltrating immune cells. Four core genes (IL1B, C1QA, CCR5, and MSR1) that significantly correlate with common immune cells (B cells, CD4+ T cells, regulatory T cells, and M2 macrophages) were identified, together with five transcription factors that regulate their expression (RELA, NFκB1, JUN, TT1, and SPI1). These findings suggest that the interplay between transcription factors, core genes, and immune cells identified in this study may be important for elucidating molecular mechanisms underlying T2DM and atherosclerosis.

Our Research Topic has taken the initial step in assembling novel research findings on potential CMD biomarkers that may stimulate further discussion. Future studies are expected to refine the understanding of the molecular landscape of CMD by further evaluating the prognostic and diagnostic value of molecular biomarkers of CMD reported in this Research Topic.

Author contributions

MM: Writing – original draft. AK: Writing – review & editing. EI: Writing – review & editing.

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Conflict of interest

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

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Reference

1. Al Ali L, van de Vegte YJ, Said MA, Groot HE, Hendriks T, Yeung MW, et al. Fetuin-A and its genetic association with cardiometabolic disease. *Sci Rep*. (2023) 13:21469. doi: 10.1038/s41598-023-48600-9

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Increased sLRP1 and decreased atrial natriuretic peptide plasma levels in newly diagnosed T2DM patients are normalized after optimization of glycemic control

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Background: Low-density lipoprotein receptor-related protein 1 (LRP1) negatively modulates circulating atrial natriuretic peptide (ANP) levels. Both molecules are involved in the regulation of cardiometabolism.

Objectives: To evaluate soluble LRP1 (sLRP1) and ANP levels in people with newly diagnosed type 2 diabetes mellitus (T2DM) and determine the effects of metabolic optimization.

Methods: This single-center longitudinal observational study recruited patients with newly diagnosed T2DM (n = 29, HbA1c > 8.5%), and 12 healthy control, age- and sex-matched volunteers. sLRP1 and ANP levels were measured by immunoassays at T2DM onset and at one year after optimization of glycemic control (HbA1c ≤ 6.5%).

Results: T2DM had higher sLRP1 levels than the control group (p = 0.014) and lower ANP levels (p =0.002). At 12 months, 23 T2DM patients reached the target of HbA1c ≤ 6.5%. These patients significantly reduced sLRP1 and increased ANP levels. Patients who did not achieve HbA1c < 6.5% failed to normalize sLRP1 and ANP levels. There was an inverse correlation in the changes in sLRP1 and ANP (p = 0.031). The extent of sLRP1 changes over 12 months of metabolic control positively correlated with those of total cholesterol, LDL cholesterol, TG, TG/HDLc, and apolipoprotein B.

Conclusions: Newly diagnosed T2DM patients have an increased sLRP1/ANP ratio, and increased sLRP1 and decreased ANP levels are normalized in the T2DM patients that reached an strict glycemic and metabolic control. sLRP1/ANP ratio could be a reliable marker of cardiometabolic function.

KEYWORDS

sLRP1, ANP, metabolic control, T2DM, hypoglycemic treatments

Introduction

Patients with type 2 diabetes mellitus (T2DM) show higher incidence of heart failure and higher cardiovascular disease (CVD) mortality risk (1). Achieving optimal glycemic control is a clinical goal in persons with T2DM to reduce CVD risk, especially at early stages of T2DM (2, 3). However, new biomarkers connecting cardiovascular and metabolic alterations need to be developed for helping to prevent CVD in T2DM.

Low-density lipoprotein receptor-related protein 1 (LRP1) plays a crucial role in atherosclerosis progression (4, 5). Its soluble form (sLRP1) comprises the α chain (515 kDa) and a fragment of the β chain (55 kDa) of the cellular receptor. sLRP1 can be detected in the circulation after either constitutive or induced cleavage (6). Previous studies from our group have shown that atherogenic lipoproteins promote sLRP1 release from vascular smooth muscle cells of human atherosclerotic plaque explants, and that circulating levels of sLRP1 are associated with carotid atherosclerosis in familial hypercholesterolemic patients (7) and with carotid plaque inflammation measured by 18F-FDG PET in patients with a recent ischemic stroke (8). In line with this, we further demonstrated that circulating sLRP1 levels are also associated with coronary artery disease risk, independently of potential confounding factors (9).

LRP1 is overexpressed in advanced human atherosclerotic lesions enriched in lipids (10), in the vascular wall of hypercholesterolemic rabbits and pigs (5, 11, 12) and in myocardium of *in vivo* models of diabetic rats (13).

In the context of cardiometabolic diseases, epicardial fat expresses and releases an altered pattern of adipokines and other molecules that contribute to vascular and cardiac dysfunction (14, 15). As LRP1 is overexpressed in epicardial fat of T2DM patients (16), it could be hypothesized that dysfunctional epicardial fat contributes to increased circulating sLRP1 levels. Supporting this, sLRP1 levels directly correlate with epicardial fat volume in type 1 diabetes mellitus (T1DM) patients and in the general population (17, 18). Together, these results suggest that sLRP1 could be a dual biomarker of cardiac and metabolic alterations in T2DM.

Atrial natriuretic peptide (ANP) is secreted by cardiomyocytes (19), and its circulating concentration decrease under insulin resistance (20, 21). Our group has previously shown in a murine model that the circulating levels of ANP are modulated by cardiac LRP1 levels, and that the reduction of cardiomyocyte LRP1 levels increases the release of ANP that coordinately inhibits hepatic fatty acid (FA) synthesis and activates FA uptake and oxidation, limiting weight gain and enhancing whole-body energy consumption (22).

LRP1 levels in heart inversely modulate ANP levels (22), which is essential in the control of metabolism. In light of this, the sLRP1/ANP ratio could be used as an indicator that integrates information about metabolic and cardiovascular alterations. Our hypothesis is that the inverse relation between the circulating levels of sLRP1 and ANP in humans are favorably influenced by the optimization of the glycemic control. The aims of this study were i) to evaluate sLRP1 and ANP levels in newly diagnosed T2DM patients as compared to healthy controls, and ii) to determine the effect of achieving metabolic optimization on these circulating biomarkers.

Methods

Patients

This single-center, observational longitudinal study included 29 patients with T2DM (mean age 56 ± 9 years) who had been referred to the Endocrinology and Nutrition Department of Hospital de la Santa Creu i Sant Pau, Barcelona (Spain), as well as 12 age- and sex-matched control subjects. The study was approved by the Ethics Committee of the Hospital de Sant Pau (reference number of the protocol IIBSP-REL-2017-27, data of approval 07/26/2017). Written informed consent was obtained from all participants. This study was performed in full compliance with the Declaration of Helsinki. T2DM patients were newly diagnosed without previous hypoglycemic, lipid-lowering or anti-inflammatory drugs. Control group were normolipemic and normoglycemic subjects, with no personal or family history of premature coronary disease, major cardiovascular risk factors, or infectious or inflammatory disease.

All T2DM patients (n = 29) were studied at onset and at 12 months after treatment started. Patients were subjected to an intensive intervention carried out to achieve recommended targets of cardiovascular risk factors that includes diet, physical

Abbreviations: Apo, apolipoprotein; ANP, atrial natriuretic peptide; BMI, body mass index; CRP, C reactive protein; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; sLRP1, soluble low-density lipoprotein receptor-related protein 1; TC, total cholesterol; TG, triglycerides; T2DM, type 2 diabetes mellitus.

activity and pharmacotherapy, in accordance with clinical practice guidelines. Lipid profile, glucose, CRP and HbA1c levels were determined by routine procedures. All T2DM patients participated in a comprehensive diabetes self-management education program, which includes individualized instruction on nutrition, physical activity and optimizing metabolic control. According to our protocol for the management of severe hyperglycemia, the initial therapy included triple therapy with metformin, dipeptidyl peptidase inhibitors and basal insulin in 90% of patients. Basal insulin was suspended after 1–2 weeks, and non-insulin drugs were modified at the discretion of the responsible physician according to the characteristics of each patient.

Immunoassays

Blood samples were obtained at disease onset and 12 months of treatment start by venipuncture in EDTA-containing or additive-free Vacutainer tubes to obtain plasma or serum, respectively. Tubes were centrifuged for 15 min at 1500*g* at room temperature for a maximum period of 30 min. The resulting plasma or serum was aliquoted and stored at −80 until use. sLRP1 levels were measured in plasma using an ELISA kit from Cloud-Clone corp (SEB010Hu), and ANP levels were measured in serum using an ELISA kit from LSBio kit (LS-F57269).

Statistical analysis

In order to justify the validity of the kits used, we have revised the ELISA data in terms of sensitivity limits and reliability of results. The Intra-Assay CV for LRP1 ELISA Kit was <10% and for ANP ELISA Kit <6.9%. The coefficients of variation Inter-Assay was CV<12% for LRP1 ELISA kit and CV<8.7% for ANP ELISA Kit. These intra-assay and inter-assay coefficient of variation (CV) support the quality and feasibility of these assays to be used as evaluation tools. The power was between 0.78 and 0.96 with N of 29 patients and 8 controls for the variables sLRP1, ANP and sLRP1/ANP. The normality of numerical data distribution was verified using the Shapiro-Wilk test, and the homoscedasticity, with the Levene's test. The categorical variables are presented as frequencies and percentages, and the quantitative variables are presented as mean ± SD. A bivariate analysis was used for paired data, and a Chi-square analysis was used for categorical data. Relationships between different Δparameters and Δbiomarkers were assessed using Spearman correlation analysis. To determine the possible confounding factors between the associations of different variables, a simple linear regression test was used. Due to the small sample size, the analysis was validated using a non-parametric approach. All statistical analyses were performed with SPSS software, version 27. A two-sided p-value < 0.05 was considered statistically significant.

Results

Clinical characteristics

Table 1 summarizes the clinical and metabolic variables of newly diagnosed T2DM patients as compared to healthy controls. There were significant differences in weight (p = 0.031), body mass index (BMI) (p = 0.005), HbA1c (p < 0.001), blood glucose (p < 0.001), C-reactive protein (CRP) (p < 0.001), triglycerides (TG) (p < 0.001), high-density lipoprotein cholesterol (HDLc) (p = 0.002), TG/HDL index (p < 0.001) and ApoB (p = 0.044) between newly diagnosed T2DM patients and control subjects. All variables, except HDLc, were higher in the T2DM group. Age and sex distribution between groups was similar.

Glycemic control optimization normalizes the main metabolic and lipidic variables at a 12-month follow-up

As summarized in Table 1, the main variables that were optimized after 12 months of T2DM metabolic control were weight, BMI, HbA1c levels, glucose, HDLc and CRP.

From the total 29 T2DM patients, 23 reached a strict glycemic control (HbA1c ≤ 6.5) while only 6 remained in less strict glycemic control (HbA1c > 6.5). As shown in Table 1, most metabolic variables including weight, BMI, glucose, CRP, TC, LDLc, ApoB, TG/HDLc index and HDLc only significantly improved in the group reaching a strict glycemic control after 1 year.

Newly diagnosed T2DM patients showed increased sLRP1 and decreased ANP plasma levels compared to controls

As shown in Figure 1, circulating sLRP1 concentrations were significantly higher in the T2DM group at onset than in the control group (p = 0.014), while those of ANP were lower (p = 0.002). Therefore, the sLRP1/ANP ratio was much higher in new-onset T2DM patients than in healthy controls (p < 0.001). Possible correlations between sLRP1, ANP, sLRP1/ANP and clinical and metabolic variables was analyzed in T2DM patients. sLRP1 directly correlated with circulating TGs (r^2 = 0.475; p = 0.009), TG/HDLc index (r^2 = 0.445; p = 0.015) and ApoB (r^2 = 0.374; p = 0.046). ANP inversely correlated with circulating ApoB (r^2=-0.374; p = 0.046), CT (r^2 = -0.378; p = 0.043) and LDLc (r^2 = -0.367; p = 0.05). The ratio sLRP1/ANP directly correlated with ApoB (r^2 = 0.506; p = 0.005), TG/HDLc index (r^2 = 0.424; p = 0.022), LDLc (r^2 = 0.375; p = 0.045), TGs (r^2 = 0.392; p = 0.035), and TC (r^2 = 0.400; p = 0.032).

Metabolic optimization normalizes circulating levels of sLRP1 and ANP, and sLRP1/ANP ratio at a 12-month follow-up

To analyze the evolution of circulating levels of sLRP1 and ANP, and of the sLRP1/ANP ratio according to the glycemic control, the changes in these variables from T2DM onset (Pre) to T2DM-1-year (Post) were analyzed in strict glycemic control (HbA1c ≤ 6.5%) *versus* less strict glycemic control (HbA1c > 6.5%) patients (Figure 1).

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TABLE 1 Data are expressed as mean ± SD.

	Control (1)	T2DM onset (2)	T2DM-1year (3)	T2DM-1year HbA1c≥6.5 (4)	T2DM-1year HbA1c<6.5 (5)	*P* (2 *vs* 1)	*P* (3 *vs* 2)	*P* (4 vs 2)	*P* (5 vs 2)
Age (years)	53.58 ± 4.81	56.52 ± 9.41	57.52 ± 9.41	57.00 ± 6.16	55.67 ± 10.09	0.155			
Sex (M/F) (%)	75/25	75.9/24.1	75.9/24.1	100/0	71.4/28.6				
Weight (kg)	78.62 ± 12.67	92.71 ± 19.02	89.56± 14.71	83.63 ± 15.48	91.12 ± 14.45	0.031	0.038	0.435	0.008
BMI (Kg/m^2)	27.26 ± 2.59	33.12 ± 6.81	31.98 ± 5.60	29.23 ± 5.10	32.55 ± 5.65	0.005	0.049	0.422	0.039
HbA1c (%)	5.37 ± 0.23	11.91 ± 2.11	6.23 ± 0.70	7.23 ± 0.51	5.95 ± 0.43	<0.001	<0.001	0.008	<0.001
Glucose (mg/dL)	87.00 ± 10.95	156.12 ± 60.10	117.98±19.46	130.68 ± 16.05	114.36 ± 19.12	<0.001	0.010	0.366	0.004
CRP (mg/L)	1.48 ± 1.02	9.02 ± 7.52	3.96± 3.77	5.35 ± 4.73	3.57 ± 3.49	<0.001	0.005	0.542	0.006
TC (mg/dL)	188.28 ± 39.20	187.67 ± 39.01	179.87 ± 49.74	247.03 ± 32.06	160.68 ± 34.92	0.819	0.606	0.065	0.042
TG (mg/dL)	78.18 ± 34.63	152.19 ± 66.55	160.87 ± 118.98	282.91 ± 187.47	126.01 ± 62.00	<0.001	0.489	0.071	0.127
HDLc (mg/dL)	54.24 ± 12.76	39.16 ± 8.37	43.67 ± 7.56	42.44 ± 9.45	44.03 ± 7.17	0.002	0.004	0.593	0.003
LDLc (mg/dL)	118.28 ± 33.22	119.18 ± 32.60	106.03 ± 39.15	156.60 ± 28.79	91.59 ± 28.26	0.586	0.086	0.294	0.008
ApoB (mg/dL)	0.89 ± 0.28	1.06 ± 0.26	0.98± 0.33	1.34 ± 0.27	0.85 ± 0.24	0.044	0.150	0.138	0.011
TG/HDLc ratio	1.56 ± 0.93	4.21 ± 2.60	3.87 ± 3.13	7.05 ± 5.04	2.96 ± 1.57	<0.001	0.749	0.114	0.042

Comparison between groups was performed by Mann-Whitney U test and Paired Samples T-Test. BMI, Body Mass Index; HbA1c, glycated hemoglobin; CRP, C reactive protein; TC, Total cholesterol; TG, triglycerides; HDLc, High density lipoprotein cholesterol; LDLc , low density lipoprotein cholesterol; ApoB, apolipoprotein B, T2DM, type 2 diabetes mellitus.

After 1 year of treatment, sLRP1 levels were significantly downregulated in T2DM patients with a strict glycemic control, but upregulated in those with less strict glycemic control (Figure 1A). ANP levels were upregulated after 1 year of treatment in patients reaching a strict glycemic control but not in those that maintained HbA1c > 6.5% over 1 year of treatment, in which ANP levels did not change (Figure 1B). Coherently, sLRP1/ANP ratio was significantly downregulated after one year of glycemic control only in those patients that reached a strict glycemic control (Figure 1C).

Correlations between classical and new variables changes over 1 year were analyzed by Spearman correlation analysis. ΔsLRP1 directly correlated with changes in metabolic variables such as ΔWeight (r^2 = 0.643; p < 0.001), ΔBMI (r^2 = 0.636; p < 0.001), ΔTG (r^2 = 0.668; p < 0.001), ΔGlucose (r^2 = 0.481; p = 0.011), ΔTG/HDL (r^2 = 0.621; p = 0.001) and inversely with the change in ΔANP (r^2 = –0.402; p = 0.031). In addition, the reduction in ΔsLRP1 also directly correlated with reductions in circulating lipids including ΔTC (r^2 = 0.673; p < 0.001), ΔLDLc (r^2 = 0.534; p = 0.004), and ApoB (r^2 = 0.736; p < 0.001).

Furthermore, we analyzed whether the correlation between ΔsLRP1 and other variables, like ΔTG, ΔTC, ΔLDL, ΔApoB and ΔTG/HDL, was maintained by considering possible confounding factors, such as weight or BMI (by simple linear regression). The sLRP1 correlation adjusted for weight was significant with TG (p < 0.000), TC (p = 0.001), LDL (p = 0.002), TG/HDL (p < 0.000) and ApoB (p < 0.000). The sLRP1 correlation adjusted by BMI was also significant for TG (p = 0.001), TC (p = 0.002), LDL (p = 0.004), TG/HDL (p < 0.000) and ApoB (p = 0.001). Therefore, the correlation between the reduction in sLRP1 and lipid variables was independent of the reduction in weight or BMI.

Discussion

This study revealed for the first time that blood sLRP1 levels, which predict cardiovascular risk (9), are upregulated, while ANP levels, which inversely predict metabolic risk (21–23), are downregulated in T2DM patients at disease onset as compared to healthy controls. In addition, we showed that sLRP1 and ANP inversely evolved after 1 year of treatment.

The elevated circulating sLRP1 levels in T2DM patients found here are in line with the documented increased LRP1 levels in the epicardial fat of T2DM patients (16) and in the myocardium of diabetic rats (13), and also with the close association between blood

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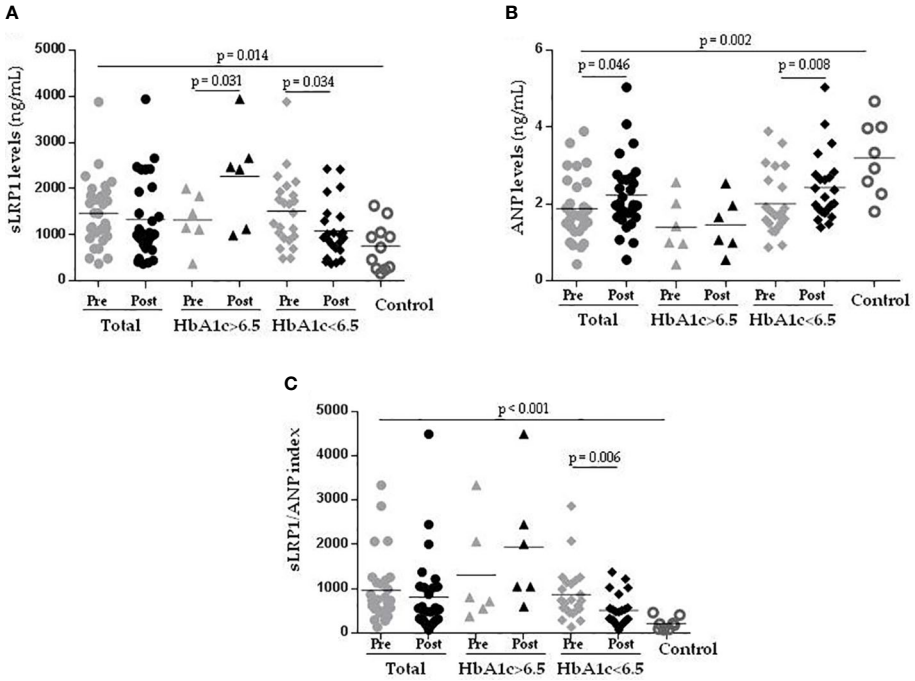

FIGURE 1
Newly diagnosed T2DM patients have increased sLRP1 and reduced ANP plasma levels that are normalized after 1 year of optimal glycemic control. Graph showing circulating levels of sLRP1 **(A)**, ANP **(B)** and sLRP1/ANP ratio **(C)** in control subjects (n = 12), Total (n = 27) (Pre =T2DM onset), (Post = T2DM-1year), T2DM-1year HbA1c>6.5 (n = 6) (Pre =T2DM onset), (Post = T2DM-1year), and T2DM-1year HbA1c ≤ 6.5 (n = 21) (Pre =T2DM onset), (Post = T2DM-1year). The horizontal line gives the mean of values from the same group. Comparisons between groups were analyzed with the Wilcoxon-test. T2DM, type 2 diabetes mellitus.

sLRP1 levels and the extension of epicardial fat in patients with type 1 diabetes and in the general population (17, 18). On the other hand, the low levels of ANP found in newly diagnosed T2DM patients are in line with the potential of low ANP levels to predict the development of diabetes in humans (23–27).

Previous studies from our group conducted in an experimental murine model evidenced that reduced levels of cardiac LRP1 promote increased circulating ANP levels, while increased levels of LRP1 favor decreased circulating ANP levels (22). Through the control of circulating ANP levels, cardiac LRP1 modulates not only fatty acid metabolism in the liver but also whole-body metabolism (22). Results from this experimental murine model highlight the presence of a functional LRP1-ANP link between heart, liver and adipose tissue. In the present study, a crucial point is that baseline sLRP1 levels are increased, while ANP are decreased, in newly diagnosed T2DM patients (without previous treatment) as compared to control subjects. In addition, if glycemic optimization is achieved after 1 year of treatment, there is a tight and inverse correlation between decreased sLRP1 levels and increased ANP levels. Taken together, these results suggest that the link between sLRP1 and ANP, previously described by our group in an *in vivo* model, is likely present in T2DM patients. In addition, our results support the concept that sLRP1 and ANP, which are key mediators of cardiometabolic mechanisms, are connected in humans and can play a key role in the interplay between cardiac and metabolic alterations.

Results from the present study show that strict glycemic control (HbA1c ≤ 6.5%) is highly efficient in reducing sLRP1 levels and increasing ANP levels in T2DM patients to the same levels found in the control group. These results suggest that an optimal glycemic control of T2DM patients may exert beneficial effects on parameters associated to cardiovascular risk, such as LRP1 (9). Currently, there is an intense debate about the potential and mechanisms of lowering HbA1c to provide protection against cardiovascular complications of T2DM (28, 29). Over the last decade, one of the proposed markers of glycemic control has been TG/HDLc (30–32). Results from the present study evidenced a positive correlation of sLRP1 with TG and with the TG/HDLc ratio in line with previous studies by our group showing the association of circulating TGs with epicardial LRP1 levels in T2DM patients (16). Here, we also showed that the decline in sLRP1 over 1-year caused by achieving a strict glycemic control correlated with the decrease in TGs and TG/HDL. This association between sLRP1 and TG remained after adjusting by weight and BMI. At this moment, the mechanisms underlying this association remain unclear. TG/HDLc has been proposed to be associated with CVD in the context of metabolic conditions (30–32), and sLRP1 has been reported to be predictive of cardiovascular risk in a case-cohort study (9). Therefore, it seems important to ascertain the mechanisms that determine the close association between sLRP1 and TG/HDLc in order to elucidate new mechanisms that are potentially involved in increasing the cardiovascular risk of patients with diabetes.

Here, we also observed that the reduction in sLRP1 after reaching optimal glycemic control correlated with the changes in other lipid parameters, such as TC, LDL-C and ApoB100. Such correlations were previously observed in different populations, in which sLRP1 is associated with cardiovascular risk (7–9). Further studies will be required to know whether sLRP1 reduction is

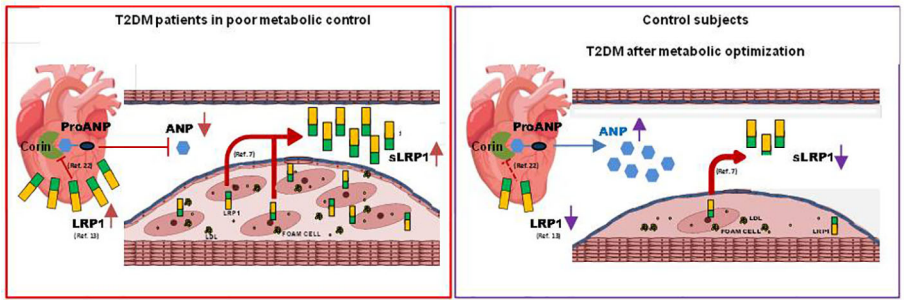

FIGURE 2
This graphical abstract combines previous findings from our group in a murine model of prediabetes with results obtained in the current study in T2DM patients. In the murine model, the reduction of cardiomyocyte LRP1 levels causes the increase of corin enzyme activity and ANP release (22). In addition, LRP1 levels are increased in the myocardium of diabetic hearts (13) and circulating sLRP1 levels correlate with atherosclerosis in humans (7). The main findings of the present study are that circulating levels of sLRP1 are increased while those of ANP are decreased in newly diagnosed type 2 diabetic patients (T2DM), and that altered sLRP1 and ANP levels are normalized in the T2DM group that reached an optimal metabolic control.

associated to a decrease of atherosclerosis in T2DM patients. In addition, further studies with increased numbers of participants and long-term follow-up are required to validate the potential of sLRP1 and sLRP1/ANP to predict the beneficial effects of lipidic/glycemic control in cardiac and metabolic alterations

Strengths and limitations

The main limitation of this study is the reduced number of participants due to the difficulty to recruit newly diagnosed untreated T2DM patients. One remarkable strength is the homogeneity of the newly diagnosed T2DM patients included in this study in terms of lack of previous hypoglycemic, lipid lowering or anti-inflammatory treatments.

Conclusion

Results from the present study show that the cardiac LRP1-ANP axis previously reported by our group in an experimental murine model of prediabetes is likely working in T2DM patients. The high levels of sLRP1 in the cardiovascular system of T2DM patients could lead to low circulating levels of ANP, explaining the high sLRP1/ANP ratio found in these patients as compared to healthy controls (summarized in Figure 2). Altered sLRP1 and ANP levels are normalized in the T2DM group that reached an optimal metabolic control. Therefore, we propose that sLRP1/ANP can be a potential marker of the cardiovascular benefits of glycemic control in T2DM patients. Further studies are required to know whether sLRP1/ANP index might improve the predictive value of other biomarkers in terms of cardiovascular and metabolic outcomes in T2DM patients.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Hospital de SantPau (protocol IIBSP-REL-2017-27). The patients/participants provided their written informed consent to participate in this study.

Author contributions

EG and PG performed ELISA assays, organized the database and did the statistical analysis. IM, JR and PG researched clinical data. AB-A collected data and contributed to the discussion. CR contributed to generation of the database. LC performed ELISA assays and collected data. JJ, SB and JS-Q designed the study and contributed to the generation of data and to discussion. XG-M and DV contributed to discussion of data. AP designed the study and wrote the manuscript. VLl-C designed the study, researched laboratory data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

References

1. Glovaci D, Fan W, Wong ND. Epidemiology of diabetes mellitus and cardiovascular disease. *Curr Cardiol Rep* (2019) 21:21. doi: 10.1007/s11886-019-1107-y

2. Llamazares Iglesias O, Sastre Marcos J, Peña Cortés V, Luque Pazos A, Cánovas Gaillemin B, Vicente Delgado A, et al. Metabolic and cardiovascular risk factor control in a diabetic cohort. *Four-year results. Endocrinol Nutr* (2012) 59:117–24. doi: 10.1016/j.endonu.2011.11.004

3. Wright AK, Suarez-Ortegon MF, Read SH, Kontopantelis E, Buchan I, Emsley R, et al. Risk factor control and cardiovascular event risk in people with type 2 diabetes in primary and secondary prevention settings. *Circulation* (2020) 142:1925–36. doi: 10.1161/CIRCULATIONAHA.120.046783

4. Llorente-Cortés V, Otero-Viñas M, Camino-López S, Llampayas O, Badimon L. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. *Circulation* (2004) 110:452–9. doi: 10.1161/01.CIR.0000136032.40666.3D

5. Bornachea O, Benitez-Amaro A, Vea A, Nasarre L, de Gonzalo-Calvo D, Escola-Gil JC, et al. Immunization with the Gly^{1127}-Cys^{1140} amino acid sequence of the LRP1 receptor reduces atherosclerosis in rabbits. Molecular, immunohistochemical and nuclear imaging studies. *Theranostics* (2020) 10:3263–80. doi: 10.7150/thno.37305

6. Quinn KA, Pye VJ, Dai YP, Chesterman CN, Owensby DA. Characterization of the soluble form of the low-density lipoprotein receptor-related protein (LRP). *Exp Cell Res* (1999) 251:433–41. doi: 10.1006/excr.1999.4590

7. de Gonzalo-Calvo D, Cenarro A, Martínez-Bujidos M, Badimon L, Bayes-Genis A, Ordonez-Llanos J, et al. Circulating soluble low-density lipoprotein receptor-related protein 1 (sLRP1) concentration is associated with hypercholesterolemia: A new potential biomarker for atherosclerosis. *Int J Cardiol* (2015) 201:20–9. doi: 10.1016/j.ijcard.2015.07.085

8. Garcia E, Camps–Renom P, Puig N, Fernandez–Leon A, Aguilera–Simon A, Benitez–Amaro A, et al. Soluble low–density lipoprotein receptor–related protein 1 as a surrogate marker of carotid plaque inflammation assessed by 18F–FDG PET in patients with a recent ischemic stroke. *J Trans Med* (2023) 21:131. doi: 10.1186/s12967-022-03867-w

9. de Gonzalo-Calvo D, Elosua R, Vea A, SubIrana I, Sayols-Baixeras S, Marrugat J, et al. Soluble low-density lipoprotein receptor-related protein 1 as a biomarker of coronary risk: Predictive capacity and association with clinical events. *Atherosclerosis* (2019) 287:93–9. doi: 10.1016/j.atherosclerosis.2019.06.904

10. Llorente-Cortés V, Otero-Viñas M, Berrozpe M, Badimon L. Intracellular lipid accumulation, low-density lipoprotein receptor-related protein expression, and cell survival in vascular smooth muscle cells derived from normal and atherosclerotic human coronaries. *Eur J Clin Invest* (2004) 34:182–90. doi: 10.1111/j.1365-2362.2004.01316.x

11. Llorente-Cortes V, Casani L, Cal R, Llenas A, Juan-Babot O, Camino-López S, et al. Cholesterol-lowering strategies reduce vascular LRP1 overexpression induced by hypercholesterolaemia. *Eur J Clin Invest* (2011) 41:1087–97. doi: 10.1111/j.1365-2362.2011.02513.x

12. Llorente-Cortés V, Otero-Viñas M, Sánchez S, Rodríguez C, Badimon L. Low-density lipoprotein upregulates low-density lipoprotein receptor-related protein expression in vascular smooth muscle cells: possible involvement of sterol regulatory element binding protein-2-dependent mechanism. *Circulation* (2002) 106:3104–10. doi: 10.1161/01.cir.0000041434.28573.0b

13. Samouillan V, Revuelta-López E, Dandurand J, Nasarre L, Badimon L, Lacabanne C, et al. Cardiomyocyte intracellular cholesteryl ester accumulation promotes tropoelastin physical alteration and degradation: Role of LRP1 and cathepsin S. *Int J Biochem Cell Biol* (2014) 55:209–19. doi: 10.1016/j.biocel.2014.09.005

14. Iacobellis G, Leonetti F. Epicardial adipose tissue and insulin resistance in obese subjects. *J Clin Endocrinol Metab* (2005) 90:6300–2. doi: 10.1210/jc.2005-1087

15. Patel VB, Shah S, Verma S, Oudit GY. Epicardial adipose tissue as a metabolic transducer: role in heart failure and coronary artery disease. *Heart Fail Rev* (2017) 22:889–902. doi: 10.1007/s10741-017-9644-1

16. Nasarre L, Juan-Babot O, Gastelurrutia P, Llucia-Valldeperas A, Badimon L, Bayes-Genis A, et al. Low density lipoprotein receptor-related protein 1 is upregulated in epicardial fat from type 2 diabetes mellitus patients and correlates with glucose and triglyceride plasma levels. *Acta Diabetol* (2014) 51:23–30. doi: 10.1007/s00592-012-0436-8

17. de Gonzalo-Calvo D, Vilades D, Nasarre L, Carreras F, Leta R, Garcia-Moll X, et al. Circulating levels of soluble low-density lipoprotein receptor-related protein 1 (sLRP1) as novel biomarker of epicardial adipose tissue. *Int J Cardiol* (2016) 223:371–3. doi: 10.1016/j.ijcard.2016.08.149

18. de Gonzalo-Calvo D, Colom C, Vilades D, Rivas-Urbina A, Moustafa AH, Pérez-Cuellar M, et al. Soluble LRP1 is an independent biomarker of epicardial fat volume in patients with type 1 diabetes mellitus. *Sci Rep* (2018) 8:16715. doi: 10.1038/s41598-018-19230-3

19. Goetze JP, Bruneau BG, Ramos HR, Ogawa T, de Bold MK, de Bold AJ. Cardiac natriuretic peptides. *Nat Rev Cardiol* (2020) 17:698–717. doi: 10.1038/s41569-020-0381-0

20. Rao S, Pena C, Shurmur S, Nugent K. Atrial natriuretic peptide: Structure, function, and physiological effects: A narrative review. *Curr Cardiol Rev* (2021) 17: e051121191003. doi: 10.2174/1573403X17666210202102210

21. Verboven K, Hansen D, Jocken JWE, Blaak EE. Natriuretic peptides in the control of lipid metabolism and insulin sensitivity. *Obes Rev* (2017) 18:1243–59. doi: 10.1111/obr.12598

22. Benitez-Amaro A, Revuelta-López E, Bornachea O, Cedó L, Vea À, Herrero L, et al. Low-density lipoprotein receptor-related protein 1 deficiency in cardiomyocytes reduces susceptibility to insulin resistance and obesity. *Metabolism* (2020) 106:154–91. doi: 10.1016/j.metabol.2020.154191

23. Magnusson M, Jujic A, Hedblad B, Engström G, Persson M, Struck J, et al. Low plasma level of atrial natriuretic peptide predicts development of diabetes: the prospective Malmo Diet and Cancer study. *J Clin Endocrinol Metab* (2012) 97:638–45. doi: 10.1210/jc.2011-2425

24. Gruden G, Landi A, Bruno G. Natriuretic peptides, heart, and adipose tissue: new findings and future developments for diabetes research. *Diabetes Care* (2014) 37:2899–908. doi: 10.2337/dc14-0669

25. Moro C. Targeting cardiac natriuretic peptides in the therapy of diabetes and obesity. *Expert Opin Ther Targets* (2016) 20:1445–52. doi: 10.1080/14728222.2016.1254198

26. Schlueter N, de Sterke A, Willmes DM, Spranger J, Jordan J, Birkenfeld AL. Metabolic actions of natriuretic peptides and therapeutic potential in the metabolic syndrome. *Pharmacol Ther* (2014) 144:12–27. doi: 10.1016/j.pharmthera.2014.04.007

27. Neeland IJ, Winders BR, Ayers CR, Das SR, Chang AY, Berry JD, et al. Higher natriuretic peptide levels associate with a favorable adipose tissue distribution profile. *J Am Coll Cardiol* (2013) 62:752–60. doi: 10.1016/j.jacc.2013.03.038

28. Taylor SI, Yazdi ZS, Beitelshees AL. Pharmacological treatment of hyperglycemia in type 2 diabetes. *J Clin Invest* (2021) 131:e142243. doi: 10.1172/JCI142243

29. Tian J, Ohkuma T, Cooper M, Harrap S, Mancia G, Poulter N, et al. Effects of intensive glycemic control on clinical outcomes among patients with type 2 diabetes with different levels of cardiovascular risk and hemoglobin A_{1c} in the ADVANCE trial. *Diabetes Care* (2020) 43:1293–9. doi: 10.2337/dc19-1817

30. Esan O, Wierzbicki AS. Triglycerides and cardiovascular disease. *Curr Opin Cardiol* (2021) 36:469–77. doi: 10.1097/HCO.0000000000000862

31. Scicali R, Giral P, D'Erasmo L, Cluzel P, Redheuil A, Di Pino A, et al. High TG to HDL ratio plays a significant role on atherosclerosis extension in prediabetes and newly diagnosed type 2 diabetes subjects. *Diabetes Metab Res Rev* (2021) 37:e3367. doi: 10.1002/dmrr.3367

32. Quispe R, Martin SS, Jones SR. Triglycerides to high-density lipoprotein-cholesterol ratio, glycemic control and cardiovascular risk in obese patients with type 2 diabetes. *Curr Opin Endocrinol Diabetes Obes* (2016) 23:150–6. doi: 10.1097/MED.0000000000000241

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Diagnostic potential of energy metabolism-related genes in heart failure with preserved ejection fraction

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Background: Heart failure with preserved ejection fraction (HFpEF) is associated with changes in cardiac metabolism that affect energy supply in the heart. However, there is limited research on energy metabolism-related genes (EMRGs) in HFpEF.

Methods: The HFpEF mouse dataset (GSE180065, containing heart tissues from 10 HFpEF and five control samples) was sourced from the Gene Expression Omnibus database. Gene expression profiles in HFpEF and control groups were compared to identify differentially expressed EMRGs (DE-EMRGs), and the diagnostic biomarkers with diagnostic value were screened using machine learning algorithms. Meanwhile, we constructed a biomarker-based nomogram model for its predictive power, and functionality of diagnostic biomarkers were conducted using single-gene gene set enrichment analysis, drug prediction, and regulatory network analysis. Additionally, consensus clustering analysis based on the expression of diagnostic biomarkers was utilized to identify differential HFpEF-related genes (HFpEF-RGs). Immune microenvironment analysis in HFpEF and subtypes were performed for analyzing correlations between immune cells and diagnostic biomarkers as well as HFpEF-RGs. Finally, qRT-PCR analysis on the HFpEF mouse model was used to validate the expression levels of diagnostic biomarkers.

Results: We selected 5 biomarkers (Chrna2, Gnb3, Gng7, Ddit4l, and Prss55) that showed excellent diagnostic performance. The nomogram model we constructed demonstrated high predictive power. Single-gene gene set enrichment analysis revealed enrichment in aerobic respiration and energy derivation. Further, various miRNAs and TFs were predicted by Gng7, such as Gng7-mmu-miR-6921-5p, ETS1-Gng7. A lot of potential therapeutic targets were predicted as well. Consensus clustering identified two distinct subtypes of HFpEF. Functional enrichment analysis highlighted the involvement of DEGs-cluster in protein amino acid modification and so on. Additionally, we identified five HFpEF-RGs (Kcnt1, Acot1, Kcnc4, Scn3a, and Gpam). Immune analysis revealed correlations between Macrophage M2, T cell CD4+ Th1 and diagnostic biomarkers, as well as an association between Macrophage and HFpEF-RGs. We further validated the expression trends of the selected biomarkers through experimental validation.

Conclusion: Our study identified 5 diagnostic biomarkers and provided insights into the prediction and treatment of HFpEF through drug predictions and network analysis. These findings contribute to a better understanding of HFpEF and may guide future research and therapy development.

KEYWORDS

heart failure with preserved ejection fraction, energy metabolism-related genes, diagnostic biomarkers, machine learning, immunoscape, targeted drug prediction

1 Introduction

Heart failure with preserved ejection fraction (HFpEF) is a type of heart failure that occurs when the diastolic function of the heart is impaired. In HFpEF, the cardiac ejection fraction remains within the normal range, but the condition is characterized by ventricular underfilling, left atrial dilatation, and elevated left atrial pressure (1). The prevalence of HFpEF is believed to be increasing and is associated with factors such as age, gender, obesity, and hypertension (2). The diagnosis of HFpEF relies on clinical symptoms, electrocardiogram, cardiac ultrasound, and other examination methods, but there are no specific diagnostic biomarkers (3). Therefore, it is crucial to identify potential biomarkers that can aid in the accurate diagnosis of HFpEF.

Energy metabolism is essential for maintaining normal physiological functions in the body, including processes like glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation. The heart's energy supply primarily relies on the metabolism of glucose, fatty acids, and lactate (4). Genes and proteins related to energy metabolism play critical roles in various human diseases, including cardiovascular disease (5), diabetes, and obesity (6). A deeper understanding of the molecular mechanisms underlying energy metabolism may provide insights into novel therapeutic approaches for HFpEF (7, 8).

To identify potential diagnostic biomarkers and therapeutic targets for HFpEF, this study conducted bioinformatics analysis using the mouse HFpEF transcriptome from the GEO database. Through screening and research, we identified five potential biomarkers and constructed a regulatory network based on energy metabolism-related genes. These biomarkers have the potential to serve as diagnostic biomarkers for HFpEF and may provide a foundation for discovering new therapeutic targets.

2 Materials and methods

2.1 Source of data

The GSE180065 dataset was sourced from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180065). The GSE180065 dataset (GPL24247) comprises RNA-seq data obtained from heart tissue samples of 10 HFpEF mice and five control mice. To construct the HFpEF mouse model in this publicly available dataset, wide-type male C57BL/6J mice were treated with a combination of high-fat diet and N[w]-nitro-l-arginine methyl ester (L-NAME) at a concentration of 0.5g/L for five weeks. The 325 energy metabolism-related genes (EMRGs) were downloaded from the NCBI and MsigDB databases.

2.2 Identification of DEGs

DEGs between the HFpEF and control groups were chosen by using the DESeq2 package (v 1.36.1) (9) in the GSE180065 dataset at P value < 0.05 and $|\log_2 FC| > 0.5$. The results of the differential analysis were illustrated by volcano map plotted by the ggplot2 package (v 3.4.1) (10). Next, the DEGs were intersected with the EMRGs to obtain DE-EMRGs.

2.3 Machine learning screening and performance evaluation of biomarkers

Three machine learning models were constructed based on DE-EMRGs by least absolute shrinkage and selection operator (LASSO), random forest (RF) and Support Vector Machine-Recursive Feature Elimination (SVM-RFE) algorithms to screen feature genes separately. LASSO regression profiling was carried out using the glmnet package (version 4.1-6) (11) to obtain LASSO-feature genes. RF analysis was performed using the caret package (v 6.0-86) based on DE-EMRGs, and genes with top 10 importance scores were screened as RF signature genes. Next, SVM analysis was performed. Finally, the genes included in the portfolio with the highest accuracy rate and lowest error rate were selected as SVM-RFE-feature genes. The biomarkers were screened by overlapping LASSO-feature genes, RF-feature genes and SVM-RFE-feature genes.

Subsequently, ROC curves were plotted using the pROC package (v 1.17.0.1) (12) to assess the diagnostic value of the biomarkers. In addition, logistic regression models were constructed using the biomarkers as a whole and the model was evaluated using ROC curves. Immediately after, the nomogram was constructed and visualized via regplot (v 1.1) and rms package

(v 4.1-1) (13). Next, the calibration curve was plotted to judge the model performance.

2.4 Single-gene GSEA analysis

In order to further explore the biomarkers related pathways and the functions they play, we performed a single-gene GSEA analysis. The single-gene GSEA analysis of biomarkers was carried out via clusterProfiler package (v 4.4.4) (14). The top 10 most significant results for each biomarker were visualized separately.

2.5 Construction of mRNA-drug interaction and TF-mRNA-miRNA networks

In order to find potential therapeutic small molecule drugs acting on biomarkers, we performed drug prediction. The drugs targeting the biomarkers (transformation into human genes) were predicted through the DrugBank database. A mRNA-drug network was constructed based on the predicted results. Then, NetworkAnalyst database was utilized to predict the targeting miRNAs and TFs of biomarkers. Lastly, the network was visualized using Cytoscape software (v 3.8.2) (15).

2.6 Consensus clustering analysis

The consensus clustering analysis was performed on the GSE180065 dataset utilizing the ConsensusClusterPlus package (v 1.60.0) (16) on the basis of biomarkers.

2.7 Screening and enrichment analysis of DEGs-cluster

DEGs-cluster between the subtypes were selected via the DESeq2 package (v 1.36.1) (9) with $P < 0.05$ and $|\log_2 FC| > 0.5$. Gene Ontology (GO) enrichment analysis of DEGs-cluster was executed via clusterProfiler package (v 4.4.4) (14) (P value < 0.05) and org.Mm.eg.db package (v 3.12.0).

2.8 Screening for HFpEF-related genes

This part of the analysis was carried out in order to obtain further information on the genes associated with the development of HFpEF. Firstly, the DEGs was intersected with the DEGs-cluster to obtain the intersected genes. Then, the protein-protein interaction (PPI) network was created on the basis of intersected genes via the STRING database (https://cn.string-db.org/). In this study, the topology of the PPI network was analyzed using the plugin cytoHubba, and the top 5 genes under the MCC algorithm were selected as HFpEF-RGs for subsequent analysis.

2.9 Immune-infiltration analysis

To obtain correlations of subtypes and biomarkers with immune cells, we performed an immune infiltration analysis. The immune score and proportions of immune cell subtypes for each sample in the GSE180065 dataset were computed via the xcell algorithm of the immunedeconv package (v 2.0.4) (17). In the first step, the differences in abundance of each immune cell between HFpEF and the control groups (differential immune cells 1) were compared and the results were presented by box plots. Then, differences in the proportion of immune cells were analyzed between subtypes (differential immune cells 2). In addition, the correlation of biomarkers with differential immune cells1 and the association of HFpEF-RGs with differential immune cells2 were computed using the Spearman method.

2.10 A 'two-hit' mouse model of HFpEF and echocardiography

Male wild-type (WT) C57BL/6 mice weighing about 20 g at the age of 8 weeks were obtained from the Hu'nan Silaikejingda Experimental Animal Co., Ltd, China. All applicable international and national guidelines for the care and use of animals were followed. Mice were divided into two treatment groups and exposed to a combination of a high-fat diet (HFD) (60% kilocalories from fat) and Nω-nitro-L-arginine methyl ester (L-NAME) (0.5 g l−1 in drinking water) or a standard (chow) diet for 15 weeks (18). The concomitant metabolic stress (obesity and metabolic syndrome) and mechanical stress (hypertension induced by constitutive NO synthases suppression) in mice—elicited by the aforementioned 'two-hit'—recapitulates the numerous systemic and cardiovascular features of HFpEF in humans. Transthoracic echocardiography was performed on mice at 15 weeks after treatment. A two-dimensional echocardiographic system (Philips iE33, Netherlands) was used to examine the cardiac function of the left ventricle by detecting and calculating the left ventricular systolic and diastolic indexes.

2.11 RNA isolation and quantitative real-time polymerase chain reaction

Eight pairs of frozen left ventricle tissue of mouse heart (8 HFpEF and 8 control samples) were collected. Afterwards, 16 samples were lysed with TRIzol reagent and total RNA was isolated following the manufacturer's instructions. The concentration of RNA was measured with a NanoPhotometer N50. Afterwards, RNA was reverse transcribed into cDNA using the SureScript First strand cDNA synthesis kit (Servicebio, Wuhan, China). The qRT-PCR reaction consisted of 3 μL of reverse transcription product, 5 μL of 2xUniversal Blue SYBR Green qPCR Master Mix, and 1 μL each of forward and reverse primer. All primer sequence information were shown in Table 1. The GAPDH gene served as an internal control, and the relative

expression of genes was determined using the $2^{-\Delta\Delta CT}$ method (19). Graphpad Prism 5 was used to make the graph and calculate the p-value.

2.12 Statistical analysis

All bioinformatics analyses were carried out in R language. And then, the data of different groups were compared by rank sum test. It was a truism that $P < 0.05$ was in significant difference, where $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***, and $P < 0.0001$: ****.

3 Results

3.1 Screening of DE-EMRGs

A total of 971 DEGs were identified through differential expression analysis based on the GSE180065 dataset (P value < 0.05 and $|\log_2 FC| > 0.5$; Supplementary Table 1). Among them, 599 genes were significantly upregulated, and 372 genes were significantly downregulated (Figure 1A). To identify the EMRGs within these DEGs, an intersection analysis was performed. The results revealed 31 overlapping genes (Figure 1B), with 18 genes showing upregulation and 13 genes showing downregulation in expression (Supplementary Table 2). Hence, these genes were defined as DE-EMRGs.

3.2 Screening and performance evaluation of biomarkers

In order to obtain biomarkers, we filtered DE-EMRGs using 3 machine learning algorithms. A total of 6 LASSO-feature genes (Chrna2, Rbp7, Gnb3, Gng7, Ddit4l, and Prss55) were screened by LASSO regression analysis (Figure 2A). Subsequently, a total of 10 RF-feature genes (Cd36, Chrna2, Nr1d1, Prss55, Cacna2d2, Selenom, Gnb3, Ddit4l, Gng7, and Cacna1d) were obtained after RF analysis (Figure 2B). The accuracy and error rate were computed and found that the SVM model had the highest accuracy rate and lowest error rate when it contained 17 genes (Figure 2C). Therefore, these 17 genes were selected as SVM-RFE-feature genes (Acot2, Cacna1d, Cacna2d2, Rbp7, Gnb3, Ddit4l, Chrna2, Selenom, Gng7, Prss55, Pparg, Ucp2, Nr1d1, Vdr, Npc1, S100a9, and 4930590J08Rik) for further analysis. Hence, a total of 5 biomarkers (Chrna2, Gnb3, Gng7, Ddit4l, and Prss55) were screened by overlapping LASSO-feature genes, RF-feature genes and SVM-RFE-feature genes (Figure 2D).

The ROC results revealed excellent diagnostic performance for both biomarkers and logistic regression models (Figures 3A–F). The nomogram on the basis of biomarkers was utilized to predict the risk of patients developing HFpEF (Figure 3G). The accuracy of the nomogram was relatively high, which was validated by the calibration curve (Figure 3H).

3.3 Single-gene GSEA analysis of biomarkers

In quick succession, single-gene GSEA was performed to explore the enriched regulatory pathways and molecular functions of each biomarker. Chrna2, Gng7, Ddit4l, and Prss55 were mainly enriched to GO terms such as aerobic respiration, ribonucleoprotein complex biogenesis, etc. (Figures 4A–D; Supplementary Tables 3-6). Gnb3 was mainly enriched to ribosome, energy derivation by oxidation of organic compounds, cellular amino acid metabolic process and so on (Figure 4E; Supplementary Table 7).

3.4 The TF-mRNA-miRNA and mRNA-drug networks of biomarkers

Considering the targeting drugs and regulatory factors of these diagnostic biomarkers, we constructed the mRNA-drug and TF-mRNA-miRNA networks. Through DrugBank database, 5 biomarkers were found that targeted by 97 therapeutic drugs (Figure 5A; Supplementary Table 8). The network included 25 drugs (Cimetidine, Nonoxynol-9, Polyethylene glycol and so on) for Chrna2, 25 drugs (Tetradecyl hydrogen sulfate (ester), Leuprolide, Cianidanol, Methyldopa and so on) for Gnb3, 25 drugs (Ursodeoxycholic acid, Caffeine, Rotavirus vaccine and so on) for Gng7, 25 drugs (Lactitol, Loxapine, Lixisenatide and so on) for Ddit4l, 15 drugs (Hydroxyethyl cellulose, Human adenovirus b serotype 7, Tyrphostin B56 and so on) for Prss55In addition, based on biomarkers, we obtained 73 miRNAs (mmu-miR-6921-5p, mmu-miR-6988-5p, mmu-miR-6998-5p, mmu-miR-7049-3p and so on) and 13 TFs (ETS1, NRF1, USF1 and so on) (Figure 5B;

TABLE 1 Primer sequences of PCR.

Gene	Forward primer (5–3)	Reverse primer (5–3)
Chrna2	AACAATGCAGACGGGGAGTTT	GGGAAGAAAGTCACGTCGATG
Gnb3	ATACTCCAGGGGCCATTCCT	GGGGAAGGGGTCCATTCTTG
Gng7	GCTTTGCTATATCGAGCCTGC	CCCAGCACTGAGGTTCCAAT
Ddit41	TGGATAGGATCGTGTGTGATGC	CGTTCCAATCAGGGAGTACAGTT
Prss55	CTGCTACTTGTTGCCCACAC	GAGGCGAGGAGAGCAGGTAT
GAPDH	CCTTCCGTGTTCCTACCCC	GCCCAAGATGCCCTTCAGT

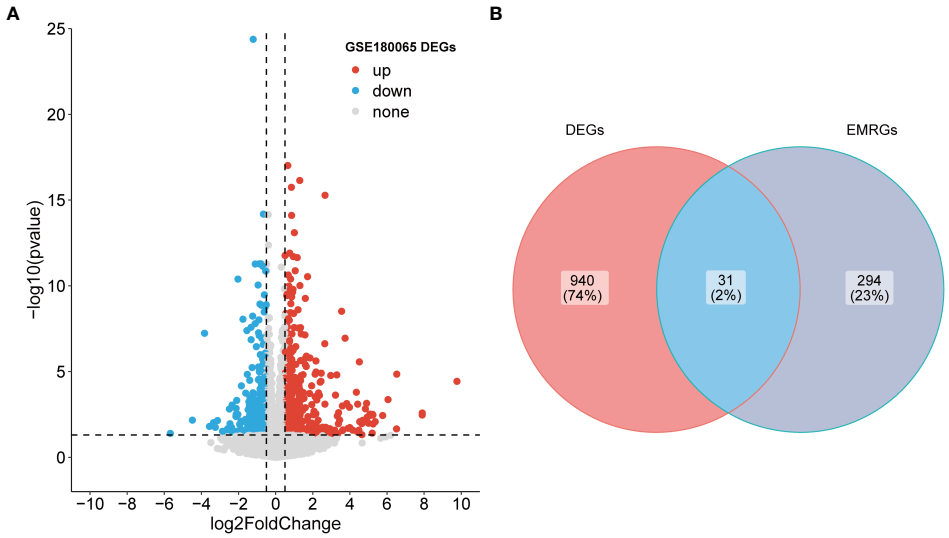

FIGURE 1
Identification of DE-EMRGs. **(A)** Volcano plot of DEGs. Red and blue dots indicate upregulated and down-regulated genes, respectively, and gray is the non-significant gene. **(B)** The intersection of DEGs with EMRGs. DE-EMRGs, differentially expressed energy metabolism-related genes; DEGs, differentially expressed genes; EMRGs, energy metabolism-related genes.

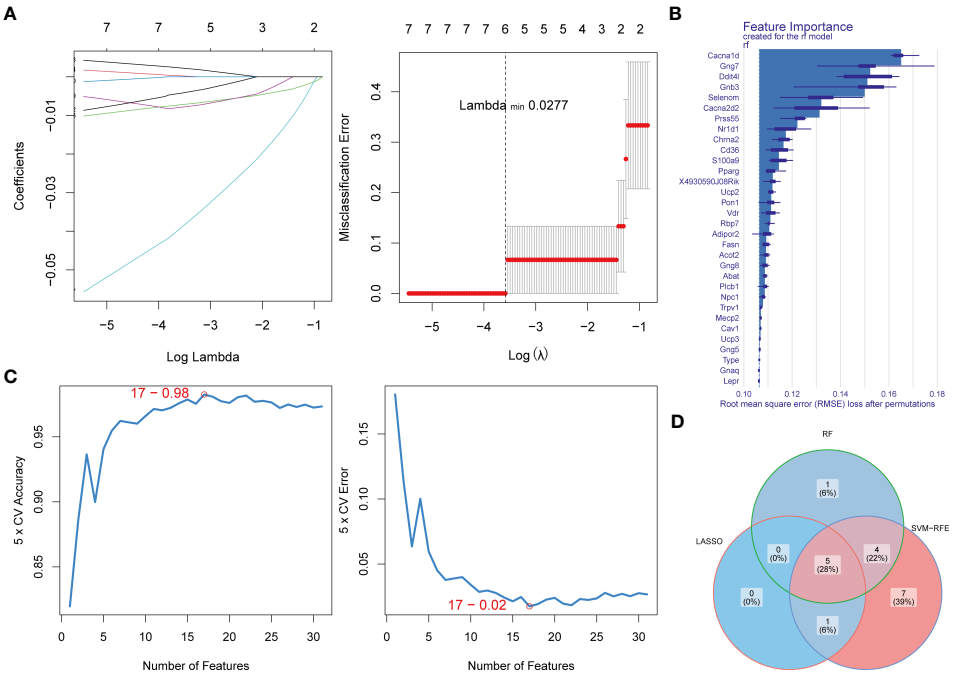

FIGURE 2
Identification of key DE-EMRGs using machine learning algorithms. **(A)** LASSO logistic regression algorithm used to screen key genes. **(B)** The importance of the variables was ranked by RMSE loss after permutations, with higher values indicating that the variable contributes greater to the accuracy of the model. **(C)** SVM-RFE algorithm to screen diagnostic biomarkers. **(D)** Venn diagram demonstrates the intersection of diagnostic biomarkers obtained by the three algorithms. DE-EMRGs, differentially expressed energy metabolism-related genes; LASSO, least absolute shrinkage and selection operator; RMSE, root mean square error; SVM-RFE, support vector machine-recursive feature elimination.

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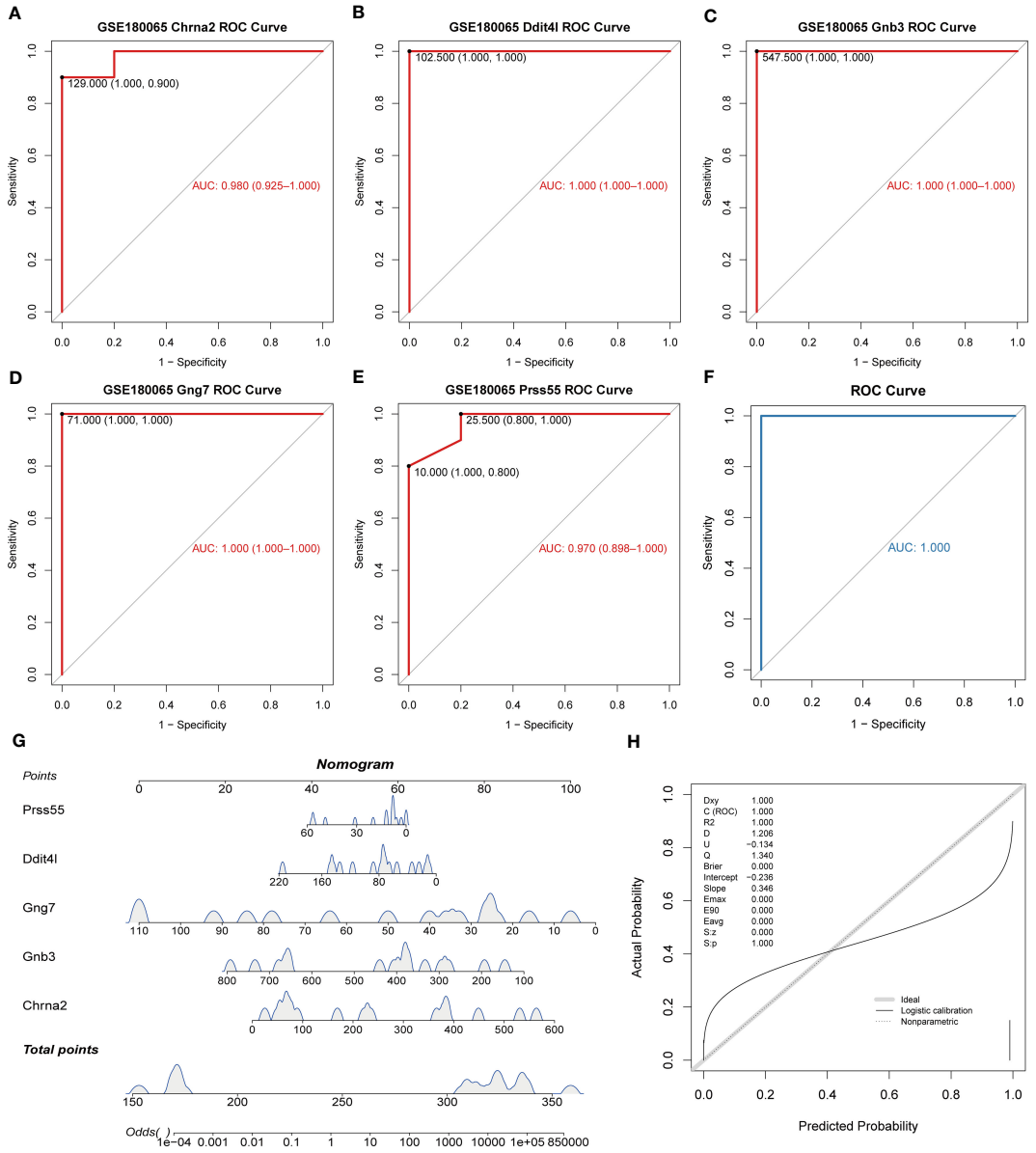

FIGURE 3
Diagnostic value of key DE-EMRGs in HFpEF. **(A–E)** The ROC curves of the key DE-EMRGs. **(F)** ROC curves of the diagnostic model in the GSE180065 dataset. **(G)** Nomogram for HFpEF samples. **(H)** Calibration curve to assess the predictive power of the nomogram. DE-EMRGs, differentially expressed energy metabolism-related genes; HFpEF, heart failure with preserved ejection fraction; ROC, receiver operating characteristic.

Supplementary Table 9). Among them, more miRNAs and TFs were predicted by Gng7. Among mmu-miR-7076-5p, mmu-miR-7030-5p, mmu-miR-7075-5p were common targets of Gng7 and Ddit4l, and mmu-miR-505-5p was shared by Gng7 and Chrna2. Besides, TFs SIN3A and MAZ might common to regulate Gng7 and Gnb3. TCF12 was related to Gnb3 and Prss55.

3.5 Identification of subtypes based on biomarkers and enrichment analysis

In order to perform a comparative analysis of the different subtypes of HFpEF, a consensus clustering analysis based on biomarkers was performed. The consensus clustering results revealed that the samples were clustered into 2 subtypes (Cluster1 and Cluster2), which had the discrimination between subtypes (Figures 6A, B). A total of 464 DEGs in different clusters were obtained. Among these clusters, Cluster 1 showed upregulation with 217 genes and downregulation with 247 genes compared to Cluster 2 (Figure 6C; Supplementary Table 10). The results of functional enrichment analysis indicated that DEGs-clusters were mainly enriched to GO entries such as the regulation of carbohydrate metabolic process and blood circulation, the N-terminal protein amino acid modification, regulatory T cell differentiation and so on (Supplementary Figure 1, Supplementary Table 11).

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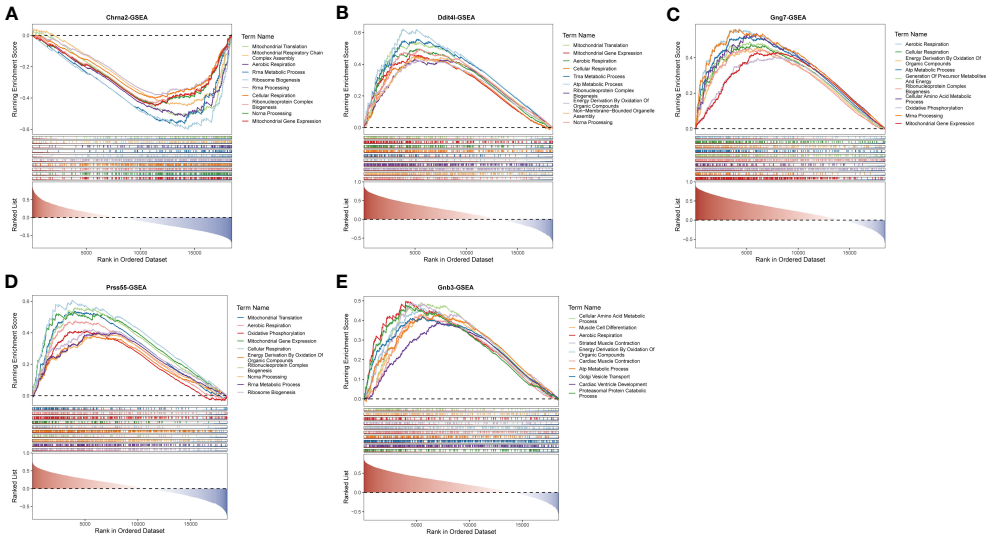

FIGURE 4
Single gene GSEA-GO for the 5 key DE-EMRGs. Enrichment in GO collection by Chrna2 **(A)**, Ddit4l **(B)**, Gng7 **(C)**, Prss55 **(D)**, and Gnb3 **(E)**. Each line represents one gene set with unique color. Gene sets were considered significant only when |NES| > 1, $P < 0.05$, and q < 0.05. Only several leading gene sets (Top 10) were displayed in the plot. GSEA, gene set enrichment analysis; GO, Gene Ontology; DE-EMRGs, differentially expressed energy metabolism-related genes; NES, normalized enrichment score.

3.6 PPI network analysis and acquisition of HFpEF-RGs

In order to further explore the crucial genes related to the occurrence and development of HFpEF based on the genes-based clusters of HFpEF, 55 intersected genes (Hmgcs2, Chrna2, Fbp2, Gpam and so on) were acquired by overlapping 971 DEGs (DEGs1) between the HFpEF and control groups and 464 DEGs (DEGs2) in different clusters (Supplementary Figure 2A). The PPI network of these 55 genes was built to understand the association among which (Supplementary Figure 2B), where Kcnt1 had the most interactions with the remaining genes. Furthermore, five core genes (Kcnt1, Acot1, Kcnc4, Scn3a, and Gpam) in the network were obtained as HFpEF-RGs using MCC algorithm (Supplementary Figure 2C).

3.7 Immune analysis of biomarkers and HFpEF-RGs

To obtain correlations between biomarkers and HFpEF-RGs with immune cells, we performed an immune-related analysis. The Xcell analysis demonstrated significant down-regulation of B cell and T cell CD4+ Th1 in the HFpEF group compared to the control group, whereas Hematopoietic stem cell and Macrophage M2 exhibited higher expression in the HFpEF group (all $P < 0.05$; Figure 7A). Additionally, the subsequent correlation analysis (Figure 7B) revealed a strong positive correlation between Macrophage M2 and Chrna2 (cor = 0.81, $P = 0.00044$). Furthermore, moderate negative correlations were observed with Gnb3 (cor = -0.7, $P = 0.0049$), Ddit4l (cor = -0.59, $P = 0.02$), and Prss55 (cor = -0.56, $P = 0.03$). As for T cell CD4+ Th1, it demonstrated strong and moderate positive correlations with Prss55 (cor = 0.81, $P = 0.00025$) and Ddit4l (cor = 0.52, $P = 0.047$), respectively, along with a moderate negative correlation with Chrna2 (cor = -0.66, $P = 0.047$). Conversely, B cell only exhibited a significant correlation with Chrna2, indicating a moderate negative relationship (cor = -0.56, $P = 0.029$).

In terms of differential expression between Cluster 1 and Cluster 2, Mast cell and Macrophage were the only cell types that exhibited significant differences, with both being significantly lower in Cluster 1 (Supplementary Figure 3A). The correlation analysis results indicated a moderate negative correlation between Acot1 and both Macrophage (cor = -0.54) and Mast cell (cor = -0.53). Furthermore, Macrophage showed a moderate negative correlation with Gpam (cor = -0.7) and Scn3a (cor = -0.53) (all $P < 0.05$; Supplementary Figure 3B).

3.8 Expression analysis of biomarkers

Echocardiography evaluation revealed significant alterations in left ventricular diastolic indexes, including interventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), and mitral ratio of peak early to late diastolic filling velocity (E/A), in the HFpEF group compared to normal mice. However, both groups exhibited preserved left ventricular ejection fraction (LVEF) (Figure 8).

To further investigate the expression changes of biomarkers in the HFpEF and control groups, eight pairs of HFpEF and control samples were collected and subjected to qRT-PCR. The results demonstrated that the expression levels of Gng7 and Prss55 were significantly lower in HFpEF samples compared to control tissues; conversely, the expression of Chrna2 was higher in HFpEF samples than in control tissues (Figures 9A–C), which aligns with findings

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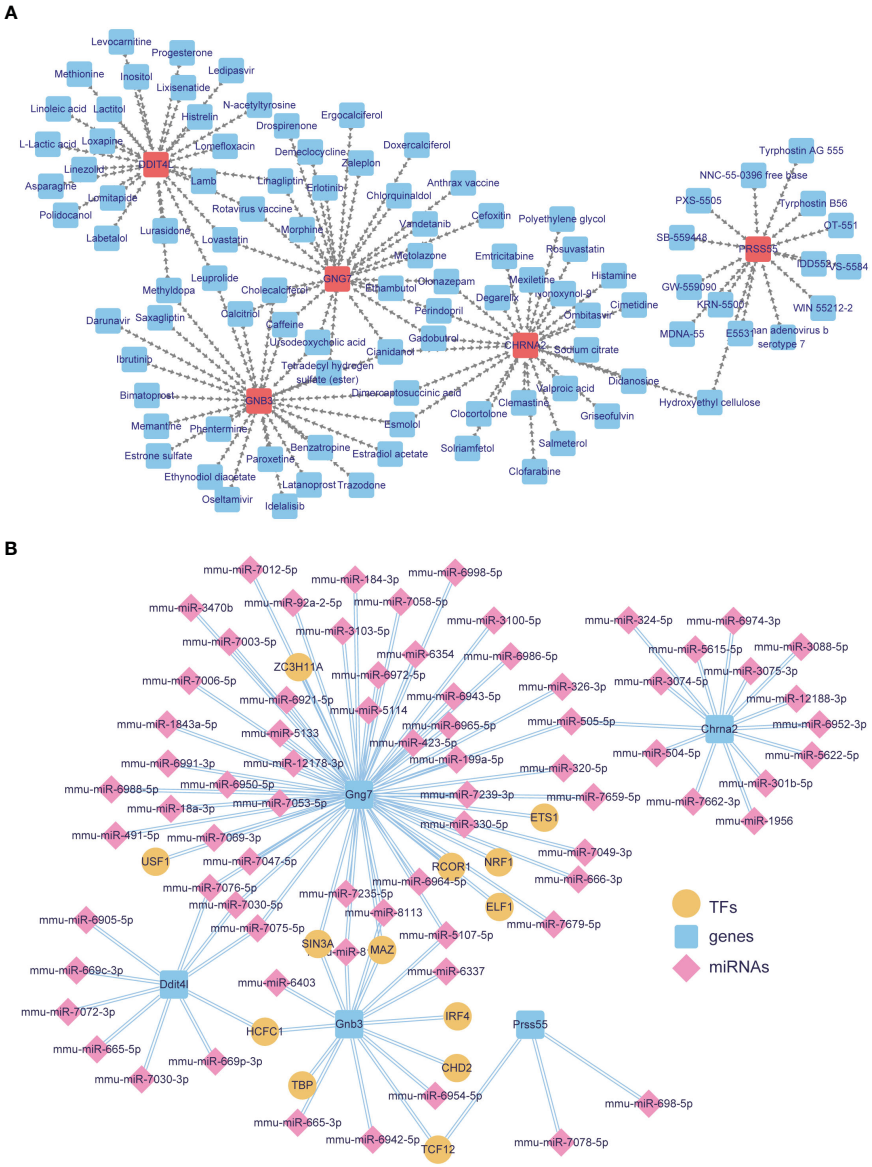

FIGURE 5
Targeted drugs and regulatory networks for key DE-EMRGs. **(A)** DrugBank-based drug-key DE-EMRGs interaction network. **(B)** Integrated miRNA-key DE-EMRGs and key DE-EMRGs-TFs interaction networks for the 5 biomarkers. Blue squares represent nine hub genes. Yellow circles represent TFs that have connectivity with biomarkers. Pink diamonds represent miRNAs associated with biomarkers. DE-EMRGs, differentially expressed energy metabolism-related genes; miRNA, microRNA; TFs, transcription factor.

from public databases. On the other hand, no significant differences in expression were observed for Gnb3 and Ddit4l between the two groups (Figures 9D, E).

4 Discussion

The pathogenesis of HFpEF remains complex and not fully understood. However, accumulating evidence suggests a significant association between energy metabolism and the development of this disease (20–22). In patients with HFpEF, abnormal energy metabolism in cardiomyocytes leads to pathological changes, including ventricular underfilling, left atrial dilatation, and elevated left atrial pressure (23). Therefore, investigating the expression changes of genes related to energy metabolism in HFpEF can shed light on their roles in disease progression and potentially offer novel targets for the diagnosis and treatment of HFpEF.

After conducting a differential gene analysis and integrating three distinct machine-learning methods in the online HFpEF mouse dataset (GSE180065), cholinergic receptor nicotinic alpha 2 subunit (Chrna2), DNA damage-inducible transcript 4-like (Ddit4l), guanine nucleotide binding protein beta 3 (Gnb3), guanine nucleotide binding protein gamma 7 (Gng7), and serine protease 55 (Pess55) emerged as promising potential diagnostic biomarkers for HFpEF associated with energy metabolism.

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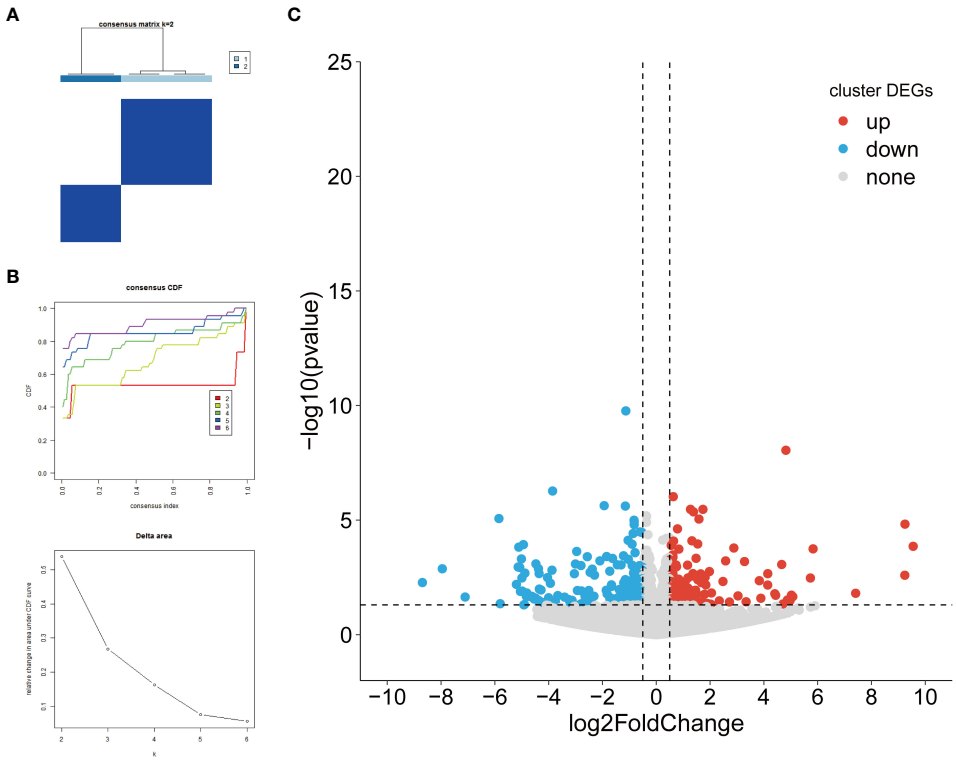

FIGURE 6
Identification of DEGs among biomarker-based subtypes. **(A)** Heatmap depicts consensus clustering solution (k = 2) for 5 biomarkers in 10 HFpEF samples; **(B)** Delta area curve of consensus clustering indicates the relative change in area under the CDF curve for k = 2 to 6. **(C)** Volcano plot of DEGs between Cluster1 and Cluster2. Red and blue dots indicate upregulated and down-regulated genes, respectively, and gray is the non-significant gene. DEGs, differentially expressed genes; HFpEF, heart failure with preserved ejection fraction; CDF, cumulative distribution function.

Subsequent qRT-PCR experiment on the our HFpEF mouse model that were collected from eight HFpEF and eight control samples validated the upregulation of Chrna2, and the downregulations of Gng7 and Prss55 in HFpEF, as did in public database.

Chrna2, the gene encoding acetylcholine receptor subunit α2, is expressed primarily in the nervous system (24). It is involved in signaling of the neurotransmitter acetylcholine and plays an important regulatory role in the nervous system. Additionally, it is closely associated with the onset and progression of a variety of neurological diseases (25). Although no direct association between Chrna2 and cardiovascular disease has been identified, acetylcholine regulates physiological processes in the cardiovascular system. For example, it controls the contraction and diastole of the heart and the dilation and contraction of blood vessels by binding to acetylcholine receptors on the heart and blood vessels (26). Furthermore, the results of Chrna2-GSEA in this study indicate its association with energy metabolism, angiogenesis and development, and cardiac ventricle morphogenesis. These findings suggest that Chrna2, as an energy metabolism-related gene, may have potential diagnostic value for HFpEF.

Ddit4l is a gene that plays an important role in cellular stress and DNA damage response. The expression of Ddit4l is regulated by a variety of factors such as oxygen levels, nutritional status, and cellular stress (27, 28). Bridget Simonson et al.'s study in mice with conditional cardiac-specific overexpression of DDiT4L (29) indicated that in the heart, DDiT4L may be an important pathway for pathological stress (such as metabolic stress) transduction to autophagy through the mTOR signaling pathway. This suggests that DDiT4L may be a therapeutic target in cardiovascular diseases when autophagy and mTOR signaling pathways play important roles. Our study also supports these findings, as the Ddit4l-GSEA results showed a close association between Ddit4l and apoptotic signaling regulation, cardiac conduction, cardiac contraction, and ventricular development. Additionally, through miRNA-Ddit4l network analysis, we identified that miR-669c-5p might regulate Ddit4l. Interestingly, previous research has shown that miR-669c-3p has a protective effect in a mouse model of ischemic stroke by enhancing alternative microglia/macrophage activation and inhibiting MyD88 signaling (30). This evidence further supports the important regulatory role of Ddit4l in the development of heart failure or HFpEF.

Gnb3 is a gene encoding the beta subunit of the G protein. It has been considered a candidate gene for hypertension, autonomic nervous system disorders, and coronary heart disease (31–33). A genetic association study has demonstrated a pathophysiological association between the genetic locus rs5443 (Gnb3) and ventricular remodeling in heart failure (34). In this study, Gnb3-GSEA results consistently showed its involvement in the development of cardiac chambers/ventricles, morphogenesis of the heart/ventricles, and development of ventricular myocardial tissue. These findings suggest that the Gnb3 gene may be associated with structural and functional abnormalities in the hearts of patients with HFpEF. On

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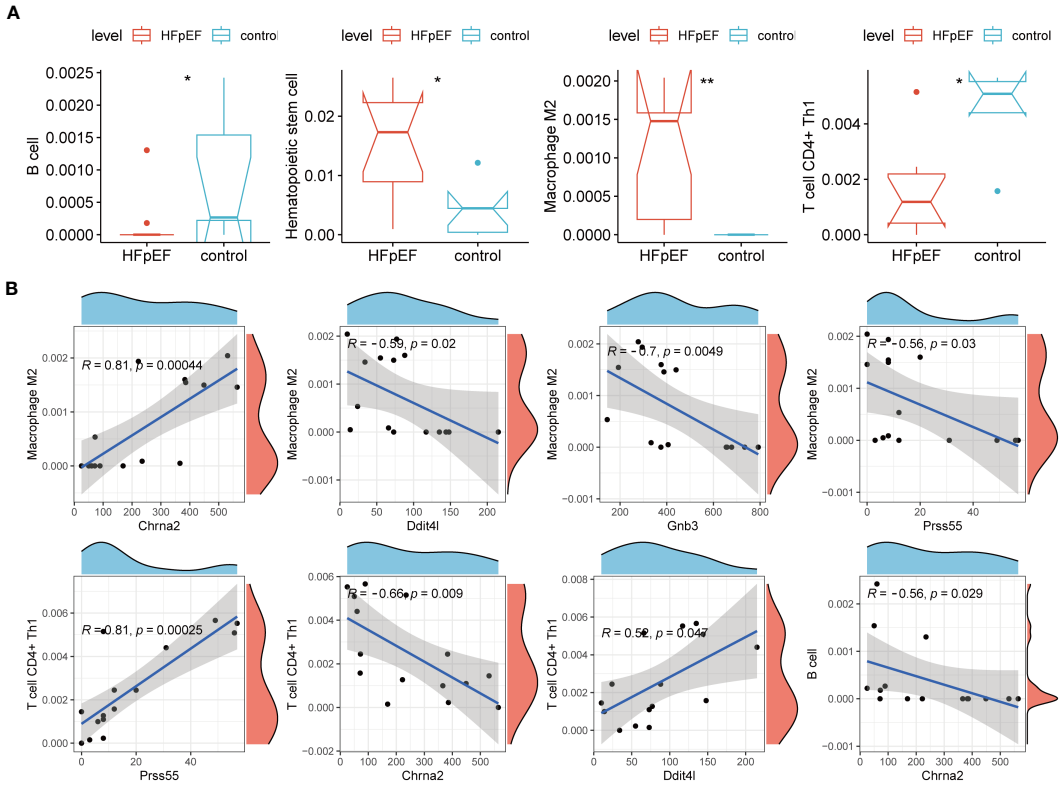

FIGURE 7
The association of biomarkers with immune microenvironment. **(A)** Immune cell infiltration between two groups by XCELL algorithms and only statistically significant ones are shown. **(B)** Scatter plots show the correlation of biomarkers with the infiltration of Macrophage M2, T cell CD4+ Th1, and B cell. *$P < 0.05$; **$P < 0.01$.

the other hand, the GNB3 825T allele might be involved in ET-1-induced vasoconstriction in the skin microcirculation (35), and there is association of GNB3 825T variant with increased renal perfusion (36), suggesting the potential relationship of Gnb3 and circulating changes as well as cardiorenal interaction in patients with HFpEF (37). Furthermore, an analysis of the TF-Gnb3 network revealed interesting results about TBP expression. When comparing the right ventricle to the left ventricle or ventricle in healthy controls and HF patients, TBP expression was found to be highly erratic (38). However, in another study conducted on the MI mouse model, TBP expression was stable (39). These findings indicate that the TBP-Gnb3 axis may play a significant role in the pathogenesis of HFpEF.

Gng7 is a gene that encodes the gamma subunit of G protein, which plays a regulatory or translational role in various transmembrane signaling systems (40). Previous studies have shown a correlation between reduced expression of Gng7 and breast cancer (41), lung cancer (42), head and neck cancer (43, 44), and esophageal cancer (45). However, there have been no studies directly linking Gng7 to HFpEF. Our study discovered a potential close association between Gng7 and ventricular development, myocardial tissue development, and cardiac contractile regulation through Gng7-GSEA results. Additionally, our analysis of the miRNA-Gng7 network revealed a close relationship between miR-199a-5p (46), miR-18a-3p (47), miR-491-5p (48), and On the other hand, the GNB3 825T allele might be involved in ET-1-induced vasoconstriction in the skin microcirculation, and there is an association of the GNB3 825T variant with increased renal perfusion, suggesting the potential relationship of Gnb3 with circulating changes, as well as cardiorenal interaction in patients with HFpEF. The predicted interaction of Gng7 and Gnb3 in the BioGRID database might provide more theoretical support to study the underlying mechanism of Gng7 in HFpEF as well (49). These findings suggest that Gng7 may play a role in the pathogenesis of HFpEF.

Prss55 is a gene that encodes a protease known as “proteinase, serine 55”. It belongs to the serine family of proteases and is primarily expressed in testicular tissue (50). Despite being relatively understudied, Prss55 is an important enzyme molecule, and its specific function and mechanism of action remain unknown. Nevertheless, emerging research suggests a potential association between Prss55 and the reproductive system (51, 52). In our study, utilizing Prss55-GSEA, we have identified its potential involvement in crucial biological processes such as angiogenesis and developmental regulation, myocardial tissue development, myocardial contraction, and cardiomyocyte development. To fully comprehend the function and biological significance of Prss55, further investigations are warranted.

In the development of HFpEF, inflammatory response plays a significant role (53). This response is triggered by the infiltration of immune cells, leading to the release of inflammatory mediators in cardiac tissue. The present study’s immunoscape analysis revealed

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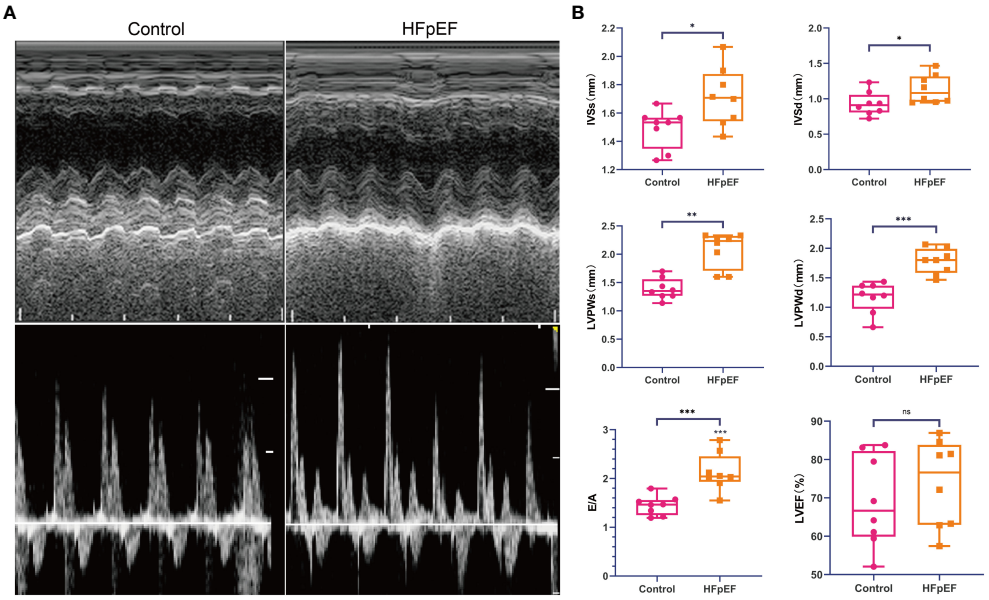

FIGURE 8
Assessment of cardiac function in the HFpEF mouse model. Echocardiography was performed at 15 weeks after a combination of a HFD (60% kilocalories from fat) and L-NAME (0.5 g l−1 in drinking water) or a standard (chow) diet in mice. **(A)** Representative recordings of echocardiographic images of the LV. **(B)** IVSs, IVSd, LVPWs, LVPWd, E/A, and LVEF were measured by echocardiography. *P < 0.05, **P < 0.01, ***P < 0.001 vs. corresponding control group. ns represents no significance. Data are means ± SEM. HFpEF, heart failure with preserved ejection fraction; HFD, high-fat diet; L-NAME, Nω-nitro-L-arginine methyl ester; LV, left ventricle; IVSs, inter-ventricular septum thickness end systolic; IVSd, inter-ventricular septum thickness end diastolic; LVPWs, left ventricular systolic posterior wall thickness; LVPWd, left ventricular posterior wall thickness end-diastolic; E/A, the early (E) wave peak velocity, representing the passive filling, to the late **(A)** wave peak velocity ratio, representing the active filling due to the atrial contraction; LVEF, left ventricular ejection fraction.

that in the HFpEF group, CD4+ Th1 expression was downregulated in B cells and T cells, while hematopoietic stem cells and M2 macrophages were dominant compared to the control group. B lymphocytes, specialized immune cells present in all jawed vertebrates, have shown increasing association with the heart (54). Although limited, available evidence suggests that B cells may be key players in the development of HFpEF. Biopsies from patients with diastolic dysfunction and controls have shown higher

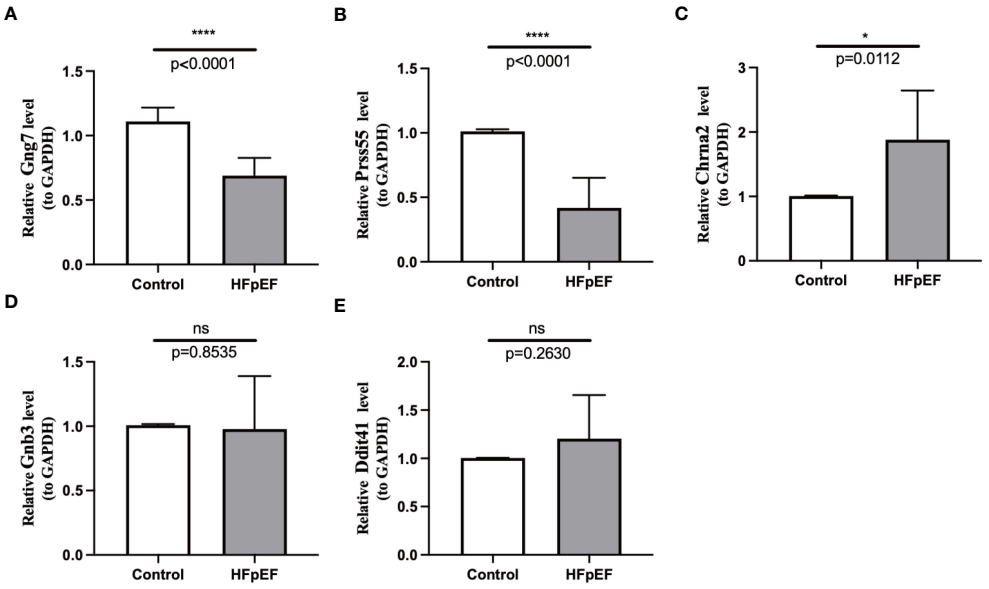

FIGURE 9
RNA expression of the 5 biomarkers was measured in HFpEF and control samples. RNA expression of Gng7 **(A)**, Prss55 **(B)**, Chrna2 **(C)**, Gnb3 **(D)**, and Ddit4l **(E)** were measured in blood samples using qRT-PCR. P-values were calculated using a two-sided unpaired Student's t-test. *P < 0.05; ****P < 0.0001; ns represents no significance. HFpEF, heart failure with preserved ejection fraction; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

circulating IgG1 and IgG3 levels in patients at higher risk of developing HFpEF (55). Anecdotal evidence points towards the potential improvement of HFpEF with immunomodulation using B-cell-targeted drugs in patients with connective tissue diseases (56). Animal models have also demonstrated the pathogenic role of CD4+ Th1 and Th17 in HF development (57, 58). Sinha et al. found that a higher proportion of CD4+ Th1 cells was associated with a lower risk of developing HF, consistent with the present study (59). Zhang et al. observed increased neutrophil and macrophage infiltration in HFpEF mice hearts, but their results showed an increase in M1 macrophages and a decrease in M2 macrophages, contrasting our findings. However, further studies are needed to explore this discrepancy and provide a rational explanation.

To promote the clinical application of the five diagnostic biomarkers, we used the DrugBank database to predict potential target drugs. Among the predictions, Cianidanol emerged as a co-targeted drug for Chrna2, Gnb3, and Gng7. Cianidanol is a member of the polyphenolic brass subfamily known for its strong antioxidant properties (60). It has been recognized for its potential therapeutic effects in cardiometabolic disorders (61, 62) and cancers (63). Animal and preclinical studies have also demonstrated the vasoprotective effects of cianidanol (64, 65). Additionally, studies suggest that it may offer a unique approach to reducing atherosclerosis (66). Esmolol, which is currently used in the treatment of cardiovascular disease, has been predicted as a co-targeted agent targeting Chrna2 and Gnb3. This cardioselective β-blocker has shown effectiveness in controlling tachycardia and acute ischemic elevated hemodynamic parameters in patients with heart disease (67). Perindopril, a co-target of Chrna2 and Gng7, is an angiotensin-converting enzyme inhibitor indicated for the treatment of hypertension (68). Furthermore, a review has shown that Perindopril, when combined with other antihypertensives, minimizes cardiovascular events (69). Methyldopa has been identified as a co-targeted agent of Ddit4l and Gng3. This drug has been used in the treatment of hypertension since the 1960s (70). Lovastatin, on the other hand, is a co-targeted drug for Ddit4l and Gng7 and is commonly used to treat coronary heart disease and hypercholesterolemia (71). Additionally, other potentially targeted drugs such as Rosuvastatin (72), Mexiletine (73), Labetalol (74), Lomitapide (75), and Metolazone (76) may be beneficial in the treatment of cardiovascular disease. However, no reports or studies have been conducted on the association of these targeted agents with HFpEF or with Chrna2, Ddit4l, Gnb3, and Gbg7. Therefore, further studies are needed to confirm their potential mechanisms of action. As for Prss55, DrugBank analysis showed that there are no potential targeted drugs directly related to cardiovascular disease treatment. This finding deserves further attention and investigation.

There are some shortcomings of this study that need to be noted. First, the study was based on bioinformatic analysis of HFpEF mouse transcriptome data from the GEO database. However, the sample size was relatively small, which may limit the statistical reliability and generalizability of the results. Second, the study identified five diagnostic biomarkers associated with energy metabolism, but expression validation in human clinical samples was not performed. It is crucial to perform clinical sample validation to determine the practical application and validity of these markers in patients with HFpEF. Although PCR validation was conducted using HFpEF mice, the results did not successfully validate the differential expression of Gnb3 and Ddit4l between the control and HFpEF groups. This lack of validation may be attributed to individual differences between samples, the variability of experimental techniques, and the complexity of gene regulation. Thirdly, although a consistent cluster analysis of HFpEF was performed based on five diagnostic biomarkers associated with energy metabolism, practical clinical significance could not be assigned to these subclasses due to the current lack of human data. Additionally, bioinformatics analysis, while important in HFpEF research, still suffers from limitations such as the quality and consistency of data, data processing, and choice of analysis methods. Furthermore, this study used a mouse model to study HFpEF, but the mouse model cannot fully reflect the complex pathological process and biological characteristics of human HFpEF, while enough public data on human HFpEF samples could not be found for analysis. Therefore, caution should be exercised when applying these findings to clinical practice. To overcome these shortcomings, future studies should aim to increase the sample size, perform human clinical sample validation, and validate and confirm the clinical application potential of these analytical results by integrating other experimental models and methods.

5 Conclusion

The analysis of HFpEF mouse transcriptome data from the GEO database successfully identified five diagnostic biomarkers, namely Chrna2, Ddit4l, Gnb3, Gng7, and Prss55, which are associated with energy metabolism. These biomarkers demonstrated a strong ability to distinguish between HFpEF samples and control samples. Furthermore, the GSEA analysis revealed their potential involvement in crucial biological processes like ventricular development and cardiac contraction. These findings have significant implications for understanding the pathogenesis of HFpEF and identifying potential disease biomarkers. Moreover, these biomarkers hold promise as early diagnostic and predictive indicators for HFpEF, offering new prospects for personalized therapy.

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 author.

Ethics statement

The animal study was approved by The Ethics Committee of Shaanxi Provincial People's Hospital, Xi'an, Shaanxi, China. The

study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. QZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. MD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review and editing. LL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. HY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1296547/full#supplementary-material

SUPPLEMENTARY FIGURE 1
GO functional enrichment analysis of 464 DEGs in Cluster1 and Cluster2 samples. **(A)** Relationship among the top 10 enriched BP terms and targets is represented in a chord plot. **(B)** Relationship among the top 10 enriched CC terms and targets is represented in a chord plot. **(C)** Relationship among the top 10 enriched mf terms and targets is represented in a chord plot. The colors of the nodes range from red to blue in descending order of logFC values. The genes are ordered according to logFC values. GO: Gene Ontology; DEGs: differentially expressed genes; BP: biological process; CC: cellular component; MF: molecular function.

SUPPLEMENTARY FIGURE 2
Construction of PPI network and identification of hub genes. **(A)** The Venn diagram of DEGs1 and DEGs2. DEGs1 represents DEGs between control and HFpEF groups, and DEGs2 represents DEGs between cluster 1 and cluster 2. **(B)** PPI network of the DEGs between cluster 1 and cluster 2. The PPI network of DEGs was constructed using Cytoscape. **(C)** The top 5 key genes were screened through the PPI network map. PPI: protein-protein interaction; DEGs: differentially expressed genes.

SUPPLEMENTARY FIGURE 3
The association of hub genes with immune microenvironment. **(A)** Immune cell infiltration between two cluster s by XCELL algorithms and only statistically significant ones are shown. **(B)** Scatter plots show the correlation of hub genes with the infiltration of Mast cell and Macrophage. *$P < 0.05$.. Primer sequences of PCR.

SUPPLEMENTARY TABLE 1
DEGs between the HFpEF and control groups in the GSE180065 dataset.

SUPPLEMENTARY TABLE 2
Expression of identified DE-EMRGs in the GSE180065 dataset.

SUPPLEMENTARY TABLE 3
GSEA-GO analysis results for Chrna2.

SUPPLEMENTARY TABLE 4
GSEA-GO analysis results for Gng7.

SUPPLEMENTARY TABLE 5
GSEA-GO analysis results for Ddit4l.

SUPPLEMENTARY TABLE 6
GSEA-GO analysis results for Prss55.

SUPPLEMENTARY TABLE 7
GSEA-GO analysis results for Gnb3.

SUPPLEMENTARY TABLE 8
Potential target drugs for 5 biomarkers predicted by DrugBank.

SUPPLEMENTARY TABLE 9
NetworkAnalyst predicted miRNA-biomarker and biomarker-TF relationship pairs.

SUPPLEMENTARY TABLE 10
DEGs between the Cluster1 and Cluster2.

SUPPLEMENTARY TABLE 11
Results of GO analysis of DEGs-clusters.

References

1. Borlaug BA, Paulus WJ. Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment. *Eur Heart J* (2011) 32(6):670–9. doi: 10.1093/eurheartj/ehq426

2. Shah SJ, Gheorghiade M. Heart failure with preserved ejection fraction: treat now by treating comorbidities. *JAMA* (2008) 300(4):431–3. doi: 10.1001/jama.300.4.431

3. Aimo A, Georgiopoulos G, Senni M, Emdin M. Searching for diagnostic biomarkers of heart failure with preserved ejection fraction: methodological issues. *Eur J Heart Fail* (2020) 22(9):1598–9. doi: 10.1002/ejhf.1977

4. Murashige D, Jang C, Neinast M, Edwards JJ, Cowan A, Hyman MC, et al. Comprehensive quantification of fuel use by the failing and nonfailing human heart. *Science* (2020) 370(6514):364–8. doi: 10.1126/science.abc8861

5. Shi J, Dai W, Hale SL, Brown DA, Wang M, Han X, et al. Bendavia restores mitochondrial energy metabolism gene expression and suppresses cardiac fibrosis in the border zone of the infarcted heart. *Life Sci* (2015) 141:170–8. doi: 10.1016/j.lfs.2015.09.022

6. Ibrahim KS, Bourwis N, Dolan S, Lang S, Spencer J, Craft JA. Characterisation of gut microbiota of obesity and type 2 diabetes in a rodent model. *Biosci Microbiota Food Health* (2021) 40(1):65–74. doi: 10.12938/bmfh.2019-031

7. Kolwicz SC Jr, Purohit S, Tian R. Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. *Circ Res* (2013) 113(5):603–16. doi: 10.1161/CIRCRESAHA.113.302095

8. Neubauer S. The failing heart–an engine out of fuel. *N Engl J Med* (2007) 356 (11):1140–51. doi: 10.1056/NEJMra063052

9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) 15(12):550. doi: 10.1186/s13059-014-0550-8

10. Ito K, Murphy D. Application of ggplot2 to pharmacometric graphics. *CPT Pharmacometrics Syst Pharmacol* (2013) 2(10):e79. doi: 10.1038/psp.2013.56

11. Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models *via* coordinate descent. *J Stat Software* (2010) 33(1):1–22. doi: 10.18637/jss.v033.i01

12. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinf* (2011) 12:77. doi: 10.1186/1471-2105-12-77

13. Yang CC, Tsai CG, Yeh JA. Miniaturization of dielectric liquid microlens in package. *Biomicrofluidics* (2010) 4(4):43006. doi: 10.1063/1.3494030

14. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation (Camb)* (2021) 2(3):100141. doi: 10.1016/j.xinn.2021.100141

15. Su G, Morris JH, Demchak B, Bader GD. Biological network exploration with Cytoscape 3. *Curr Protoc Bioinf* (2014) 47(8.13):1–24. doi: 10.1002/0471250953.bi0813s47

16. Wilkerson MD, Hayes DN. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. *Bioinformatics* (2010) 26(12):1572–3. doi: 10.1093/bioinformatics/btq170

17. Sturm G, Finotello F, List M. Immunedeconv: an R package for unified access to computational methods for estimating immune cell fractions from bulk RNA-sequencing data. *Methods Mol Biol* (2020) 2120:223–32. doi: 10.1007/978-1-0716-0327-7_16

18. Schiattarella GG, Altamirano F, Tong D, French KM, Villalobos E, Kim SY, et al. Nitrosative stress drives heart failure with preserved ejection fraction. *Nature* (2019) 568(7752):351–6. doi: 10.1038/s41586-019-1100-z

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001) 25 (4):402–8. doi: 10.1006/meth.2001.1262

20. Kumar AA, Kelly DP, Chirinos JA. Mitochondrial dysfunction in heart failure with preserved ejection fraction. *Circulation* (2019) 139(11):1435–50. doi: 10.1161/CIRCULATIONAHA.118.036259

21. Capone F, Sotomayor-Flores C, Bode D, Wang R, Rodolico D, Strocchi S, et al. Cardiac metabolism in HFpEF: from fuel to signalling. *Cardiovasc Res* (2023) 118 (18):3556–75. doi: 10.1093/cvr/cvac166

22. Adams V, Wunderlich S, Mangner N, Hommel J, Esefeld K, Gielen S, et al. Ubiquitin-proteasome-system and enzymes of energy metabolism in skeletal muscle of patients with HFpEF and HFrEF. *ESC Heart Fail* (2021) 8(4):2556–68. doi: 10.1002/ehf2.13405

23. Lam CS, Donal E, Kraigher-Krainer E, Vasan RS. Epidemiology and clinical course of heart failure with preserved ejection fraction. *Eur J Heart Fail* (2011) 13 (1):18–28. doi: 10.1093/eurjhf/hfq121

24. Wada K, Ballivet M, Boulter J, Connolly J, Wada E, Deneris ES, et al. Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. *Science* (1988) 240(4850):330–4. doi: 10.1126/science.2832952

25. Trivisano M, Terracciano A, Milano T, Cappelletti S, Pietrafusa N, Bertini ES, et al. Mutation of CHRNA2 in a family with benign familial infantile seizures: Potential role of nicotinic acetylcholine receptor in various phenotypes of epilepsy. *Epilepsia* (2015) 56(5):e53–7. doi: 10.1111/epi.12967

26. Heusch G. The coronary circulation as a target of cardioprotection. *Circ Res* (2016) 118(10):1643–58. doi: 10.1161/CIRCRESAHA.116.308640

27. Cuaz-Pérolin C, Furman C, Larigauderie G, Legedz L, Lasselin C, Copin C, et al. REDD2 gene is upregulated by modified LDL or hypoxia and mediates human macrophage cell death. *Arterioscler Thromb Vasc Biol* (2004) 24(10):1830–5. doi: 10.1161/01.ATV.0000142366.69080.c3

28. Ellisen LW, Ramsayer KD, Johannessen CM, Yang A, Beppu H, Minda K, et al. REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. *Mol Cell* (2002) 10(5):995–1005. doi: 10.1016/S1097-2765(02)00706-2

29. Simonson B, Subramanya V, Chan MC, Zhang A, Franchino H, Ottaviano F, et al. DDiT4L promotes autophagy and inhibits pathological cardiac hypertrophy in response to stress. *Sci Signal* (2017) 10(468):eaaf5967. doi: 10.1126/scisignal.aaf5967

30. Kolosowska N, Gotkiewicz M, Dhungana H, Giudice L, Giugno R, Box D, et al. Intracerebral overexpression of miR-669c is protective in mouse ischemic stroke model by targeting MyD88 and inducing alternative microglial/macrophage activation. *J Neuroinflamm* (2020) 17(1):194. doi: 10.1186/s12974-020-01870-w

31. Siffert W, Rosskopf D, Siffert G, Busch S, Moritz A, Erbel R, et al. Association of a human G-protein beta3 subunit variant with hypertension. *Nat Genet* (1998) 18(1):45–8. doi: 10.1038/ng0198-45

32. Klintschar M, Stiller D, Schwaiger P, Kleiber M. DNA polymorphisms in the tyrosine hydroxylase and GNB3 genes: association with unexpected death from acute myocardial infarction and increased heart weight. *Forensic Sci Int* (2005) 153(2-3):142–6. doi: 10.1016/j.forsciint.2004.09.103

33. Matsunaga T, Nagasumi K, Yamamura T, Gu N, Nishikino M, Ueda Y, et al. Association of C825T polymorphism of G protein beta3 subunit with the autonomic nervous system in young healthy Japanese individuals. *Am J Hypertens* (2005) 18(4 Pt 1):523–9. doi: 10.1016/j.amjhyper.2004.11.008

34. Schmitz B, De Maria R, Gatsios D, Chrysanthakopoulou T, Landolina M Gasparini M, et al. Identification of genetic markers for treatment success in heart failure patients: insight from cardiac resynchronization therapy. *Circ Cardiovasc Genet* (2014) 7(6):760–70. doi: 10.1161/CIRCGENETICS.113.000384

35. Mitchell A, Rushentsova U, Siffert W, Philipp T, Wenzel RR. The angiotensin II receptor antagonist valsartan inhibits endothelin 1-induced vasoconstriction in the skin microcirculation in humans in *vivo*: influence of the G-protein beta3 subunit (GNB3) C825T polymorphism. *Clin Pharmacol Ther* (2006) 79:274–81. doi: 10.1016/j.clpt.2005.11.008

36. Zeltner R, Delles C, Schneider M, Siffert W, Schmieder RE. G-protein beta(3) subunit gene (GNB3) 825T allele is associated with enhanced renal perfusion in early hypertension. *Hypertension* (2001) 37:882–6. doi: 10.1161/01.hyp.37.3.882

37. Jung S, Bosch A, Kolwelter J, Striepe K, Kannenkeril D, Schuster T, et al. Renal and intraglomerular haemodynamics in chronic heart failure with preserved and reduced ejection fraction. *ESC Heart Fail* (2021) 8:1562–70. doi: 10.1002/ehf2.13257

38. Molina CE, Jacquet E, Ponien P, Muñoz-Guijosa C, Baczkó I, Maier LS, et al. Identification of optimal reference genes for transcriptomic analyses in normal and diseased human heart. *Cardiovasc Res* (2018) 114(2):247–58. doi: 10.1093/cvr/cvx182

39. Li Q, Hu T, Chen L, Sun J, Xie J, Li R, et al. Selection of reference genes for normalization of quantitative polymerase chain reaction data in mouse models of heart failure. *Mol Med Rep* (2015) 11(1):393–9. doi: 10.3892/mmr.2014.2750

40. Zhang Y, Yang J, Wang X, Li X. GNG7 and ADCY1 as diagnostic and prognostic biomarkers for pancreatic adenocarcinoma through bioinformatic-based analyses. *Sci Rep* (2021) 11(1):20441. doi: 10.1038/s41598-021-99544-x

41. Mei J, Wang T, Zhao S, Zhang Y. Osthole inhibits breast cancer progression through upregulating tumor suppressor GNG7. *J Oncol* (2021) 2021:6610511. doi: 10.1155/2021/6610511

42. Sheng H, Li X, Xu Y. Knockdown of FOXP1 promotes the development of lung adenocarcinoma. *Cancer Biol Ther* (2019) 20(4):537–45. doi: 10.1080/15384047.2018.1537999

43. Hartmann S, Szaumkessel M, Salaverria I, Simon R, Sauter G, Kiwerska K, et al. Loss of protein expression and recurrent DNA hypermethylation of the GNG7 gene in squamous cell carcinoma of the head and neck. *J Appl Genet* (2012) 53(2):167–74. doi: 10.1007/s13353-011-0079-4

44. Demokan S, Chuang AY, Chang X, Khan T, Smith IM, Pattani KM, et al. Identification of guanine nucleotide-binding protein γ-7 as an epigenetically silenced gene in head and neck cancer by gene expression profiling. *Int J Oncol* (2013) 42 (4):1427–36. doi: 10.3892/ijo.2013.1808

45. Ohta M, Mimori K, Fukuyoshi Y, Kita Y, Motoyama K, Yamashita K, et al. Clinical significance of the reduced expression of G protein gamma 7 (GNG7) in oesophageal cancer. *Br J Cancer* (2008) 98(2):410–7. doi: 10.1038/sj.bjc.6604124

46. Garcia-Elias A, Tajes M, Yañez-Bisbe L, Enjuanes C, Comín-Colet J, Serra SA, et al. Atrial fibrillation in heart failure is associated with high levels of circulating microRNA-199a-5p and 22-5p and a defective regulation of intracellular calcium and cell-to-cell communication. *Int J Mol Sci* (2021) 22(19):10377. doi: 10.3390/ijms221910377

47. Yang L, Cheng CF, Li ZF, Huang XJ, Cai SQ, Ye SY, et al. Berberine blocks inflammasome activation and alleviates diabetic cardiomyopathy via the miR−18a−3p/Gsdmd pathway. *Int J Mol Med* (2023) 51(6):49 [pii]. doi: 10.3892/ijmm.2023.5252

48. Yuan M, Zhan Q, Duan X, Song B, Zeng S, Chen X, et al. A functional polymorphism at miR-491-5p binding site in the 3'-UTR of MMP-9 gene confers increased risk for atherosclerotic cerebral infarction in a Chinese population. *Atherosclerosis* (2013) 226(2):447–52. doi: 10.1016/j.atherosclerosis.2012.11.026

49. Yan K, Kalyanaraman V, Gautam N. Differential ability to form the G protein betagamma complex among members of the beta and gamma subunit families. *J Biol Chem* (1996) 271:7141–6. doi: 10.1074/jbc.271.12.7141

50. Neth P, Profanter B, Geissler C, Nägler DK, Nerlich A, Sommerhoff CP, et al. T-SP1: a novel serine protease-like protein predominantly expressed in testis. *Biol Chem* (2008) 389(12):1495–504. doi: 10.1515/BC.2008.170

51. Liu M, Jiang C, Zhang X, Zhang G, Liu M, Zheng R, et al. PRSS55 is a novel potential causative gene for human male infertility. *Reprod BioMed Online* (2022) 45 (3):553–62. doi: 10.1016/j.rbmo.2022.05.016

52. Shang X, Shen C, Liu J, Tang L, Zhang H, Wang Y, et al. Serine protease PRSS55 is crucial for male mouse fertility *via* affecting sperm migration and sperm-egg binding. *Cell Mol Life Sci* (2018) 75(23):4371–84. doi: 10.1007/s00018-018-2878-9

53. Schiattarella GG, Rodolico D, Hill JA. Metabolic inflammation in heart failure with preserved ejection fraction. *Cardiovasc Res* (2021) 117(2):423–34. doi: 10.1093/cvr/cvaa217

54. Bermea K, Bhalodia A, Huff A, Rousseau S, Adamo L. The role of B cells in cardiomyopathy and heart failure. *Curr Cardiol Rep* (2022) 24(8):935–46. doi: 10.1007/s11886-022-01722-4

55. van den Hoogen P, de Jager SCA, Huibers MMH, Schoneveld AH, Puspitasari YM, Valstar GB, et al. Increased circulating IgG levels, myocardial immune cells and IgG deposits support a role for an immune response in pre- and end-stage heart failure. *J Cell Mol Med* (2019) 23(11):7505–16. doi: 10.1111/jcmm.14619

56. Baniaamam M, Voskuyl AE, Nurmohamed MT, Handoko ML. Clinical improvement of cardiac function in a patient with systemic lupus erythematosus and heart failure with preserved ejection fraction treated with belimumab. *BMJ Case Rep* (2021) 14(1):e237549. doi: 10.1136/bcr-2020-237549

57. Bansal SS, Ismahil MA, Goel M, Patel B, Hamid T, Rokosh G, et al. Activated T lymphocytes are essential drivers of pathological remodeling in ischemic heart failure. *Circ Heart Fail* (2017) 10(3):e003688. doi: 10.1161/CIRCHEARTFAILURE.116.003688

58. Laroumanie F, Douin-Echinard V, Pozzo J, Lairez O, Tortosa F, Vinel C, et al. CD4+ T cells promote the transition from hypertrophy to heart failure during chronic pressure overload. *Circulation* (2014) 129(21):2111–24. doi: 10.1161/CIRCULATIONAHA.113.007101

59. Sinha A, Sitlani CM, Doyle MF, Fohner AE, Buzkova P, Floyd JS, et al. Association of immune cell subsets with incident heart failure in two population-based cohorts. *ESC Heart Fail* (2022) 9(6):4177–88. doi: 10.1002/ehf2.14140

60. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev* (2009) 2(5):270–8. doi: 10.4161/oxim.2.5.9498

61. Bernatova I. Biological activities of (-)-epicatechin and (-)-epicatechin-containing foods: Focus on cardiovascular and neuropsychological health. *Biotechnol Adv* (2018) 36(3):666–81. doi: 10.1016/j.biotechadv.2018.01.009

62. Barteková M, Adameová A, Görbe A, Ferenczyová K, Pecháňová O, Lazou A, et al. Natural and synthetic antioxidants targeting cardiac oxidative stress and redox signaling in cardiometabolic diseases. *Free Radic Biol Med* (2021) 169:446–77. doi: 10.1016/j.freeradbiomed.2021.03.045

63. Sharifi-Rad M, Pezzani R, Redaelli M, Zorzan M, Imran M, Ahmed Khalil A, et al. Preclinical pharmacological activities of epigallocatechin-3-gallate in signaling pathways: an update on cancer. *Molecules* (2020) 25(3):467. doi: 10.3390/molecules25030467

64. Kluknavsky M, Balis P, Puzserova A, Radosinska J, Berenyiova A, Drobna M, et al. (-)-epicatechin prevents blood pressure increase and reduces locomotor hyperactivity in young spontaneously hypertensive rats. *Oxid Med Cell Longev* (2016) 2016:6949020. doi: 10.1155/2016/6949020

65. Galleano M, Bernatova I, Puzserova A, Balis P, Sestakova N, Pechanova O, et al. (-)-Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanism. *IUBMB Life* (2013) 65(8):710–5. doi: 10.1002/iub.1185

66. Naito Y, Yoshikawa T. Green tea and heart health. *J Cardiovasc Pharmacol* (2009) 54(5):385–90. doi: 10.1097/FJC.0b013e3181b6e7a1

67. Garnock-Jones KP. Esmolol: a review of its use in the short-term treatment of tachyarrhythmias and the short-term control of tachycardia and hypertension. *Drugs* (2012) 72(1):109–32. doi: 10.2165/11208210-000000000-00000

68. Alfakih K, Hall AS. Perindopril. *Expert Opin Pharmacother* (2006) 7(1):63–71. doi: 10.1517/14656566.7.1.63

69. Campbell DJ. A review of Perindopril in the reduction of cardiovascular events. *Vasc Health Risk Manag* (2006) 2(2):117–24. doi: 10.2147/vhrm.2006.2.2.117

70. Mah GT, Tejani AM, Musini VM. Methyldopa for primary hypertension. *Cochrane Database Syst Rev* (2009) 2009(4):CD003893. doi: 10.1002/14651858.CD003893.pub3

71. Duong H, Bajaj T. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing (2023).

72. Rahhal A, Khir F, Orabi B, Chbib S, Al-Khalaila O, Abdelghani MS, et al. A comparative study of high-intensity rosuvastatin versus atorvastatin therapy post-acute coronary syndrome using real-world data. *Curr Probl Cardiol* (2022) 47(7):100956. doi: 10.1016/j.cpcardiol.2021.100956

73. Moak JP, Smith RT, Garson A Jr. Mexiletine: an effective antiarrhythmic drug for treatment of ventricular arrhythmias in congenital heart disease. *J Am Coll Cardiol* (1987) 10(4):824–9. doi: 10.1016/S0735-1097(87)80276-0

74. Fongemie J, Felix-Getzik E. A review of nebivolol pharmacology and clinical evidence. *Drugs* (2015) 75(12):1349–71. doi: 10.1007/s40265-015-0435-5

75. Palazzuoli A, Ruocco G, Severino P, Gennari L, Pirrotta F, Stefanini A, et al. Effects of metolazone administration on congestion, diuretic response and renal function in patients with advanced heart failure. *J Clin Med* (2021) 10(18):4207. doi: 10.3390/jcm10184207

76. Cox ZL, Hung R, Lenihan DJ, Testani JM. Diuretic strategies for loop diuretic resistance in acute heart failure: the 3T trial. *JACC Heart Fail* (2020) 8(3):157–68. doi: 10.1016/j.jchf.2019.09.012

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Genetic association of lipid-lowering drug target genes with erectile dysfunction and male reproductive health

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Objective: The effect of hypolipidemic drugs on male erectile function is still controversial. This Mendelian randomization (MR) study aimed to explore the potential impact of lipid-lowering drug targets on ED.

Methods: We collected seven genetic variants encoding lipid-lowering drug targets (LDLR, HMGCR, NPC1L1, PCSK9, APOB, APOC3 and LPL) from published genome-wide association study (GWAS) statistics, and performed drug target MR analysis. The risk of ED was defined as the primary outcome, sex hormone levels and other diseases as the secondary outcomes. Mediation analyses were performed to explore potential mediating factors.

Results: The results showed that LDLR, LPL agonists and APOC3 inhibitors were significantly associated with a reduced risk of ED occurrence. APOB inhibitors were associated with an increased risk of ED occurrence. In terms of sex hormone levels, LDLR and LPL agonists were significantly associated with increased TT levels, and HMGCR was associated with decreased TT and BT levels significantly. In terms of male-related disease, MR results showed that LDLR agonists and PCSK9 inhibitors were significantly associated with an elevated risk of PH; HMGCR, NPC1L1 inhibitors were associated with a reduced risk of PCa; and LDLR agonists were significantly associated with a reduced risk of AS and MI; in addition, HMGCR inhibitors were associated with a reduced risk of PCa.

Conclusion: After performing drug-targeted MR analysis, we found that that there was a causal relationship between lipid-lowering drug targets and ED. APOC3, APOB, LDLR and LPL may be new candidate drug targets for the treatment of ED.

KEYWORDS

erectile dysfunction, lipids, sex hormone, male diseases, Mendelian randomization analysis

Introduction

Erectile dysfunction (ED), defined as the persistent inability of the penis to achieve and/or maintain an erection sufficient for a satisfactory sex life, is one of the most common diseases in urology (1). Although ED does not pose a threat to life, it poses a significant safety hazard to society. It not only affects the physical and mental health of patients, but also causes great distress to sexual partners, leading to a decrease in the quality of life for patients and their partners, disharmony in the family, and more seriously, a decrease in work productivity, an increase in domestic violence, and an increase in medical burden. ED is highly related to cardiovascular risk factors such as hyperlipidemia, diabetes and abnormal blood pressure. Previous studies have found that the pathogenesis of ED and cardiovascular disease is basically the same, both centered on vascular endothelial dysfunction, ultimately leading to vascular atherosclerosis (2–4). Therefore, ED and cardiovascular disease share common risk factors. Lipids, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), play a crucial role in this process.

Nicotinic acid, statins, fibrates and novel Lipid Lowering drugs are commonly used in the treatment of hyperlipidemia (5–8). There is clinical evidence that lipid-lowering drug therapy can significantly improve erectile function in patients with organic ED caused by hyperlipidemia (9, 10). However, some scholars have found that patients with hyperlipidemia may experience a decrease in testosterone levels during the use of lipid-lowering drugs, which in turn can lead to the occurrence of ED. Several meta-analyses have also shown similar conclusions (11, 12). In addition, some studies suggest that statins may indirectly lead to the occurrence of ED by affecting autonomic nervous function or psychological factors (13). Randomized controlled trials (RCTs) are the standard methods for determining drug efficacy and adverse reactions. However, there is currently a lack of large-scale randomized controlled trials between lipid-lowering drugs and ED. The impact of lipid-lowering drugs on the occurrence of ED and sex hormone levels is still unclear, and further exploration is needed.

With the increasing popularity of genome-wide association studies (GWAS), Mendelian randomization (MR) may be an effective alternative to RCT studies for problem solving. Because genetic variants (alleles) are randomly assigned during meiosis, participants in MR studies are "randomized" based on the presence of alleles. This is similar to a randomized controlled trial, where participants are randomly assigned to either an experimental treatment group or a control group (14, 15). Thus, MR analysis has the advantage of being less susceptible to confounding factors than other research methods. In recent years, drug target MR analysis has emerged as an effective tool. It is used to infer the effect of drugs targeting protein-coding genes, antagonists, agonists, or inhibitors on disease risk (16). This tool is very helpful in deciphering the potential of drug therapy and facilitating drug development.

In this study, we performed drug-targeted MR analysis to determine the effect of lipid-lowering drugs on ED and to explore the potential impact of lipid-lowering drug targets on sex hormone levels and other common male disorders.

Materials and methods

Study design

To explore the relationship between lipid-lowering drug target genes and male reproductive health at the genetic level, a two-sample Mendelian randomization approach was used in this study.

Genetic variant selection

Based on the latest guidelines for the treatment of dyslipidemia, we selected common lipid-lowering drugs and novel therapeutic approaches such as statins, ezetimibe, PCSK9 inhibitors, mipomersen and antisense oligonucleotides targeting apolipoprotein C-III (APOC3) mRNA. The genes encoding the pharmacological targets of these drugs were identified using the DrugBank database. These target genes were further classified into LDL-c reducing target genes (i.e.; LDLR, HMGCR, NPC1L1, PCSK9 and APOB) and TG reducing target genes (i.e., LPL and APOC3) based on the primary pharmacological effect (Table 1).

The summary data of LDL-C and TG were from two GWAS summary statistics containing 440,546 and 441,016 European individuals respectively (17). By obtaining instrumental variables that target each drug target to lower LDL-C or TC, they can be used to model the effects of each lipid-lowering drug. The instrumental variables were selected to be single nucleotide polymorphisms (SNP) located within ±100kb of the drug target locus and associated with LDL-C or TG levels ($P< 10\times5^{-8}$). To avoid the influence of strong linkage disequilibrium (LD) on the results, a threshold for LD was set ($r^2 < 0.3$).

Outcome

We used coronary heart disease (CHD) and ED as the results of drug-targeted MR analyses, where CHD was the positive control dataset to validate the feasibility and efficacy of lipid-lowering drug targets. The CHD dataset was derived from a GWAS summary statistic with a total of 184,305 cases, which contained 60,801 cases and 123,504 controls (18). ED as the main outcome, the data were from two independent GWAS datasets, respectively from FinnGen and ebi databases (19). Total testosterone (TT), bioavailable testosterone (BT), estrogen (E2) and SHBG were used as secondary outcomes, and their gender specific genetic instruments were from previously published UKB studies (20). Prostatitis (PI), prostate hyperplasia (PH), prostate cancer (PCa),

TABLE 1 Basic information on lipid-lowering drugs.

Drug name	Pharmacological action	Drug targets	Target genes	Chromosome
—	Reduce LDL-C	LDL Receptor	LDLR	chr19:11,200,038-11,244,492
Simvastatin, Atorvastatin, Rosuvastatin	Reduce LDL-C	HMG-CoA reductase	HMGCR	chr5:74,632,154-74,657,929
Ezetimibe	Reduce LDL-C	Niemann-Pick C1-like protein 1	NPC1L1	chr7:44,552,134-44,580,914
Alirocumab, Evolocumab	Reduce LDL-C	Proprotein convertase subtilisin/kexin type 9	PCSK9	chr1:55,505,221-55,530,525
Mipomersen	Reduce LDL-C	Apolipoprotein B-100	APOB	chr2:21,224,301-21,266,945
—	Reduce TG	Lipoprotein Lipase	LPL	chr8:19,759,228-19,824,769
Volanesorsen	Reduce TG	Apolipoprotein C-III	APOC3	chr11:116,700,422-116,703,788

LDLR, low density lipoprotein receptor; HMGCR, HMG-CoA reductase; NPC1L1, Niemann-Pick C1-Like 1; PCSK9, proprotein convertase subtilisin-kexin type 9; APOB, Apolipoprotein (apo) B; LPL, Lipoprotein lipase; APOC3, antisense oligonucleotides targeting apolipoprotein C-III.

aberrant spermatogenesis (AS), and male infertility (MI) equally performed as secondary outcomes with genetic instrumentation from previously published studies and the FinnGen database (21). Detailed information can be found in Supplementary Table 1.

Estimation of causal effects

We used the inverse variance weighted method (IVW) to estimate the causal effect between the drug targets and ED. In addition, we performed additional analyses by weighted median method and weighted model (22–24). Sufficient evidence of a causal effect was consistently provided by statistically significant IVW results plus direction of results across all 3 analyses. We also conducted Steiger filtering to ensure directional correlation between medication and outcomes.

Meta-analysis

The 'metafor' package was used to analyze the data indicators, and the Risk ratio OR was used as the merging statistic (25). When I^2>50%, the study was considered to have greater heterogeneity, and the results of the combined analysis were analyzed using the random effects model for Meta-analysis, and when I^2<50%, the study was considered to have homogeneity, and the results of the combined analysis were analyzed using the fixed effects model.

Quality controls

Heterogeneity was tested using MR Egger and IVW methods. Cochrane's Q was used to evaluate the heterogeneity of the genetic tools, and p>0.05 indicated the absence of significant heterogeneity. MR Egger regression equation was used to evaluate the horizontal multiplicity of genetic tools and p > 0.05 indicated the absence of horizontal multiplicity. To ensure that our results were not significantly affected by a particular SNP, we also removed each SNP in turn using the leave-one-out method and compared the results of the IVW method with all variants.

Mediation MR analysis

To determine whether there was a direct relationship between the observed associations between drugs and outcomes, we used a "two-sample" approach to assess potential intermediate effects (exposure-mediated-outcome pathways) of drugs on established outcome variables (Figure 1). This method reduces the bias of high LD correlations between genetic variants in MR analyses compared with multivariate MR methods. The method uses a "product of coefficients" approach to evaluate indirect effects and a delta method to derive standard errors for indirect effects (26).

The statistical analysis in this study was conducted using RStudio software (version 4.1.2). The resources used were mainly obtained from the TwoSampleMR R package developed by Hemani et al.

Results

Positive control analysis

We identified 48 SNPs associated with LDLR agonists, 19 SNPs associated with HMGCR inhibitors, 6 SNPs associated with NPC1L1 inhibitors, 33 SNPs associated with PCSK9 inhibitors, and 32 SNPs associated with apoB inhibitors from the genetic tools of LDL-C; Forty SNPs associated with APOC3 inhibitors and 58 SNPs associated with LPL agonists were identified from the genetic tools of TG. In the MR analysis with CHD as the outcome, as expected, the results of IVW showed that the seven drugs significantly reduced the risk of CHD (Figure 2).

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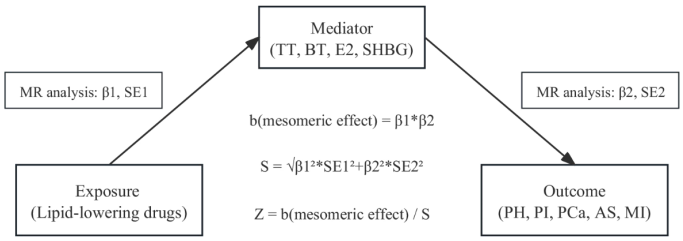

FIGURE 1
Mediation analysis of the effect of drug targets on male diseases.

The causal relationship between lipid-lowering drug targets and primary outcomes

According to the IVW results of the primary analysis method, LDLR agonists showed a significant association with a reduced risk of ED occurrence in the ebi database (OR [95%] = 0.755 [0.559-0.951], p=0.005). In the FinnGen database, the APOC3 inhibitors reduced the risk of ED occurrence (OR [95%] = 0.733 [0.439-1.026], p=0.038). In contrast, APOB inhibitors showed a significant association with an elevated risk of ED occurrence (OR [95%] = 2.77 [2.289-3.251], p<0.001). Results of MR analysis are shown in Table 2.

Meta-analysis was performed on the MR results obtained from each of the two databases. The combined analysis showed that LDLR, LPL agonists and APOC3 inhibitors were significantly associated with a reduced risk of ED occurrence (OR [95%] = 0.766 [0.586 - 0.947], p=0.004 vs OR [95%] = 0.806 [0.643-0.968], p=0.009 vs OR [95%] = 0.866 [0.749-0.984], p=0.016) (Figure 3).APOB inhibitors were significantly associated with an increased risk of ED occurrence (OR[95%]=1.331 [1.087-1.574], p=0.021). Other inhibitors were not significantly associated with the risk of ED occurrence.

The causal relationship between lipid-lowering drug targets and secondary outcomes

In the results of MR analysis with sex hormone levels as an outcome, LDLR and LPL agonists were significantly associated with increased TT levels (OR[95%] = 1.048 [1.019-1.078], p=0.001 vs OR [95%] = 2.77 [2.289-3.251], p<0.001), and HMGCR was significantly associated with decreased TT and BT levels significantly (OR[95%] = 1.08 [1.060-1.1006], p<0.001 vs OR [95%] = 0.824 [0.764-0.884], p<0.001).LDLR, LPL agonists, and PCSK9, APOC3 inhibitors were significantly correlated with elevated SHBG levels, while HMGCR was decreased significantly. In addition, no significant causal relationship was found between drugs and E2 levels (Supplementary Figures 12, 13).

In studies with male-related disease as an outcome, MR results showed that LDLR agonists and PCSK9 inhibitors were significantly

Outcome	Target	method	nsnp	pval	OR (95% CI)
CHD	LDLR	Weighted median	42	<0.001	0.4653 (0.2990 - 0.6317)
		Inverse variance weighted	42	<0.001	0.4384 (0.2938 - 0.5831)
		Weighted mode	42	<0.001	0.4712 (0.2480 - 0.6944)
CHD	HMGCR	Weighted median	19	<0.001	0.6204 (0.3862 - 0.8546)
		Inverse variance weighted	19	<0.001	0.6183 (0.4425 - 0.7941)
		Weighted mode	19	<0.001	0.6226 (0.4065 - 0.8388)
CHD	NPC1L1	Weighted median	6	0.0082	0.4977 (-0.0193 - 1.0147)
		Inverse variance weighted	6	<0.001	0.4575 (0.0176 - 0.8973)
		Weighted mode	6	0.0910	0.5287 (-0.0692 - 1.1266)
CHD	PCSK9	Weighted median	28	<0.001	0.4701 (0.2427 - 0.6974)
		Inverse variance weighted	28	<0.001	0.4355 (0.2738 - 0.5972)
		Weighted mode	28	<0.001	0.4719 (0.2416 - 0.7023)
CHD	APOB	Weighted median	29	<0.001	0.7353 (0.5799 - 0.8906)
		Inverse variance weighted	29	<0.001	0.7132 (0.5819 - 0.8446)
		Weighted mode	29	0.0053	0.7498 (0.5633 - 0.9363)
CHD	APOC3	Weighted median	37	<0.001	0.8577 (0.7741 - 0.9413)
		Inverse variance weighted	37	<0.001	0.8168 (0.7609 - 0.8726)
		Weighted mode	37	<0.001	0.8524 (0.7808 - 0.9240)
CHD	LPL	Weighted median	56	<0.001	0.6621 (0.5605 - 0.7637)
		Inverse variance weighted	56	<0.001	0.6414 (0.5761 - 0.7068)
		Weighted mode	56	<0.001	0.6712 (0.5587 - 0.7837)

FIGURE 2
MR analysis of association between drug targets and CHD.

TABLE 2 The effect of drug targets on ED.

Drug target	Methods	ED (FinnGen database)		ED (ebi database)	
		OR (95% CI)	P value	OR (95% CI)	P value
LDLR	Inverse variance weighted	0.834 (0.375, 1.294)	0.44	0.755 (0.559, 0.951)	0.005
	Weighted median	1.109 (0.426, 1.791)	0.767	0.852 (0.563, 1.14)	0.275
	Weighted mode	1.222 (0.532, 1.913)	0.572	0.896 (0.562, 1.23)	0.523
HMGCR	Inverse variance weighted	1.768 (1.032, 2.504)	0.129	0.716 (0.364, 1.068)	0.063
	Weighted median	1.63 (0.653, 2.608)	0.327	0.821 (0.339, 1.304)	0.425
	Weighted mode	1.715 (0.73, 2.7)	0.297	0.762 (0.282, 1.242)	0.282
NPC1L1	Inverse variance weighted	2.12 (0.412, 3.827)	0.389	1.191 (0.346, 2.037)	0.685
	Weighted median	1.544 (-0.481, 3.569)	0.674	0.911 (-0.208, 2.03)	0.871
	Weighted mode	1.327 (-0.845, 3.5)	0.808	0.882 (-0.406, 2.169)	0.855
PCSK9	Inverse variance weighted	0.933 (0.494, 1.373)	0.759	1.222 (0.935, 1.509)	0.170
	Weighted median	0.818 (0.24, 1.396)	0.495	1.466 (1.069, 1.863)	0.059
	Weighted mode	0.836 (0.291, 1.381)	0.524	1.481 (1.108, 1.853)	0.048
APOB	Inverse variance weighted	2.77 (2.289, 3.251)	<0.001	1.034 (0.752, 1.316)	0.816
	Weighted median	3.097 (2.529, 3.665)	<0.001	0.818 (0.438, 1.197)	0.298
	Weighted mode	3.182 (2.562, 3.803)	0.001	0.796 (0.385, 1.206)	0.289
LPL	Inverse variance weighted	0.932 (0.641, 1.223)	0.635	0.906 (0.775, 1.037)	0.138
	Weighted median	1.152 (0.729, 1.576)	0.511	0.96 (0.775, 1.146)	0.67
	Weighted mode	1.092 (0.676, 1.509)	0.679	0.958 (0.775, 1.141)	0.647
APOC3	Inverse variance weighted	0.733 (0.439, 1.026)	0.038	0.894 (0.767, 1.022)	0.087
	Weighted median	0.804 (0.410, 1.197)	0.276	0.92 (0.745, 1.095)	0.352
	Weighted mode	0.801 (0.393, 1.210)	0.296	0.909 (0.743, 1.075)	0.267

LDLR, low density lipoprotein receptor; HMGCR, HMG-CoA reductase; NPC1L1, Niemann-Pick C1-Like 1; PCSK9, proprotein convertase subtilisin-kexin type 9; APOB, Apolipoprotein (apo) B; LPL, Lipoprotein lipase; APOC3, antisense oligonucleotides targeting apolipoprotein C-III.

associated with an elevated risk of PH occurrence (OR [95%]=1.362 [1.196-1.527], p<0.001 vs OR [95%]=1.261 [1.095-1.427], p=0.006); HMGCR, NPC1L1 inhibitors were significantly associated with a reduced risk of developing PCa (OR [95%] = 0.616 [0.399-0.833], p<0.001 vs OR [95%] = 0.427 [-0.23-1.088], p=0.01); and LDLR agonists were significantly associated with a reduced risk of developing AS and MI (OR [95%] = 0.473 [-0.028 - 0.974], p=0.003 vs OR[95%] = 0.412 [-0.192 - 1.015], p=0.003); in addition, HMGCR inhibitors were significantly associated with a reduced risk of developing PCa (OR[95%] = 7.239 [6.286-8.193], p< 0.001) (Supplementary Figures 14, 15).

Sensitivity analysis

Cochrane's Q and MR Egger regression equations were used to assess levels of heterogeneity and horizontal pleiotropy. When we examined the causal relationship between APOC3 inhibitors and ED (FennGen), we found significant horizontal pleiotropy (p=0.03) (Supplementary Table 11). When we examined the causal relationship between PCSK9, APOB inhibitors and ED(ebi), we found significant horizontal pleiotropy (p=0.03 vs p=0.03). Therefore, in order to obtain more reliable results, we used more stringent criteria for selecting instrumental variables, changing the LD parameters from r^2<0.3 to r^2<0.2 (PCSK9 and APOC3) and r^2<0.1 (APOB). MR analysis was performed again, and the updated results did not show significant heterogeneity or horizontal pleiotropy, and the updated results will be used for subsequent Meta-analysis (Supplementary Table 12).

The results of the sensitivity analysis also showed no heterogeneity or horizontal pleiotropy (p>0.05) in all other outcomes (MR analysis between drugs and hormones, MR analysis between drugs and other male diseases) (Supplementary Tables 14, 16).

The leave-one-out method showed that there would be no significant difference in the results after removing any SNP (Supplementary Figures 1–11).

Mediation analysis

Given the close relationship between sex hormones and male erectile function, reproductive function, and some male disease

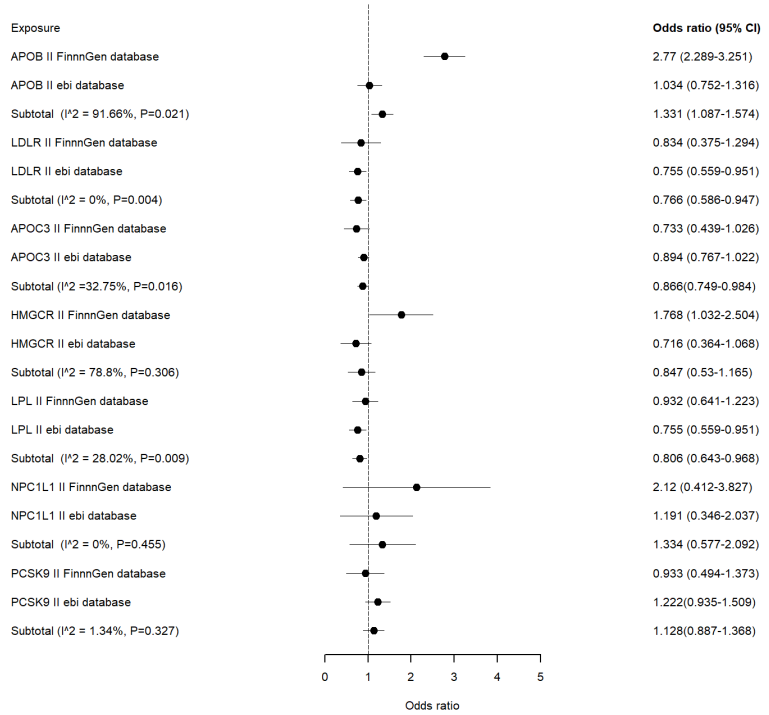

FIGURE 3
Meta analysis of association between drug targets and ED.

interest, they may mediate the effect of lipid-lowering drugs on male disease risk. We therefore used the coefficient product method of mediation analysis to examine the mediating pathway from drug to disease. The results of the mediation analyses suggest that lipid-lowering drugs do not influence the risk of disease development by altering hormone levels. Sex hormone levels were not a mediator between lipid-lowering drugs and disease occurrence (Supplementary Table 18).

Discussion

Erectile dysfunction is a common disease in urology and andrology, which not only brings great pain to the patient and his family, but also is not conducive to the stability of society and family. Therefore, the epidemiology and pathogenesis of ED have attracted more and more attention from scholars all over the world. A large number of experimental and clinical data show that hyperlipidemia is an important risk factor for erectile dysfunction. Current studies have shown that hyperlipidemia induced arterial stenosis and occlusion may only be the late mechanism of hyperlipidemia induced ED. Hyperlipidemia can affect the endothelial cells, smooth muscle cells and peripheral nerves of the penis in the early stage and damage the erectile function of the penis (27–29).

Firstly this study identified two potential targets, low density lipoprotein receptor (LDLR) and Lipoprotein lipase (LPL), which are significantly associated with the development of ED by drug target MR analysis. LPL is a key enzyme regulating lipid fuel processing, and decreased levels or activity can alter LPL lipolytic function, leading to hyperlipidemia and metabolic disorders in the body, causing damage to the vascular endothelium (30). Lorentzen et al.'s study concluded that high blood lipids can increase endothelial cell activity or sensitivity (31). *In vitro* experiments also demonstrated that high plasma TG can damage vascular endothelial cells (32). Therefore, we hypothesized that LPL gene agonists could increase LPL enzyme activity, reduce plasma TG levels, and protect vascular endothelial function, thereby achieving a reduction in the risk of ED. Similar to LPL, LDLR is a key receptor for the body to remove LDL-C from plasma by endocytosis, and it is one of the 3 genes known to be associated with autosomal dominant inheritance of familial hypercholesterolemia (33, 34). Increased LDLR expression reduces plasma cholesterol levels. Musicki et al. found that LDLR-deficient mice fed a high cholesterol diet had a significantly reduced erectile response, and further studies found significantly increased levels of oxidative stress in the penises of mice (35). Similar results were obtained in the present study that LDLR agonists significantly reduced the risk of CHD development and were significantly associated with a reduced risk of ED development.

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) inhibitors, also known as statins, are the first-line drugs used for the prevention and treatment of cardiovascular diseases (36, 37). Currently, the main statins used in clinical practice are lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin. As early as 1996, Bruckert et al. reported the first case of statin-induced ED, and subsequently divided 678 hyperlipidemic male patients aged 30-70 years into a lipid-lowering drug group and a placebo group

(339 cases in each group), and found that the incidence of ED in the lipid-lowering drug group was 12.1%, and the placebo group was 5.6%, with the difference statistically significant (P=0.0029) (38). Rizvi et al. have also reported 5 patients with ED triggered by simvastatin (10 mg/d and 20 mg/d), which returned to normal after 1 week of discontinuation of the drug (2 of them had ED recurrence in the provocation test) (11). However, in the present study, MR analysis did not reveal a significant causal relationship between HMGCR inhibitors and ED occurrence. We believe that most of the patients reported in the previous literature had ED risk factors such as hyperlipidemia and essential hypertension, and the increased incidence of ED may be a result of the combined effect of the above multifactorial factors, which should not be simply attributed to lipid-lowering drugs alone. And simvastatin listed earlier, can not completely represent other statin drugs. Some scholars believe that statins cause ED may be related to their inhibition of HMG-CoA reductase to reduce cholesterol synthesis, affecting the synthesis of steroid hormones (including testosterone).Corona et al. found that 244 cases of statin users serum total testosterone and free testosterone concentrations were significantly lower than normal values (39). In the present study, MR analysis showed that HMGCR inhibitors were associated with significantly lower levels of TT, BT, and SHBG, consistent with previous studies. In addition, the non-lipid-lowering effects of statins (e.g., antioxidant effects) have received increasing attention. The results of foreign studies in recent years have shown that statins can reduce the risk of prostate cancer, decrease the recurrence rate of prostate cancer, and delay the progression of the disease (40, 41). The results of this study are consistent with previous findings, but the specific mechanism of action is not clear.

Currently lipid-lowering drugs still recommend statins as the first-line therapeutic drugs, but there are some patients who are intolerant to statins or have poor therapeutic effects (8). With the development of new drugs, some non-statin lipid-lowering drugs have been gradually applied to clinical practice. Since the identification of the causative gene, proprotein convertase subtilisin-kexin type 9 (PCSK9), in familial hypercholesterolemia family lines in 2003, the development of PCSK9-targeted inhibitors has progressed rapidly (42, 43). PCSK9 inhibitors are now widely used as a new class of lipid-lowering drugs in a wide range of patients with cardiovascular diseases, including familial hypercholesterolemia. A study by Mostaza et al. found that the R46L variant of PCSK9 was significantly associated with the development of ED, and that the prevalence of ED was higher in carriers of the T allele of the R46L gene (44). And in a real-world study, Scicali et al. evaluated the effect of PCSK9-i on sexual function as assessed by the Men's Sexual Health Questionnaire (MSHQ) and the International Index of Erectile Function (IIEF-5) questionnaires, and found that patients' MSHQs improved with the addition of PCSK9-i treatment, whereas there was no significant difference in the IIEF-5 before and after use (45). In the present study, MR analysis similarly did not find a significant causal relationship between PCSK9-i and the development of ED. In addition the present study also found that PCSK9-i and SHBG levels were associated with an elevated risk of PH development, but the exact mechanism still needs to be explored subsequently.

ApoC III is a strong inhibitor of LPL, which hinders the breakdown and clearance of TRLs and leads to the aggregation of circulating pro-atherosclerotic factors such as coeliac particles and VLDL. Numerous studies at home and abroad have shown that elevated serum ApoC III levels can cause hypertriacylglycerolemia, and that inhibiting ApoC III synthesis or lowering blood (46–48). Levels of ApoC III is an effective strategy for the prevention of cardiovascular disease. In the present study, ApoC III inhibitors were found to significantly reduce the risk of ED. APOB also plays a central role in human lipoprotein metabolism. The APOB gene produces two forms of apoB through a unique post-transcriptional editing process: apoB-48 and apoB-100.ApoB-100 is an important structural component of very low-density lipoproteins (VLBIs) and their metabolites, intermediate-density lipoproteins (IDL) and ApoB-100 is an important structural component of LDL and its metabolites, intermediate density lipoprotein (IDL) and LDL, as well as a ligand for receptor-mediated endocytosis of LDL (49). Increased plasma concentrations of ApoB have been shown to be a key risk factor for the development of atherosclerosis. Mipomersen, an antisense oligodeoxynucleotide inhibitor of apolipoprotein B-100, has been approved for the treatment of familial hypercholesterolemia (50). Some studies have suggested a significant negative correlation between ApoB and testosterone levels (51, 52), but the present study did not find a significant causal relationship between ApoB inhibitors and testosterone levels. It is noteworthy that ApoB inhibitors were the only lipid-lowering agents found to be associated with an increased risk of ED development in the MR analysis results of this study, and this result and the specific mechanism still need to be verified in subsequent clinical trials.

Our study has some unavoidable limitations. First, MR analysis is only a method for analyzing causal relationships between exposures and outcomes, and it is more useful for determining the direction of the association than for quantifying the magnitude of the association. It cannot completely replace clinical trials in the objective world. Secondly, drug-targeted MR analysis may not accurately reflect the effects of short-term and different routes of administration. Finally, due to insufficient GWAS data resources, we only performed MR analysis on European populations and our findings may not be applicable to other ethnicities.

Conclusion

In conclusion, after performing drug-targeted MR analysis, we found that APOC3 inhibitors among lipid-lowering drugs significantly reduced the risk of ED occurrence, while on the contrary APOB inhibitors may be associated with an elevated risk of ED occurrence. In addition, the results also found that PCSK9 inhibitors increased the risk of PH, and NPC1L1, HMGCR inhibitors decreased the risk of PCa, but at the same time HMGCR inhibitors increased the risk of male infertility. Notably, this study suggests that LDLR and LPL may be new candidate drug targets for the treatment of ED, and their agonists significantly reduced the risk of ED occurrence in MR analysis, but the results still need to be further validated in basic, clinical studies.

Data availability statement

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

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

QS: Writing – original draft, Writing – review & editing. RW: Writing – review & editing. YL: Writing – review & editing. QT: Writing – original draft. KW: Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1362499/full#supplementary-material

References

1. Shamloul R, Ghanem H. Erectile dysfunction. *Lancet* (2013) 381(9861):153–65. doi: 10.1016/S0140-6736(12)60520-0

2. Miner M, Parish SJ, Billups KL, Paulos M, Sigman M, Blaha MJ. Erectile dysfunction and subclinical cardiovascular disease. *Sex Med Rev* (2019) 7(3):455–63. doi: 10.1016/j.sxmr.2018.01.001

3. Randrup E, Baum N, Feibus A. Erectile dysfunction and cardiovascular disease. *Postgrad Med* (2015) 127(2):166–72. doi: 10.1080/00325481.2015.992722

4. Mostafaei H, Mori K, Hajebrahimi S, Abufaraj M, Karakiewicz PI, Shariat SF. Association of erectile dysfunction and cardiovascular disease: an umbrella review of systematic reviews and meta-analyses. *BJU Int* (2021) 128(1):3–11. doi: 10.1111/bju.15313

5. Wierzbicki AS, Kim EJ, Esan O, Ramachandran R. Hypertriglyceridaemia: An update. *J Clin Pathol* (2022) 75(12):798–806. doi: 10.1136/jclinpath-2021-207719

6. Aguilar-Salinas CA, Gomez-Diaz RA, Corral P. New therapies for primary hyperlipidemia. *J Clin Endocrinol Metab* (2022) 107(5):1216–24. doi: 10.1210/clinem/dgab876

7. Parhofer KG, Laufs U. The diagnosis and treatment of hypertriglyceridemia. *Dtsch Arztebl Int* (2019) 116(49):825–32. doi: 10.3238/arztebl.2019.0825

8. Bosco G, Di Giacomo Barbagallo F, Spampinato S, Lanzafame L, Di Pino A, Piro S, et al. Management of statin intolerant patients in the era of novel lipid lowering therapies: A critical approach in clinical practice. *J Clin Med* (2023) 12(6). doi: 10.3390/jcm12062444

9. Esposito K, Giugliano F, Di Palo C, Giugliano G, Marfella R, D'Andrea F, et al. Effect of lifestyle changes on erectile dysfunction in obese men: a randomized controlled trial. *JAMA* (2004) 291(24):2978–84. doi: 10.1001/jama.291.24.2978

10. Kim SC. Hyperlipidemia and erectile dysfunction. *Asian J Androl* (2000) 2 (3):161–6.

11. Rizvi K, Hampson JP, Harvey JN. Do lipid-lowering drugs cause erectile dysfunction? A systematic review. *Fam Pract* (2002) 19(1):95–8. doi: 10.1093/fampra/19.1.95

12. Cai X, Tian Y, Wu T, Cao CX, Bu SY, Wang KJ. The role of statins in erectile dysfunction: a systematic review and meta-analysis. *Asian J Androl* (2014) 16(3):461–6. doi: 10.4103/1008-682X.123678

13. Tuccori M, Montagnani S, Mantarro S, Capogrosso-Sansone A, Ruggiero E, Saporiti A, et al. Neuropsychiatric adverse events associated with statins: epidemiology, pathophysiology, prevention and management. *CNS Drugs* (2014) 28(3):249–72. doi: 10.1007/s40263-013-0135-1

14. Sekula P, Del Greco MF, Pattaro C, Kottgen A. Mendelian randomization as an approach to assess causality using observational data. *J Am Soc Nephrol* (2016) 27 (11):3253–65. doi: 10.1681/ASN.2016010098

15. Birney E. Mendelian randomization. *Cold Spring Harb Perspect Med* (2022) 12 (4). doi: 10.1101/cshperspect.a041302

16. Evans DS. Target discovery for drug development using mendelian randomization. *Methods Mol Biol* (2022) 2547:1–20. doi: 10.1007/978-1-0716-2573-6_1

17. Richardson TG, Sanderson E, Palmer TM, Ala-Korpela M, Ference BA, Davey Smith G, et al. Evaluating the relationship between circulating lipoprotein lipids and apolipoproteins with risk of coronary heart disease: A multivariable Mendelian randomisation analysis. *PloS Med* (2020) 17(3):e1003062. doi: 10.1371/journal.pmed.1003062

18. Nikpay M, Goel A, Won HH, Hall LM, Willenborg C, Kanoni S, et al. A comprehensive 1,000 Genomes-based genome-wide association meta-analysis of coronary artery disease. *Nat Genet* (2015) 47(10):1121–30. doi: 10.1038/ng.3396

19. Bovijn J, Jackson L, Censin J, Chen CY, Laisk T, Laber S, et al. GWAS identifies risk locus for erectile dysfunction and implicates hypothalamic neurobiology and diabetes in etiology. *Am J Hum Genet* (2019) 104(1):157–63. doi: 10.1016/j.ajhg.2018.11.004

20. Ruth KS, Day FR, Tyrrell J, Thompson DJ, Wood AR, Mahajan A, et al. Using human genetics to understand the disease impacts of testosterone in men and women. *Nat Med* (2020) 26(2):252–8. doi: 10.1038/s41591-020-0751-5

21. Sakaue S, Kanai M, Tanigawa Y, Karjalainen J, Kurki M, Koshiba S, et al. A cross-population atlas of genetic associations for 220 human phenotypes. *Nat Genet* (2021) 53(10):1415–24. doi: 10.1038/s41588-021-00931-x

22. Burgess S, Scott RA, Timpson NJ, Davey Smith G, Thompson SG, Consortium E-I. Using published data in Mendelian randomization: a blueprint for efficient identification of causal risk factors. *Eur J Epidemiol* (2015) 30(7):543–52. doi: 10.1007/s10654-015-0011-z

23. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet Epidemiol* (2016) 40(4):304–14. doi: 10.1002/gepi.21965

24. Hartwig FP, Davey Smith G, Bowden J. Robust inference in summary data Mendelian randomization *via* the zero modal pleiotropy assumption. *Int J Epidemiol* (2017) 46(6):1985–98. doi: 10.1093/ije/dyx102

25. Lortie CJ, Filazzola A. A contrast of meta and metafor packages for meta-analyses in R. . *Ecol Evol* (2020) 10(20):10916–21. doi: 10.1002/ece3.6747

26. Woolf B, Zagkos L, Gill D. TwoStepCisMR: A novel method and R package for attenuating bias in cis-mendelian randomization analyses. *Genes (Basel)* (2022) 13(9). doi: 10.3390/genes13091541

27. Gandaglia G, Briganti A, Jackson G, Kloner RA, Montorsi F, Montorsi P, et al. A systematic review of the association between erectile dysfunction and cardiovascular disease. *Eur Urol* (2014) 65(5):968–78. doi: 10.1016/j.eururo.2013.08.023

28. Vlachopoulos C, Rokkas K, Ioakeimidis N, Stefanadis C. Inflammation, metabolic syndrome, erectile dysfunction, and coronary artery disease: common links. *Eur Urol* (2007) 52(6):1590–600. doi: 10.1016/j.eururo.2007.08.004

29. Vlachopoulos C, Ioakeimidis N, Rokkas K, Angelis A, Terentes-Printzios D, Kratiras Z, et al. Central haemodynamics and prediction of cardiovascular events in patients with erectile dysfunction. *Am J Hypertens* (2017) 30(3):249–55. doi: 10.1093/ajh/hpw150

30. Li Y, He PP, Zhang DW, Zheng XL, Cayabyab FS, Yin WD, et al. Lipoprotein lipase: from gene to atherosclerosis. *Atherosclerosis* (2014) 237(2):597–608. doi: 10.1016/j.atherosclerosis.2014.10.016

31. Lorentzen B, Henriksen T. Plasma lipids and vascular dysfunction in preeclampsia. *Semin Reprod Endocrinol* (1998) 16(1):33–9. doi: 10.1055/s-2007-1016250

32. Birkeland KI, Kilhovd B, Thorsby P, Torjesen PA, Ganss R, Vaaler S, et al. Heterogeneity of non-insulin-dependent diabetes expressed as variability in insulin sensitivity, beta-cell function and cardiovascular risk profile. *Diabetes Med* (2003) 20 (1):37–45. doi: 10.1046/j.1464-5491.2003.00838.x

33. Go GW, Mani A. Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. *Yale J Biol Med* (2012) 85(1):19–28.

34. Santos PC, Pereira AC. Type of LDLR mutation and the pharmacogenetics of familial hypercholesterolemia treatment. *Pharmacogenomics* (2015) 16(15):1743–50. doi: 10.2217/pgs.15.113

35. Musicki B, Liu T, Lagoda GA, Strong TD, Sezen SF, Johnson JM, et al. Hypercholesterolemia-induced erectile dysfunction: endothelial nitric oxide synthase (eNOS) uncoupling in the mouse penis by NAD(P)H oxidase. *J Sex Med* (2010) 7 (9):3023–32. doi: 10.1111/j.1743-6109.2010.01880.x

36. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* (2001) 292(5519):1160–4. doi: 10.1126/science.1059344

37. Friesen JA, Rodwell VW. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. *Genome Biol* (2004) 5(11):248. doi: 10.1186/gb-2004-5-11-248

38. Bruckert E, Giral P, Heshmati HM, Turpin G. Men treated with hypolipidaemic drugs complain more frequently of erectile dysfunction. *J Clin Pharm Ther* (1996) 21 (2):89–94. doi: 10.1111/j.1365-2710.1996.tb00006.x

39. Corona G, Boddi V, Balercia G, Rastrelli G, De Vita G, Sforza A, et al. The effect of statin therapy on testosterone levels in subjects consulting for erectile dysfunction. *J Sex Med* (2010) 7(4 Pt 1):1547–56. doi: 10.1111/j.1743-6109.2009.01698.x

40. Ramos JD, Yu EY. Progress in understanding what is being statin(ed) in prostate cancer. *JAMA Oncol* (2015) 1(4):428–30. doi: 10.1001/jamaoncol.2015.0833

41. Peltomaa AI, Raittinen P, Talala K, Taari K, Tammela TLJ, Auvinen A, et al. Prostate cancer prognosis after initiation of androgen deprivation therapy among statin users. A population-based cohort study. *Prostate Cancer Prostatic Dis* (2021) 24 (3):917–24. doi: 10.1038/s41391-021-00351-2

42. Melendez QM, Krishnaji ST, Wooten CJ, Lopez D. Hypercholesterolemia: the role of PCSK9. *Arch Biochem Biophys* (2017) 625-626:39–53. doi: 10.1016/j.abb.2017.06.001

43. Pasta A, Cremonini AL, Pisciotta L, Buscaglia A, Porto I, Barra F, et al. PCSK9 inhibitors for treating hypercholesterolemia. *Expert Opin Pharmacother* (2020) 21 (3):353–63. doi: 10.1080/14656566.2019.1702970

44. Mostaza JM, Lahoz C, Salinero-Fort MA, de Dios O, Castillo E, Gonzalez-Alegre T, et al. R46L polymorphism in the PCSK9 gene: Relationship to lipid levels, subclinical vascular disease, and erectile dysfunction. *J Clin Lipidol* (2018) 12(4):1039–46.e3. doi: 10.1016/j.jacl.2018.04.004

45. Scicali R, Russo GI, Di Mauro M, Manuele F, Di Marco G, Di Pino A, et al. Analysis of arterial stiffness and sexual function after adding on PCSK9 inhibitor treatment in male patients with familial hypercholesterolemia: A single lipid center real-world experience. *J Clin Med* (2020) 9(11). doi: 10.3390/jcm9113597

46. Jin JL, Guo YL, Li JJ. Apoprotein C-III: A review of its clinical implications. *Clin Chim Acta* (2016) 460:50–4. doi: 10.1016/j.cca.2016.06.016

47. de la Parra Soto LG, Gutierrez-Uribe JA, Sharma A, Ramirez-Jimenez AK. Is Apo-CIII the new cardiovascular target? An analysis of its current clinical and dietetic therapies. *Nutr Metab Cardiovasc Dis* (2022) 32(2):295–308. doi: 10.1016/j.numecd.2021.09.035

48. Mehta A, Shapiro MD. Apolipoproteins in vascular biology and atherosclerotic disease. *Nat Rev Cardiol* (2022) 19(3):168–79. doi: 10.1038/s41569-021-00613-5

49. Whitfield AJ, Barrett PH, van Bockxmeer FM, Burnett JR. Lipid disorders and mutations in the APOB gene. *Clin Chem* (2004) 50(10):1725–32. doi: 10.1373/clinchem.2004.038026

50. Parham JS, Goldberg AC. Mipomersen and its use in familial hypercholesterolemia. *Expert Opin Pharmacother* (2019) 20(2):127–31. doi: 10.1080/14656566.2018.1550071

51. Rezanezhad B, Borgquist R, Willenheimer R, Elzanaty S. Association between serum levels of testosterone and biomarkers of subclinical atherosclerosis. *Aging Male* (2018) 21(3):182–6. doi: 10.1080/13685538.2017.1412422

52. Rezanezhad B, Borgquist R, Willenheimer R, Elzanaty S. The association between serum testosterone and risk factors for atherosclerosis. *Curr Urol* (2019) 13 (2):101–6. doi: 10.1159/000499285

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The impact of SLCO1B1 rs4149056 on LDL-C target achievement after lipid lowering therapy optimization in men and women with familial hypercholesterolemia

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Background and aims: FH women are less likely to receive intensive statin treatment and to obtain a 50% reduction of LDL-C from baseline compared to men with FH. SLCO1B1 rs4149056 might influence statin therapy compliance and thus LDL-C target achievement. Our aim was to evaluate the impact of SLCO1B1 rs4149056 on LDL-C target achievement after lipid lowering therapy (LLT) optimization in men and women with FH.

Methods: This was a retrospective observational study involving 412 FH subjects with a probable or defined clinical diagnosis of FH who had had genetic analysis from June 2016 to September 2022. Biochemical analysis was obtained from all subjects at baseline and at the last follow-up after LLT optimization.

Results: After LLT optimization the percentage of FH subjects on high-intensity statins decreased from the M/SLCO1B1- group to the W/SLCO1B1+ group and the same was found in LDL-C target distribution (for both p for trend < 0.01). The prevalence of SASE fear increased from the M/SLCO1B1- group to the W/SLCO1B1+ group and the same was observed in reported myalgia distribution (for both p for trend < 0.01). Logistic regression analysis showed that the W/SCLO1B1-, M/SCLO1B1+ and W/SCLO1B1+ groups were inversely associated with LDL-C target achievement (p for trend < 0.001) and the W/SCLO1B1+ group exhibited the strongest association.

Conclusion: A low prevalence of FH women with SLCO1B1 rs4149056 were on high intensity statins and they rarely achieved LDL-C target. The genotype effect of SLCO1B1 rs4149056 could be more pronounced in FH women than men.

KEYWORDS

familial hypercholesterolemia, LDL-C target, lipid lowering therapy, SLCO1B1 rs4149056, cardiovascular risk

Introduction

Familial hypercholesterolemia (FH) is the most frequent monogenic lipid disorder characterized by an increased plasma level of low-density lipoprotein cholesterol (LDL-C) since childhood (1). FH subjects have a high risk of premature atherosclerotic cardiovascular disease (ASCVD) mainly due to a lifelong elevated LDL-C plasma level that promotes the development and the progression of atherosclerotic injury in the arterial wall (2); however, among FH subjects ASCVD risk is highly heterogeneous and it seems to be also influenced by other risk factors beyond LDL-C (3).

Early diagnosis and lipid lowering treatment optimization can considerably reduce the risks of premature atherosclerotic cardiovascular disease (ASCVD) in FH subjects (4). Statin treatment is the cornerstone of lipid-lowering therapies (LLT) and it should be initiated as soon as possible, even during childhood, as it has been shown to decrease the risk of CVD in adults (5, 6). Although the efficacy and safety of statins have already been demonstrated (7), statin treatment discontinuation is frequent in clinical practice, especially among patients on high intensity or long-term statin therapies (8, 9). The most frequently observed disorder is the onset of statin associated muscle symptoms (SAMS) that leads to statin discontinuation (10); moreover, a higher SAMS prevalence was reported in women than in men and this could be explained by the different effects of gender on pharmacokinetics and pharmacodynamics of statins (11). Previous findings from the CAscade SCreening for Awareness and DEtection of Familial Hypercholesterolemia (CASCADE-FH) registry reported that FH women were less likely to receive an intensive statin treatment as well as not obtaining a 50% reduction from baseline LDL-C compared to FH men (12). Thus, a different statin approach could partially explain the high percentage of premature ASCVD reported both in men and women with FH in contrast to the sex related cardiovascular injury onset observed in the general population (13). Beyond the impact of sex on LLT use, genetic polymorphisms associated with statin trafficking into the liver might influence the adherence as well as the efficacy of statins (14).

The solute carrier organic anion transporter 1B1 (*SLCO1B1*) gene encodes organic anion transporter polypeptide 1b1 (OATP1B1) that carries statins into tissues (15). It has been shown that the single nucleotide polymorphism SLCO1B1 521T>C (rs4149056) enhanced statin plasma levels and it was associated with an increased risk of SAMS in the general population (16). However, the liver concentration of statins as well as their LDL-C lowering effect are reduced in subjects with SLCO1B1 rs4149056 (17). Thus, the achievement of the recommended LDL-C target could be difficult in FH subjects with SLCO1B1 rs4149056. There is no data regarding the impact of SLCO1B1 rs4149056 on LDL-C target achievement in FH subjects.

In this study we aimed to evaluate the impact of SLCO1B1 rs4149056 on LDL-C target achievement after lipid lowering therapy optimization in men and women with FH.

Methods

Study design and population

This was a retrospective observational study involving subjects with a probable or defined clinical diagnosis of FH (Dutch Lipid Clinical Network score ≥ 6) who had had genetic analysis (18) from June 2016 to September 2022. All subjects were enrolled from the referral lipid center of the University Hospital of Catania and were aged between 18 and 70 years at the time of enrollment. At baseline, all participants underwent a physical examination and review of their clinical history. All subjects had biochemical analysis at baseline and at the last follow-up (January 2023-June 2023) after at least 3 month's lipid lowering therapy optimization that was performed according to LDL-C values as well as a physician's decision and Italian reimbursement rules. According to 2019 ESC/EAS guidelines for the management of dyslipidemias, all FH subjects obtained lipid lowering therapy optimization that was defined as a daily intake of high intensity statins plus ezetimibe +/- proprotein convertase subtilisin/kexin type 9 monoclonal antibodies (PCSK9-mAb). Based on the recommendations of the ESC/EAS guidelines for the management of dyslipidemias, baseline LDL-C target was defined as the following: LDL-C < 70 mg/dL or < 100 mg/dL in FH subjects with or without ASCVD enrolled from June 2016 to August 2019 or LDL-C < 55 mg/dL or < 70 mg/dL in FH subjects with or without ASCVD enrolled from September 2019 to September 2022 (19, 20). At the last follow-up, LDL-C target was defined as an LDL-C < 55 mg/dL or < 70 mg/dL for FH subjects with or without ASCVD, respectively.

Body weight and height were measured, and body mass index (BMI) was calculated as weight divided by the squared value of height

(kg/m2). Arterial hypertension was defined as brachial blood pressure (BP) ≥ 140 mm Hg (systolic) and/or 90 mm Hg (diastolic) on at least two different occasions, or if the subjects were on antihypertensive therapy. Lipid lowering therapy was defined as a daily intake of one of the following drugs: statins, ezetimibe, or PCSK9-i. According to drug intensity, statin therapy was classified as low-intensity (fluvastatin 20–40 mg, lovastatin 20 mg, pravastatin 20 mg, simvastatin 10 mg) moderate-intensity (fluvastatin XL 80 mg, lovastatin 40 mg, pravastatin 40 mg, simvastatin 20–40 mg, atorvastatin 10–20 mg, rosuvastatin 5–10 mg) or high-intensity (atorvastatin 40–80 mg, rosuvastatin 20–40 mg) (21). When they occurred, the fear of statin associated side effects (SASE) or myalgia were reported by FH subjects on low to moderate intensity statins at the last follow-up. PCSK9-mAb therapy included alirocumab or evolocumab. Type 2 diabetes (T2D) was defined as a fasting plasma glucose (FPG) ≥ 126 mg/dL on two consecutive readings and/or glycated hemoglobin (HbA1c) ≥ 6.5% or the use of anti-diabetic medications (22). Smoking habits were divided into either current smoking (defined as a minimum of one cigarette in the last month) or not (23). ASCVD was defined as a documented myocardial infarction, acute coronary syndrome, coronary revascularization (percutaneous coronary intervention or coronary artery bypass graft surgery) or other arterial revascularization procedures, stroke or transient ischemic attack, or peripheral arterial disease (24).

The study population was stratified into two groups according to sex. The study was approved by the local ethics committee in accordance with the ethical standards of the institutional and national research committees and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from each subject enrolled in the study.

Biochemical analysis

FPG was measured with the glucose oxidase method. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), hs-CRP, aspartate transaminase (AST), alanine transaminase (ALT), and creatine phosophokinase (CPK) were assessed by available enzymatic methods. Apolipoprotein B (ApoB), and Apolipoprotein A1 (ApoA1) were evaluated with a nephelometer assay (Siemens AG Healthcare Sector, Erlangen, Germany). Levels of lipoprotein(a) [Lp(a)] were measured with the latex agglutination immunoassay. LDL-C was calculated using the Friedewald formula. HbA1c was measured with high-performance liquid chromatography using a National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial assay reference (22). Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.; normal range 4.25-5.9% [23-41 mmol/mol]).

Statistical analysis

The distributional characteristics of each variable, including normality, were assessed by the Kolmogorov-Smirnov test. Data are reported as mean ± standard deviation (SD) for continuous parametric and median (interquartile range-IQR) for continuous non-parametric variables and as frequency (percentage) for categorical variables. When necessary, continuous non-parametric variables (TG, Lp(a), hs-CRP, CPK) were logarithmically transformed for statistical analysis to reduce skewness. The Chi square (χ^2) test was used for categorical variables. To test differences in clinical and biochemical characteristics between the groups Student's t test was used.

In a secondary analysis, the study population was stratified into four groups according to sex and SLCO1B1 rs4149056 presence: men without SLCO1B1 rs4149056 (M/SLCO1B1- group), women without SLCO1B1 rs4149056 (W/SLCO1B1- group), men with SLCO1B1 rs4149056 (M/SCLO1B1+ group), women with SLCO1B1 rs4149056 (W/SCLO1B1+ group). A χ^2 test was performed to assess the distributions of high-intensity statins, LDL-C target, fear of SASE and reported myalgia in the four groups. In order to evaluate the impact of sex and SLCO1B1 rs4149056 on LDL-C target achievement, we performed a logistic regression analysis adjusted for age, statin intensity, ezetimibe, and PCSK9-i. The variance inflation factor (VIF) was used to check for the problem of multicollinearity in multivariate analysis. All statistical analyses were performed using IBM SPSS Statistics for Windows version 23. For all tests, $p < 0.05$ was considered significant.

Results

A total of 488 probable/defined FH subjects who had had genetic analysis were evaluated; of these, 412 FH subjects (210 men and 202 women) satisfied the inclusion criteria and participated in this retrospective observational study (Figure 1).

The genetic profile of the study population is presented in Table 1. While fewer than one third of subjects did not present a genetic variant, the prevalence of mutation positive FH was 72.6% and it was similar both in men and women. The majority of subjects were heterozygous FH and the most frequent genetic variant was LDLR mutation with no difference between the two groups. Finally, the proportion of FH subjects with SLCO1B1 rs4149056 was 24.5% and it was similar between FH men and women.

Table 2 shows the baseline characteristics of the study population. No differences in age and BMI were found between the two groups and the prevalence of FH subjects with ASCVD history was similar both in men and women. Pretreatment TC and LDL-C as well as baseline TC, LDL-C, Non-HDL-C, ApoB and Apo AI plasma levels were significantly higher in women compared to men (for pretreatment TC 339.26 ± 26.19 *vs* 356.15 ± 25.97 $p < 0.05$; for pretreatment LDL-C 255.21 ± 24.47 *vs* 271.14 ± 23.87 $p < 0.05$; for TC 217.93 ± 25.71 *vs* 235.52 ± 24.93 $p < 0.05$; for LDL-C 149.54 ± 21.84 *vs* 164.21 ± 21.13 $p < 0.05$; for Non-HDL-C 165.76± 22.08 *vs* 180.69 ± 21.93 $p < 0.05$; for ApoB 114.64 ± 12.44 *vs* 128.22 ± 12.01 $p < 0.05$; for Apo AI 140.65 ± 13.8 *vs* 151.22 ± 12.81 $p < 0.001$). Moreover, the percentage of FH subjects on lipid lowering therapy was significantly lower in women than men (42.6% *vs* 61%, $p < 0.01$); of these, while a higher prevalence of FH subjects on moderate or high-intensity statins were found in men than women (45.0% *vs* 31.9% and 25.4% *vs* 19.1% respectively, for both $p < 0.05$) the distribution of subjects on

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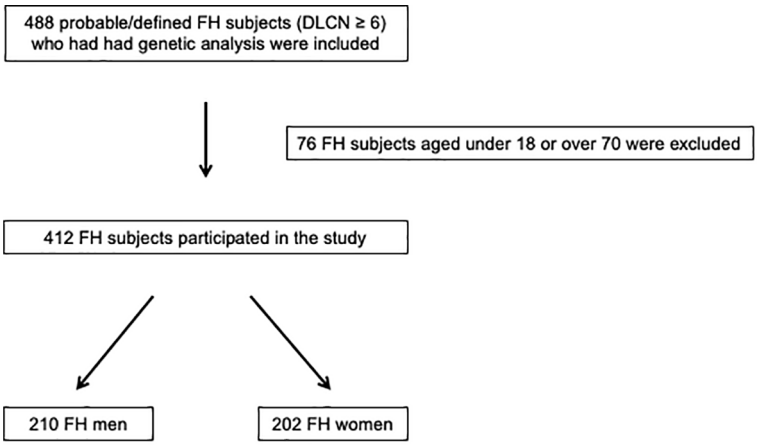

FIGURE 1
Enrollment flowchart of the study population. DLCN, Dutch Lipid Clinic Network; FH, familial hypercholesterolemia.

TABLE 1 Genetic profile of the Study Population stratified according to sex.

	Men (n = 210)	Women (n = 202)	*p* Value between two groups
FH Genotype			
Mutation-negative, n (%)	58 (27.6)	55 (27.2)	0.87
Mutation-positive, n (%)	152 (72.4)	147 (72.8)	0.87
- LDLR, n (%)	149 (98.0)	144 (97.9)	0.91
- LDLR defective, n (%)	83 (55.7)	77 (53.5)	0.23
- LDLR null, n (%)	66 (44.3)	67 (46.5)	0.23
- ApoB, n (%)	3 (2.0)	1 (0.7)	–
- PCSK9, n (%)	–	1 (0.7)	–
- ApoE, n (%)	–	1 (0.7)	–
FH Phenotype			
Heterozygous FH, n (%)	147 (98.7)	142 (98.6)	–
Compound heterozygous FH, n (%)	2 (1.3)	1 (0.7)	–
Homozygous FH, n (%)	–	1 (0.7)	–
SLCO1B1 Polymorphism			
rs4149056, n (%)	48 (22.9)	53 (26.2)	0.26
- Heterozygous rs4149056, n (%)	47 (97.9)	51 (96.2)	0.72
- Homozygous rs4149056, n (%)	1 (2.1)	2 (3.8)	–

Data are presented as mean ± standard deviation or percentages. FH, familial hypercholesterolemia; LDLR, low-density lipoprotein receptor; ApoB, apolipoprotein B; PCSK9, proprotein convertase subtilisin/kexin type 9; ApoE, apolipoprotein E; SLCO1B1, solute carrier organic anion transporter family member 1B1.

low-intensity statin was higher in FH women than men (49.0% *vs* 29.6%, $p < 0.01$). As concerns lipid lowering combination therapy, while a higher prevalence of FH men was on moderate intensity statins plus ezetimibe compared to women (39.5% *vs* 32.0%, $p < 0.05$), the percentage of subjects on low intensity statins plus ezetimibe was higher in FH women than men (40.0% *vs* 31.6%, $p < 0.05$).

At the last follow-up, after LLT optimization, a significant improvement of lipid profile was observed in the study population; however, FH women exhibited a higher LDL-C than men (104.82 ± 20.05 *vs* 92.83 ± 19.79, $p < 0.05$) and the proportion of subjects on LDL-C target was lower in FH women than men (27.7% *vs* 38.1%, $p < 0.05$). The glucose profile was similar between FH men and women and only 3 new cases of T2D occurred. All FH subjects were on lipid lowering therapy but the majority of them were on low to moderate intensity statins and they were more prevalent in FH women than men (70.3% *vs* 48.6% $p < 0.001$). Among these, the percentages of FH subjects who reported the fear of SASE or myalgia were higher in women than men (48.0% *vs* 32.9%, $p < 0.01$ and 22.3% *vs* 15.7%, $p < 0.05$, respectively). While a higher prevalence of FH men were on high-intensity statins plus ezetimibe compared to women (53.7% *vs* 32.0%, $p < 0.01$), an increased percentage of subjects on low-intensity statins plus ezetimibe was found in FH women than men (30.5% *vs* 13.2%, $p < 0.01$) and the same prevalence was reported in subjects on statins plus ezetimibe plus PCSK9i (for high-intensity statins plus ezetimibe plus PCSK9i 51.5% *vs* 29.2%, $p < 0.01$; for low-intensity statins plus ezetimibe plus PCSK9i 33.9% *vs* 10.3%, $p < 0.01$) (Table 3).

In a secondary analysis, the study population was stratified into four groups according to sex and SLCO1B1 rs4149056 presence: men without SLCO1B1 rs4149056 (M/SLCO1B1- group), women without SLCO1B1 rs4149056 (W/SLCO1B1- group), men with SLCO1B1 rs4149056 (M/SCLO1B1+ group), women with SLCO1B1 rs4149056 (W/SCLO1B1+ group). After LLT optimization, the percentage of FH subjects on high-intensity statins decreased from the M/SLCO1B1- group to the W/SLCO1B1+ group and the same was found in LDL-C target distribution (for both *p* for trend < 0.01) (Figure 2); however, the prevalence of SASE fear increased from the M/SLCO1B1- group to

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TABLE 2 Baseline characteristics of the Study Population stratified according to sex.

	Men (n = 210)	Women (n = 202)	*p* Value between two groups
Demographic Characteristics			
Age, years	52.4 ± 8.3	51.9 ± 8.7	0.61
Body mass index, kg/m^2	25.2 ± 3.2	25.1 ± 3.2	0.83
History of ASCVD, n (%)	39 (18.6)	26 (12.9)	0.07
Glucose Profile			
Type 2 diabetes, n (%)	4 (1.9)	3 (1.5)	–
FPG, mg/dL	89.5 ± 5.4	88.8± 5.4	0.59
HbA1c, %	5.5 ± 0.3	5.5 ± 0.3	0.54
Lipid Profile			
Pretreatment TC, mg/dL	339.3 ± 26.2	356.2 ± 26	< 0.05
Pretreatment LDL-C, mg/dL	255.2 ± 24.5	271.1 ± 23.9	< 0.05
TC, mg/dL	217.9 ± 25.7	235.5 ± 24.9	< 0.05
HDL-C, mg/dL	49.5 ± 9.4	56.4 ± 9.3	< 0.001
Triglycerides, mg/dL	95 (77-120)	87 (73-115)	0.06
LDL-C, mg/dL	149.5 ± 21.8	164.2 ± 21.1	< 0.05
Non-HDL-C, mg/dL	165.8 ± 22.1	180.7 ± 21.9	< 0.05
ApoB, mg/dL	114.6 ± 12.4	128.2 ± 12	< 0.05
ApoAI, m g/dL	140.7 ± 13.8	151.2 ± 12.8	< 0.001
ApoB to ApoAI ratio	0.8 ± 0.2	0.9 ± 0.3	0.14
Lp(a), mg/dL	19.9 (10.4-40.4)	22.7 (10.4-45.1)	0.19
LDL-C target, n (%)	11 (5.2)	7 (3.5)	0.11
Liver and Muscle Enzymes			
AST, U/L	25.1 ± 6.9	24.4 ± 7	0.18
ALT, U/L	27.7 ± 8.4	25.8 ± 8.6	0.21
CPK, U/L	126 (94-166)	121 (92-161.5)	0.17
Risk Factors			
Systolic BP, mmHg	120 ± 9.9	118.6 ± 9.8	0.39
Diastolic BP, mmHg	72.1 ± 9.1	70.6 ± 9.5	0.28
Smoking, n (%)	53 (25.2)	42 (20.8)	0.11
hs-CRP, mg/dL	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.51
Treatment			
Antihypertensive therapy, n (%)	56 (26.6)	45 (22.3)	0.12
Lipid lowering therapy, n (%)	128 (61.0)	86 (42.6)	< 0.01
Statin monotherapy, n (%)	71 (33.8)	47 (23.3)	< 0.01
- Low-intensity statin, n (%)	21 (29.6)	23 (49.0)	< 0.01
- Moderate-intensity statin, n (%)	32 (45.0)	15 (31.9)	< 0.05
- High-intensity statin, n (%)	18 (25.4)	9 (19.1)	< 0.05
Ezetimibe monotherapy, n (%)	19 (9.0)	14 (6.9)	0.13
Statin plus ezetimibe, n (%)	38 (18.1)	25 (12.4)	< 0.05
- Low-intensity statin plus ezetimibe, n (%)	12 (31.6)	10 (40.0)	< 0.05
- Moderate-intensity statin plus ezetimibe, n (%)	15 (39.5)	8 (32.0)	< 0.05
- High-intensity statin plus ezetimibe, n (%)	11 (28.9)	7 (28.0)	0.51

Data are presented as mean ± standard deviation, percentages, or median (interquartile range). ASCVD, atherosclerotic cardiovascular disease; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoAI, apolipoprotein AI; Lp(a), lipoprotein (a); BP, blood pressure; hs-CRP, high sensitivity C-reactive protein.

the W/SLCO1B1+ group and the same was observed in reported myalgia distribution (for both *p* for trend < 0.01) (Figure 3).

Logistic regression analysis showed that the W/SCLO1B1-, M/SCLO1B1+ and W/SCLO1B1+ groups were inversely associated with LDL-C target achievement (*p* for trend < 0.001) and the F/SCLO1B1+ group exhibited the strongest association (Table 4).

Discussion

In this study, we investigated the impact of SLCO1B1 rs4149056 on LDL-C target achievement after lipid lowering therapy optimization in FH men and women; to the best of our knowledge, this is the first study exploring the SLCO1B1 genotype-sex interaction in this population. We found that the prevalence of subjects on LDL-C target as well as on high intensity statin therapy was significantly lower in FH women with SLCO1B1

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TABLE 3 Metabolic profile and adverse events of the Study Population stratified according to sex at the last follow-up after lipid lowering therapy optimization.

	Men (n = 210)	Women (n = 202)	*p* Value between two groups
Glucose Profile			
FPG, mg/dL	92.5 ± 5.4	90.4 ± 5.8	0.39
HbA1c, %	5.6 ± 0.3	5.6 ± 0.3	0.24
Lipid Profile			
TC, mg/dL	164.6 ± 20.9	175.2 ± 21.3	< 0.05
HDL-C, mg/dL	50.5 ± 9.8	57.2 ± 9.7	< 0.001
Triglycerides, mg/dL	89 (73-115)	82 (66-110)	0.07
LDL-C, mg/dL	92.8 ± 19.8	104.8 ± 20.1	< 0.05
Non-HDL-C, mg/dL	122.5 ± 18.6	124.1 ± 19.5	0.46
ApoB, mg/dL	83.7 ± 13.2	87 ± 12.6	0.38
ApoAI, m g/dL	141.7 ± 13.8	154.9 ± 13.6	< 0.001
ApoB to ApoAI ratio	0.7 ± 0.3	0.7 ± 0.3	0.44
Lp(a), mg/dL	22.4 (10.4-42.5)	26.9 (10.4-51.4)	0.1
LDL-C target, n (%)	80 (38.1)	56 (27.7)	< 0.05
Liver and Muscle Enzymes			
AST, U/L	25.2 ± 6.7	25.5 ± 7.2	0.67
ALT, U/L	27.9 ± 9.5	28 ± 9.9	0.74
CPK, U/L	130 (99-171.5)	144 (107.5-186)	0.09
Treatment			
Lipid lowering therapy, n (%)	210 (100.0)	202 (100.0)	–
High-intensity statin, n (%)	108 (51.4)	60 (29.7)	< 0.001
Low-to-moderate-intensity statin, n (%)	102 (48.6)	142 (70.3)	< 0.001
- Fear of SASE, n (%)	69 (32.9)	97 (48.0)	< 0.01
- Myalgia, n (%)	33 (15.7)	45 (22.3)	< 0.05
Statin plus ezetimibe, n (%)	136 (64.8)	128 (63.4)	0.82
- Low-intensity statin plus ezetimibe, n (%)	18 (13.2)	39 (30.5)	< 0.01
- Moderate-intensity statin plus ezetimibe, n (%)	45 (33.1)	48 (37.5)	0.09
- High-intensity statin plus ezetimibe, n (%)	73 (53.7)	41 (32.0)	< 0.01
Statin plus ezetimibe plus PCSK9i, n (%)	68 (32.4)	65 (32.2)	0.92
- Low-intensity statin plus ezetimibe plus PCSK9i, n (%)	7 (10.3)	22 (33.9)	< 0.01
- Moderate-intensity statin plus ezetimibe plus PCSK9i, n (%)	26 (38.2)	24 (36.9)	0.37
- High-intensity statin plus ezetimibe plus PCSK9i, n (%)	35 (51.5)	19 (29.2)	< 0.01
Ezetimibe plus PCSK9i, n (%)	6 (2.8)	9 (4.4)	0.12
Adverse events			
Newly diagnosed T2D, n (%)	2 (1.0)	1 (0.5)	–
Newly diagnosed cardiovascular events, n (%)	5 (2.4)	7 (3.5)	0.36

Data are presented as mean ± standard deviation, percentages, or median (interquartile range). ASCVD, atherosclerotic cardiovascular disease; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoAI, apolipoprotein AI; Lp(a), lipoprotein (a); BP, blood pressure; hs-CRP, high sensitivity C-reactive protein; SASE, statin associated side effects; PCSK9-i, proprotein convertase subtilisin/kexin type 9 inhibitors; T2D, type 2 diabetes.

rs4149056 than the other groups; moreover, FH women with SLCO1B1 rs4149056 exhibited the strongest inverse association with LDL-C target. Thus, our findings suggest that the genotype effect of SLCO1B1 rs4149056 could be more pronounced in women than men and it is in line with a previous finding by Turkmen et al. who found that, in a large cohort of subjects in a primary care setting, women with SLCO1B1 rs4149056 had elevated cholesterol levels compared to men and this was largely explained by a higher prevalence of women who discontinued the prescribed statins (25). A possible explanation of this finding may be that the sex difference of SLCO1B1 rs4149056 effect could be due to biological differences as well as to a lower percentage of muscle mass in women than men leading to an increased plasma level of statins with a possible higher risk of muscle symptoms (26). In this context, in our study a higher prevalence of myalgia as well as of SASE fear were observed in FH women with SLCO1B1 rs4149056 than men with the same polymorphism or subjects without SLCO1B1 rs4149056. Our results are in line with previous findings that evaluated the sex difference of statin therapy management in the general population (27, 28). In fact, Voora et al. found that in the STRENGHT Study SASE were more prevalent in women than men and that SLCO1B1 rs4149056 and female sex were significantly associated with SASE; moreover, Bradley et al. showed that in the PALM Registry the fear of side effects was the main reason for statin discontinuation and this was largely observed in women. However, in our study we found that after lipid lowering therapy optimization the majority of

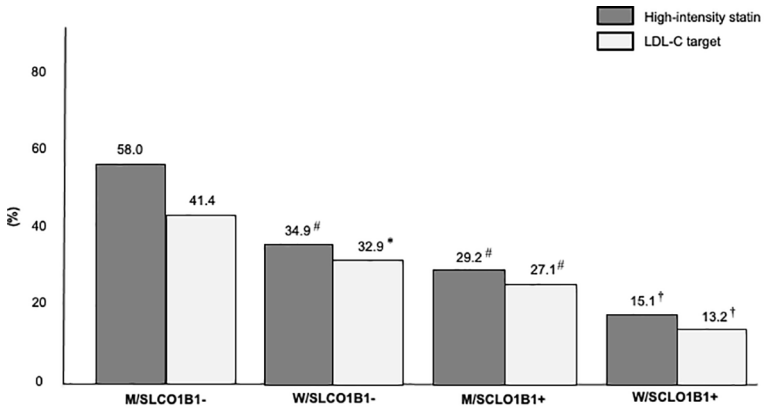

FIGURE 2
Percentages of high-intensity statin use and LDL-C target achievement in the Study Population stratified according to sex and SLCO1B1 rs4149056 presence after lipid lowering therapy optimization. M/SLCO1B1-, men without SLCO1B1 rs4149056; W/SLCO1B1-, women without SLCO1B1 rs4149056; M/SCLO1B1+, men with SLCO1B1 rs4149056; W/SCLO1B1+, women with SLCO1B1 rs4149056. *, p <0.05; #, p < 0.01; †, p < 0.001.

FH women were on low to moderate intensity statins plus other lipid lowering drugs and this was in line with a recent finding by Schreuder et al. who found that in a multicenter cohort of FH subjects, more than half of the women were on low to moderate intensity statins and only 26.9% of them achieved the recommended LDL-C target (29). Accordingly, in our study after lipid lowering therapy optimization the percentage of FH women who reached the specified LDL-C target was 27.7%.

In the last few years, it has been shown that in FH the cardiovascular risk is heterogeneous and the identification of FH subjects who are more vulnerable to cardiovascular injury is needed to better improve their management and treatment (30, 31). In this context, sex related differences of cardiovascular prevention and LLT management have been observed in FH subjects (32, 33); these findings could have a deleterious impact on the long-term cardiovascular health in this population. This could be attributable to different behavioral characteristics, life course lipoprotein distribution or hormone related lipid fluctuations (34, 35); however, genetic polymorphisms involved in pharmacokinetic and pharmacodynamic pathways could also influence the sex differences of LLT adherence (14, 36). In this context, in our study the genetic evaluation of SLCO1B1 rs4149056 presence was able to detect FH subjects who reported myalgia, discontinued high intensity statins and did not achieve the recommended LDL-C target. Thus, the application of a genetic tool able to identify subjects at higher risk of statin intolerance could be useful to ameliorate LLT management in FH subjects more vulnerable to cardiovascular injury (37).

There are several limitations to our study; first this was a retrospective observational study and thus causal relationship and temporality cannot be established between starting lipid lowering therapy optimization and reported myalgia or SASE fear. Moreover, based on the type of study the lipid lowering therapy optimization after the addition of inclisiran or bempedoic acid was not evaluated due to the restricted time of follow-up. Furthermore, no data on muscle mass as well as on plasma levels of sex hormones, menopausal

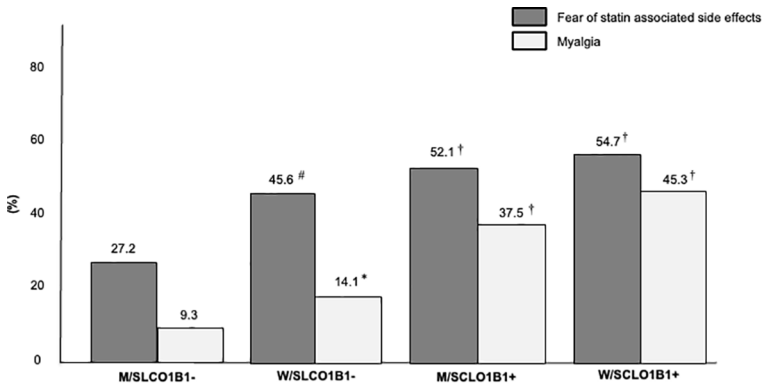

FIGURE 3
Percentages of FH subjects with fear of statin associated side effects and reported myalgia in the Study Population stratified according to sex and SLCO1B1 rs4149056 presence after lipid lowering therapy optimization. M/SLCO1B1-, men without SLCO1B1 rs4149056; W/SLCO1B1-, women without SLCO1B1 rs4149056; M/SCLO1B1+, men with SLCO1B1 rs4149056; W/SCLO1B1+, women with SLCO1B1 rs4149056. *, p <0.05; #, p < 0.01; †, p < 0.001.

TABLE 4 Logistic regression of LDL-C Target Achievement in the Study Population stratified according to sex and SLCO1B1 rs4149056 presence.

Quartiles	No. of Participants	Multivariate ORs (95% CIs)
		Model
M/SLCO1B1-	162	1.00 (reference)
W/SLCO1B1-	149	0.72 (0.6 – 0.85)
M/SLCO1B1+	48	0.69 (0.58 – 0.81)
W/SLCO1B1+	53	0.39 (0.3 – 0.52)
P for trend		< 0.001

Logistic regression model was used to estimate ORs and 95% CIs. The model was adjusted for age, statin intensity, ezetimibe, and PCSK9-i. M/SLCO1B1-, men without SLCO1B1 rs4149056; W/SLCO1B1-, women without SLCO1B1 rs4149056; M/SCLO1B1+, men with SLCO1B1 rs4149056; W/SCLO1B1+, women with SLCO1B1 rs4149056.

status or estrogen supplementation were available in our cohort of subjects; further studies are needed to better evaluate the impact of these variables on lipid lowering therapy optimization in FH subjects with SLCO1B1 rs4149056. Finally, data on nutritional counseling as well as on physical activity were not available.

In conclusion, the adherence of intensive lipid lowering therapy was low in FH women with SLCO1B1 rs4149056 and these subjects rarely achieved the recommended LDL-C target in clinical practice. The genotype effect of SLCO1B1 rs4149056 could be more pronounced in women than men; further prospective studies are needed to evaluate the applicability of a genetic tool able to identify FH subjects who are more vulnerable to cardiovascular injury.

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 author/s.

Ethics statement

The studies involving humans were approved by Catania 2, Piazza Santa Maria di Gesù n° 5, Catania, Italy. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

GB: Conceptualization, Data curation, Methodology, Writing – original draft, Software. FB: Data curation, Investigation, Writing – review & editing. MD: Data curation, Investigation, Writing – review & editing. NM: Data curation, Investigation, Writing – review & editing. SSc: Data curation, Investigation, Writing – review & editing. SSp: Data curation, Investigation, Writing – review & editing. AV: Data curation, Investigation, Writing – review & editing. FD: Data curation, Investigation, Writing – review & editing. MM: Data curation, Investigation, Writing – review & editing. SD: Data curation, Investigation, Writing – review & editing. AF: Data curation, Investigation, Writing – review & editing. AS: Data curation, Investigation, Writing – review & editing. AM: Data curation, Investigation, Methodology, Visualization, Writing – review & editing. AD: Data curation, Investigation, Methodology, Visualization, Writing – review & editing. LF: Data curation, Investigation, Methodology, Visualization, Writing – review & editing. FP: Data curation, Investigation, Methodology, Visualization, Writing – review & editing. SP: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – review & editing. RS: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: Consensus Statement of the European Atherosclerosis Society. *Eur Heart J* (2013) 34:3478–90. doi: 10.1093/eurheartj/eht273

2. Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur Heart J* (2017) 38:2459–72. doi: 10.1093/eurheartj/ehx144

3. Sharifi M, Rakhit RD, Humphries SE, Nair D. Cardiovascular risk stratification in familial hypercholesterolaemia. *Heart* (2016) 102:1003–8. doi: 10.1136/heartjnl-2015-308845

4. Drexel H, Coats AJS, Spoletini I, Bilato C, Mollace V, Perrone Filardi P, et al. An expert opinion paper on statin adherence and implementation of new lipid-lowering medications by the ESC Working Group on Cardiovascular Pharmacotherapy: Barriers to be overcome. *Eur Heart J Cardiovasc Pharmacother* (2020) 6:115–21. doi: 10.1093/EHJCVP/PVZ079

5. Luirink IK, Wiegman A, Kusters DM, Hof MH, Groothoff JW, de Groot E, et al. 20-year follow-up of statins in children with familial hypercholesterolemia. *New Engl J Med* (2019) 381:1547–56. doi: 10.1056/NEJMoa1816454

6. Zambon A, Mello E Silva A, Farnier M. The burden of cholesterol accumulation through the lifespan: why pharmacological intervention should start earlier to go further? *Eur Heart J Cardiovasc Pharmacother* (2021) 7:435–41. doi: 10.1093/EHJCVP/PVAA123

7. Adhyaru BB, Jacobson TA. Safety and efficacy of statin therapy. *Nat Rev Cardiol* (2018) 15:757–69. doi: 10.1038/s41569-018-0098-5

8. Stroes ES, Thompson PD, Corsini A, Vladutiu GD, Raal FJ, Ray KK, et al. Statin-associated muscle symptoms: impact on statin therapy—European Atherosclerosis Society Consensus Panel Statement on Assessment, Aetiology and Management. *Eur Heart J* (2015) 36:1012–22. doi: 10.1093/eurheartj/ehv043

9. Khalaf K, Johnell K, Austin PC, Tyden P, Midlöv P, Perez-Vicente R, et al. Low adherence to statin treatment during the 1st year after an acute myocardial infarction is associated with increased 2nd-year mortality risk—an inverse probability of treatment weighted study on 54 872 patients. *Eur Heart J Cardiovasc Pharmacother* (2021) 7:141–7. doi: 10.1093/EHJCVP/PVAA010

10. Bosco G, Di Giacomo Barbagallo F, Spampinato S, Lanzafame L, Di Pino A, Piro S, et al. Management of statin intolerant patients in the era of novel lipid lowering therapies: A critical approach in clinical practice. *J Clin Med* (2023) 12:2444. doi: 10.3390/jcm12062444

11. Rosano GMC, Lewis B, Agewall S, Wassmann S, Vitale C, Schmidt H, et al. Gender differences in the effect of cardiovascular drugs: a position document of the Working Group on Pharmacology and Drug Therapy of the ESC. *Eur Heart J* (2015) 36:2677–80. doi: 10.1093/eurheartj/ehv161

12. Amrock SM, Duell PB, Knickelbine T, Martin SS, O'Brien EC, Watson KE, et al. Health disparities among adult patients with a phenotypic diagnosis of familial hypercholesterolemia in the CASCADE-FH™ patient registry. *Atherosclerosis* (2017) 267:19–26. doi: 10.1016/j.atherosclerosis.2017.10.006

13. Agarwala A, Deych E, Jones LK, Sturm AC, Aspry K, Ahmad Z, et al. Sex-related differences in premature cardiovascular disease in familial hypercholesterolemia. *J Clin Lipidol* (2023) 17:150–6. doi: 10.1016/j.jacl.2022.11.009

14. Sakkers TR, Mokry M, Civelek M, Erdmann J, Pasterkamp G, Diez Benavente E, et al. Sex differences in the genetic and molecular mechanisms of coronary artery disease. *Atherosclerosis* (2023) 384:117279. doi: 10.1016/j.atherosclerosis.2023.117279

15. Romaine SPR, Bailey KM, Hall AS, Balmforth AJ. The influence of SLCO1B1 (OATP1B1) gene polymorphisms on response to statin therapy. *Pharmacogenomics J* (2010) 10:1–11. doi: 10.1038/tpj.2009.54

16. SEARCH Collaborative Group, Link E, Parish S, Armitage J, Bowman L, Heath S, et al. SLCO1B1 variants and statin-induced myopathy–a genomewide study. *N Engl J Med* (2008) 359:789–99. doi: 10.1056/NEJMoa0801936

17. Canestaro WJ, Austin MA, Thummel KE. Genetic factors affecting statin concentrations and subsequent myopathy: A HuGENet systematic review. *Genet Med* (2014) 16:810–9. doi: 10.1038/gim.2014.41

18. Averna M, Cefalù AB, Casula M, Noto D, Arca M, Bertolini S, et al. Familial hypercholesterolemia: the Italian atherosclerosis society network (LIPIGEN). *Atherosc Suppl* (2017) 29:11–6. doi: 10.1016/j.atherosclerosissup.2017.07.001

19. Catapano AL, Graham I, De Backer G, Wiklund O, Chapman MJ, Drexel H, et al. 2016 ESC/EAS guidelines for the management of dyslipidaemias. *Eur Heart J* (2016) 37:2999–3058. doi: 10.1093/eurheartj/ehw272

20. Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur Heart J* (2020) 41:111–88. doi: 10.1093/eurheartj/ehz455

21. Masana L, Pedro-Botet J, Civeira F. IMPROVE-IT clinical implications. Should the "high-intensity cholesterol-lowering therapy" strategy replace the "high-intensity statin therapy"? *Atherosclerosis* (2015) 240:161–2. doi: 10.1016/j.atherosclerosis.2015.03.002

22. Scicali R, Di Pino A, Urbano F, Ferrara V, Marchisello S, Di Mauro S, et al. Analysis of steatosis biomarkers and inflammatory profile after adding on PCSK9 inhibitor treatment in familial hypercholesterolemia subjects with nonalcoholic fatty liver disease: A single lipid center real-world experience. *Nutr Metab Cardiovasc Dis* (2021) 31:869–79. doi: 10.1016/j.numecd.2020.11.009

23. Scicali R, Di Pino A, Platania R, Purrazzo G, Ferrara V, Giannone A, et al. Detecting familial hypercholesterolemia by serum lipid profile screening in a hospital setting: Clinical, genetic and atherosclerotic burden profile. *Nutr Metab Cardiovasc Dis* (2018) 28:35–43. doi: 10.1016/j.numecd.2017.07.003

24. Visseren FLJ, Mach F, Smulders YM, Carballo D, Koskinas KC, Bäck M, et al. 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. *Eur Heart J* (2021) 42:3227–337. doi: 10.1093/eurheartj/ehab484

25. Türkmen D, Masoli JAH, Kuo C, Bowden J, Melzer D, Pilling LC. Statin treatment effectiveness and the *SLCO1B1* *5 reduced function genotype: Long-term outcomes in women and men. *Br J Clin Pharmacol* (2022) 88:3230–40. doi: 10.1111/bcp.15245

26. Franconi F, Campesi I. Pharmacogenomics, pharmacokinetics and pharmacodynamics: interaction with biological differences between men and women. *Br J Pharmacol* (2014) 171:580–94. doi: 10.1111/bph.12362

27. Voora D, Shah SH, Spasojevic I, Ali S, Reed CR, Salisbury BA, et al. The SLCO1B1*5 genetic variant is associated with statin-induced side effects. *J Am Coll Cardiol* (2009) 54:1609–16. doi: 10.1016/j.jacc.2009.04.053

28. Bradley CK, Wang TY, Li S, Robinson JG, Roger VL, Goldberg AC, et al. Patient-reported reasons for declining or discontinuing statin therapy: Insights from the PALM registry. *J Am Heart Assoc* (2019) 8(7):e011765. doi: 10.1161/JAHA.118.011765

29. Schreuder MM, Hamkour S, Siegers KE, Holven KB, Johansen AK, van de Ree MA, et al. LDL cholesterol targets rarely achieved in familial hypercholesterolemia patients: A sex and gender-specific analysis. *Atherosclerosis* (2023) 384:117117. doi: 10.1016/j.atherosclerosis.2023.03.022

30. Romero-Jiménez MJ, Mansilla-Rodríguez ME, Gutiérrez-Cortizo EN. Predictors of cardiovascular risk in familial hypercholesterolemia. *Curr Opin Lipidol* (2023) 34:272–7. doi: 10.1097/MOL.0000000000000903

31. Scicali R, Bosco G, Scamporrino A, Di Mauro S, Filippello A, Di Giacomo Barbagallo F, et al. Evaluation of high-density lipoprotein-bound long non-coding RNAs in subjects with familial hypercholesterolaemia. *Eur J Clin Invest* (2023) 54(1): e1408. doi: 10.1111/ECI.14083

32. Iyen B, Qureshi N, Kai J, Akyea RK, Leonardi-Bee J, Roderick P, et al. Risk of cardiovascular disease outcomes in primary care subjects with familial hypercholesterolaemia: A cohort study. *Atherosclerosis* (2019) 287:8–15. doi: 10.1016/j.atherosclerosis.2019.05.017

33. Myasoedova VA, Rimbert A, Camera M, Le May C, Capoulade R, Cariou B, et al. LDL lowering effect of PCSK9 inhibition is reduced in women. *Eur Heart J Cardiovasc Pharmacother* (2023) 9:337–42. doi: 10.1093/EHJCVP/PVAD009

34. Antza C, Gallo A, Boutari C, Ershova A, Gurses KM, Lewek J, et al. Prevention of cardiovascular disease in young adults: Focus on gender differences. A collaborative review from the EAS Young Fellows. *Atherosclerosis* (2023) 384:117272. doi: 10.1016/j.atherosclerosis.2023.117272

35. Holven KB, Roeters van Lennep J. Sex differences in lipids: A life course approach. *Atherosclerosis* (2023) 384:117270. doi: 10.1016/j.atherosclerosis.2023.117270

36. Ahmed S, Zhou Z, Zhou J, Chen S-Q. Pharmacogenomics of drug metabolizing enzymes and transporters: relevance to precision medicine. *Genomics Proteomics Bioinf* (2016) 14:298–313. doi: 10.1016/j.gpb.2016.03.008

37. Bigossi M, Maroteau C, Dawed AY, Taylor A, Srinivasan S, Melhem AL, et al. A gene risk score using missense variants in *SLCO1B1* is associated with earlier onset statin intolerance. *Eur Heart J Cardiovasc Pharmacother* (2023) 9:536–45. doi: 10.1093/ehjcvp/pvad040

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Analysis of the association between testosterone and cardiovascular disease potential risk factor apolipoprotein B in adult males without cancer: national health and nutrition examination survey 2011-2016

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Background: Over the years, there has been extensive exploration of the association between testosterone and lipid profiles, yet the precise mechanisms underlying their interaction remain incompletely elucidated. Similarly, there is a dearth of research on the correlation between serum apolipoprotein B (apoB) and serum total testosterone (TT), particularly within specific populations.

Methods: We conducted a cross-sectional study to assess the relationship between serum TT concentration and serum apoB concentration. Using the National Health and Nutrition Examination Survey (NHANES) from 2011 to 2016, we employed weighted generalized linear models, weighted univariate, weighted multivariate analysis, and smooth curve fitting to assist in exploring the relationship between serum TT and apoB. Serum apoB concentration served as the independent variable, and serum TT concentration as the dependent variable. ApoB was divided into four quartiles—Q1 (<0.7g/L, N=691), Q2 (≥0.7g/L to <0.9g/L, N=710), Q3 (≥0.9g/L to <1.1g/L, N=696), and Q4 (≥1.1g/L, N=708)—thereby further solidifying the stable association between the two. Additionally, the application of smooth curve fitting will contribute to a more detailed elucidation of the specific relationship between serum TT concentration and serum apoB concentration under different factors (Drinking, Smoke, Diabetes, Hypertension, and High cholesterol level.).

Results: The results indicate a negative correlation between serum TT concentration and apoB concentration (β=-113.4; 95% CI: -146.6, -80.2; P<0.001). After adjusting for confounding variables, the negative correlation between apoB concentration and TT concentration remains significant (β=-61.0; 95% CI: -116.7, -5.2; P=0.040). When apoB concentration was converted from a continuous variable to a categorical variable (quartiles: Q1<0.7g/L; Q2:≥0.7g/L to<0.9g/L; Q3:≥0.9g/L to <1.1g/L; Q4: ≥1.1g/L), TT level of

participants in the highest quartile (≥1.1g/L) was -47.2 pg/mL (95% CI: -91.2, -3.3; P=0.045) lower than that in the lowest quartile (<0.7g/L). The smooth curve fitting diagram revealed differences in the relationship between TT concentration and apoB among individuals with different cardiovascular disease (CVD) risk factors.

Conclusions: This study elucidates a robust inverse correlation between serum TT concentration and apoB concentration, maintaining statistical significance even upon adjustment for confounding factors. These findings present a promising avenue for addressing the prevention and treatment of low testosterone and CVD.

KEYWORDS

testosterone, ApoB, LDL-C, CVD, NHANES

Introduction

Testosterone is produced by Leydig cells within the testis, playing a crucial role in the differentiation of the male reproductive tract and masculinization of the external genitalia during fetal development (1). The release of testosterone is regulated via the hypothalamus-pituitary-testis interstitial cell axis (2). Decreased levels of serum testosterone (at or below 300 ng/dL) often present with diminished sexual thoughts and frequency, weight gain, and erectile dysfunction (3, 4). Gonadotropin-releasing hormone (GnRH) binds to membrane receptors on the pituitary gonadotropes, stimulating the biosynthesis and secretion of luteinizing hormone (LH) (5, 6). LH binds to LHR on the surface of interstitial cells, initiating intracellular signal transduction. Dufau and Catt (7) demonstrated that cAMP is generated in response to LH. The cAMP pathway through protein kinase A (PKA) is crucial in steroidogenesis. Furthermore, the chronic stimulation of interstitial cells by LH and cAMP is vital for regulating the expression levels of proteins and enzymes involved in steroidogenesis, thus playing a crucial role in the nutritional regulation of steroid production responsible for sustained steroidogenesis over an extended period. The intricate link between male testosterone and lipid metabolism is evident. Lee et al. observed a negative correlation between testosterone and one-tenth of triglycerides, a positive correlation between testosterone and one-tenth of high-density lipoprotein cholesterol, and an inverted U-shaped correlation between testosterone and one-tenth of low-density lipoprotein (8). In a cross-sectional study conducted by Liu et al., elevated lipid accumulation products were found to be associated with a higher incidence of testosterone loss and deficiency, particularly in individuals with hypertension and non-smokers (9). Lipid accumulation products have a certain significance in predicting testosterone deficiency (9). A study by Hurley demonstrated that the use of high-dose androgens resulted in a 50% reduction in HDL-C and an increase of over 50% in LDL-C (10). Furthermore, the biosynthesis and secretion of testosterone exhibit a tight correlation with age. During puberty, testosterone levels peak in response to luteinizing hormone stimulation and subsequently decline with advancing age (11). A study conducted by Mohr BA et al. (12) established percentile thresholds for testosterone level fluctuations in distinct age cohorts, thereby revealing substantial horizontal and vertical associations between serum TT, CFT, bioactive testosterone levels, and age. Additionally, several observational studies suggest an association between low testosterone levels in males and adverse cardiovascular outcomes (13, 14).

Recent evidence suggests that while lipid-lowering therapies targeting serum LDL-C levels reduce the risk of atherosclerotic cardiovascular disease (ASCVD) in the general population, some individuals with normal or low LDL-C concentrations still experience ASCVD-related events, and some may even show the progression of atherosclerosis (15). Some researchers have constructed Cox proportional hazard models to analyze data, revealing that for each increase of one standard unit in non-HDL-C cholesterol and apoB levels, there is a 19% escalation in the incidence rate of major cardiovascular events, surpassing the impact of LDL-C levels (15%). The predictive capability of apoB (HR = 1.24) and non-HDL-C cholesterol (HR = 1.31) was more pronounced than that of LDL-C. Subsequent research further indicated that in assessing residual cardiovascular risk following statin therapy, apoB, and non-HDL-C cholesterol levels exhibited higher predictive value (16). A meta-analysis by Sniderman et al. (17) in 2011 found that compared to a 40% reduction in LDL-C levels, a 40% reduction in non-HDL-C cholesterol would reduce cardiovascular events by 200,000 over ten years, while a 40%

Abbreviations: ApoB, Apolipoprotein B; TT, Total testosterone; TG, Triglyceride; TC, Total cholesterol; CVD, Cardiovascular Disease; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; CI, Confidence interval; OR, Odds ratio; Ref, Reference.

reduction in apoB would prevent 500,000 cardiovascular events. This highlights the superior predictive value of apoB compared to LDL-C and non-HDL-C in assessing cardiovascular benefits of lipid-lowering therapy, further endorsing apoB as a crucial treatment target, especially post-achievement of LDL-C targets.

Exploring the relationship between testosterone and apoB dates back to Stefanick's study in 1987 (18). In our study, we investigated the relationship between testosterone and apoB in diverse populations and exposure factors. Among individuals without cancer, we explored the linear relationship between serum TT and serum apoB in various CVD, and visually depicted the relationship using a smooth curve fitting plot. Investigating the correlation between serum apoB and serum TT offers valuable insights into screening individuals with potential CVD within the population exhibiting normal or low LDL-C levels. Notably, certain guidelines currently propose apoB as a secondary target for intervention in blood lipid management, aiming to reduce the residual risk of ASCVD in patients (19, 20). Therefore, exploring the association between apoB and TT contributes significantly to the comprehensive understanding and management of CVD.

Methods

Study design and population

The NHANES is an extensive health and nutrition survey conducted by the National Center for Health Statistics in the United States. Since its initiation in the early 1960s, NHANES has been dedicated to evaluating the health and nutritional status of individuals throughout the country. By conducting family interviews and physical examinations, this survey gathers comprehensive information encompassing biological, social, psychological, behavioral, and demographic aspects, all provided at no cost to participants. In this cross-sectional study, we utilized NHANES data from 2011 to 2016 to explore the relationship between apoB and total TT within the general population.

Sample selection

We conducted our analysis using the NHANES database, which comprised a total of 29,902 participants from the years 2011 to 2016. The dataset encompassed various demographic variables, including age, race, ratio of family income to poverty, marital status, and education level. Additionally, we considered several cardiovascular disease risk factors such as hypertension, high cholesterol levels, diabetes, BMI, drinking habits, and smoking variables. It also includes Vigorous work activity. All data can be found at the following URL: www.cdc.gov/nchs/nhanes/ (Date of access online: January 12, 2024).

From the initial pool of 29,902 participants, we excluded those who were female (n=15,151), individuals below 18 years or above 65 years of age (n=7,820), participants with a cancer diagnosis (n=267), and those with missing data for apoB (n=3,827) and total testosterone (n=32). Consequently, a total of 2,805 participants were included in this cross-sectional study (Figure 1). It is important to note that all NHANES research participants from 2011 to 2016 provided informed consent, and the study protocol obtained approval from the Research Ethics Review Committee of the National Health Statistics Center.

Variables

The demographic variables considered in this study were age, race (including Mexican American, other Hispanic, non-Hispanic white, non-Hispanic black, or other race), the ratio of family income to poverty, marital status, and education level. For this study, the age range of 18 to 65 years was chosen due to the known variations in testosterone levels across different age groups. Testosterone levels are typically low in children under 10 years of age, start to increase during the ages of 10 to 15 with the onset of puberty (21), and reach their peak around the age of 19 (22). However, as men continue to age, testosterone levels gradually decline, particularly in older men above the age of 65 (23). To classify the education level variable, individuals with educational attainment below the 9th grade, those with 9th-11th grade education (including 12th grade without a diploma), and high school graduates or equivalent were categorized as the "High School Grad and Less Than" group. On the other hand, individuals with some college or associate degrees, as well as college graduates or above, were classified as the "Above" group. Thus, the education level variable was dichotomized as "High School Grad and Less Than" versus "Above."

The questionnaire variables in this study encompassed several factors. These included hypertension (categorized as "No" or "Yes"), high cholesterol levels ("No" or "Yes"), diabetes ("No" or "Yes"), drinking ("No" or "Yes"), smoke ("Not at all," "Every day," or "Some days"), and vigorous work activity ("No" or "Yes"). To define alcohol users, individuals who consumed a minimum of 12 drinks within the past 12 months were considered (24). Therefore, we used the threshold of 12 drinks in the past 12 months as the

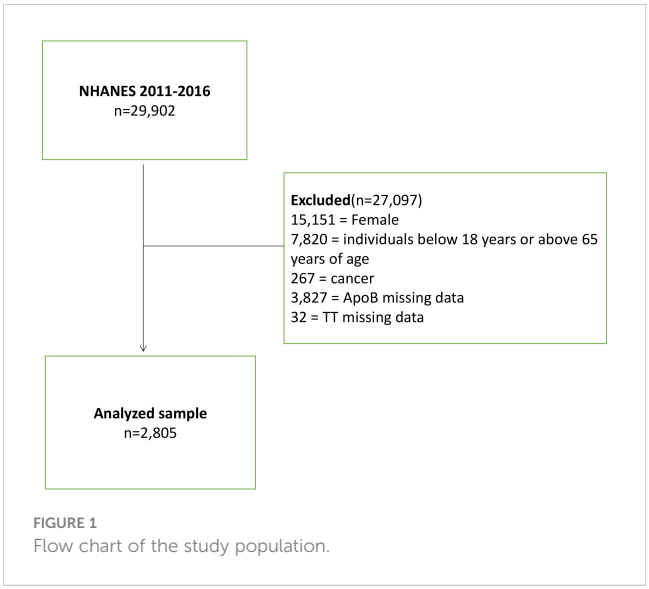

FIGURE 1
Flow chart of the study population.

criterion to categorize the drinking variable into a binary form, namely "Drinking" ("No" or "Yes").

The examination variable included body mass index (BMI), which was treated as a continuous variable. For analysis, BMI was categorized into two groups: < 25 kg/m² and ≥ 25 kg/m².

For the NHANES laboratory methodology regarding the determination of TT, further information can be accessed at: https://wwwn.cdc.gov/Nchs/Nhanes/2011-2012/TST_G.htm (Date of access online: January 12, 2024). Similarly, detailed information regarding the NHANES laboratory methodology for apoB determination is available at: https://wwwn.cdc.gov/Nchs/Nhanes/2011-2012/APOB_G.htm (Date of access online: January 12, 2024). Additional covariates can be found at the following URL: www.cdc.gov/nchs/nhanes/ (Date of access online: January 12, 2024).

Data analysis

Given the intricate probability cluster design of NHANES, individual sample weights were assigned to each survey participant, and all statistical analyses in this study took into account these weights. We conducted statistical analyses in accordance with the guidelines outlined by the Centers for Disease Control (CDC) (25). To study the associations of participant characteristics, we utilized weighted linear regression and the Chi-square test where appropriate. Initially, we conducted a weighted univariate analysis to examine the relationship between TT and the covariates. Subsequently, we conducted weighted multifactor analyses while adjusting for covariates such as age, race, education level, ratio of family income to poverty, marital status, hypertension, high cholesterol levels, BMI, diabetes, drinking, smoking, and vigorous work activity. Furthermore, to confirm the relationship between apoB and TT, we performed smooth curve fitting using apoB (g/L) as the independent variable and TT (ng/dL) levels as the dependent variable. This analysis allowed us to visualize and assess the relationship between apoB and TT.

All statistical analyses were carried out using Empowerstats (https://www.empowerstats.net/cn/) and R software. All estimates were weighted using appropriate NHANES sample weights. Following guidelines from the Centers for Disease Control and Prevention, weighted models were used to address oversampling of minority ethnicities, ensuring fair and accurate estimates of the population impact. Results were considered statistically significant when p-values were less than 0.05.

Results

Table 1 presents 14 study variables, including the independent variable ApoB, the dependent variable TT, and covariates Age, Ratio of family income to poverty, Race, Education level, Marital status, Diabetes, Smoking, Drinking, Hypertension, High cholesterol level, Vigorous work activity, and BMI. The analysis sample comprises 2,805 male participants. We divided ApoB into quartiles Q1-Q4, corresponding to mean ages of 36.3, 40.9, 43.1, and 43.9 years, and corresponding TT levels of 501.1, 472.1, 442.7, and 427.1 ng/dL, respectively. For continuous variables, we calculated weighted means (95% CI) and determined P-values using survey-weighted linear regression (svyglm). For categorical variables, we calculated weighted percentages (95% CI) and obtained P-values using survey-weighted chi-square tests (svytable).

Table 2 presents the univariate analysis results for 13 variables related to the dependent variable TT. The table reveals a close and statistically significant association between serum ApoB and TT. Additionally, Ratio of family income to poverty, Age, Marital status, Hypertension, High cholesterol level, Diabetes, Smoking, and BMI are also linked to TT. For each unit increase in Ratio of family income to poverty, TT decreases by 6.2 ng/dL. With every year's increment in Age, TT decreases by 1.2 ng/dL. Regarding Marital status, the "unmarried" group exhibits a 53.3 ng/dL increase in TT compared to the "married or with partners" group. Hypertension is associated with a 56.7 ng/dL decrease in TT compared to individuals without Hypertension. High cholesterol level is linked to a TT reduction of 42.5 ng/dL compared to those without High cholesterol level. Each 1 kg/m² increase in BMI corresponds to a 13.1 ng/dL decrease in TT. When dichotomized, individuals with a BMI ≥25 kg/m² experience a 151.6 ng/dL decrease in TT compared to those with a BMI <25 kg/m². Diabetes individuals witness a TT decrease of 72.5 ng/dL compared to non-Diabetes individuals. In the Smoking category, compared to "Not at all," the "Every day" and "Some days" groups show TT increases of 46.2 ng/dL and 51.3 ng/dL, respectively.

Table 3 presents the model results, indicating that in the unadjusted model, there is a negative correlation between apoB concentration and serum TT concentration (β=-113.4; 95% CI: -146.6, -80.2; P<0.001). This implies that for every 1g/L increase in apoB, serum TT decreases by 113.4ng/dL, with statistical significance. In the adjusted model (β=-61.0; 95% CI: -116.7, -5.2; P=0.040), each 1g/L increase in apoB is associated with a 61.0ng/dL decrease in serum TT, and this association is statistically significant. The conversion of the apoB concentration from a continuous variable to a categorical variable (quartile: Q1<0.7g/L; Q2≥0.7g/L to <0.9g/L; Q3≥0.9g/L to <1.1g/L; Q4≥1.1g/L) revealed that the level of TT of the participants in the highest quartile (≥1.1g/L) was -47.2 pg/mL (95% CI: -91.2, -3.3; P=0.045) lower than that in the lowest quartile (<0.7g/L).

It can be seen that after adjusting the relevant variables, apoB was linearly correlated with TT, and TT decreased with the increase of apoB.

In the drinking population, TT showed a downward trend with increasing apoB, and then the downward trend tended to slow down. In the non-alcohol drinkers, apoB was linearly correlated with TT, and TT decreased with the increase of apoB. The results were obtained after adjusting for Age, Race, Education level, Ratio of family income to poverty, Marital status, Hypertension, High cholesterol level, BMI, Diabetes, Smoke, and Vigorous work activity (Figure 2C).

In the smoker(Every day and Some days smoke)population, apoB was linearly correlated with TT, and TT decreased with the increase of apoB. In the non-smoker population, The relationship

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TABLE 1 Characteristics of participants, Weighted (N =2,805).

ApoB (g/L) quartile	Q1 <0.7g/L	Q2 ≥0.7g/L to <0.9g/L	Q3 ≥0.9g/L to <1.1g/L	Q4 ≥1.1g/L	P-value
N	691	710	696	708	
ApoB (g/L)	0.6 (0.6,0.6)	0.8 (0.8,0.8)	1.0 (1.0,1.0)	1.3 (1.3,1.3)	<0.001
TT (ng/dL)	501.1 (481.6,520.5)	472.1 (454.9,489.4)	442.7 (424.6,460.8)	427.1 (411.7,442.4)	<0.001
Age (years)	36.3 (34.8,37.7)	40.9 (39.4,42.4)	43.1 (41.9,44.3)	43.9 (42.8,45.0)	<0.001
Ratio of family income to poverty	2.7 (2.5,3.0)	2.9 (2.7,3.2)	3.1 (2.9,3.3)	2.8 (2.6,3.0)	0.010
Race					<0.001
Mexican American	8.8 (6.3,12.2)	9.1 (6.2,13.3)	11.1 (8.0,15.2)	13.5 (10.1,17.8)	
Other Hispanic	6.0 (4.2,8.5)	5.9 (4.0,8.5)	7.6 (5.5,10.3)	9.4 (6.6,13.2)	
Non-Hispanic White	63.8 (57.3,69.8)	65.4 (59.2,71.1)	65.0 (58.8,70.8)	60.8 (54.1,67.0)	
Non-Hispanic Black	13.2 (9.9,17.4)	9.9 (7.7,12.7)	7.4 (5.8,9.4)	8.2 (6.2,10.8)	
Other Race	8.3 (6.7,10.2)	9.8 (7.6,12.5)	8.9 (6.7,11.6)	8.1 (6.2,10.7)	
Education level					0.058
High School Grad and Less Than	36.9 (31.6,42.6)	37.6 (32.0,43.5)	39.0 (32.1,46.4)	46.7 (39.9,53.6)	
Above	63.1 (57.4,68.4)	62.4 (56.5,68.0)	61.0 (53.6,67.9)	53.3 (46.4,60.1)	
Marital status					<0.001
married or with partners	53.3 (48.0,58.5)	63.7 (57.5,69.4)	69.2 (65.0,73.2)	68.1 (63.0,72.8)	
widowed or divorced	9.0 (6.5,12.3)	10.8 (7.7,15.0)	9.1 (6.5,12.7)	12.5 (10.0,15.4)	
unmarried	36.5 (31.9,41.4)	23.8 (19.5,28.7)	20.1 (16.7,24.1)	16.8 (13.1,21.2)	
separated	1.2 (0.6,2.2)	1.7 (1.1,2.6)	1.5 (0.7,3.4)	2.7 (1.7,4.3)	
Diabetes					0.686
No	90.8 (87.4,93.4)	92.2 (89.4,94.4)	92.9 (89.4,95.4)	92.7 (90.3,94.5)	
Yes	9.2 (6.6,12.6)	7.8 (5.6,10.6)	7.1 (4.6,10.6)	7.3 (5.5,9.7)	
Smoke					0.402
Not at all	45.3 (36.8,54.2)	54.9 (47.3,62.2)	54.4 (46.8,61.8)	48.0 (41.0,55.1)	
Every day	43.6 (35.9,51.7)	35.0 (28.3,42.4)	33.3 (26.9,40.4)	40.5 (33.0,48.4)	
Some days	11.0 (7.0,16.8)	10.1 (7.0,14.5)	12.3 (8.4,17.8)	11.5 (7.3,17.7)	
Drinking					0.653
No	96.9 (93.2,98.6)	97.7 (95.8,98.8)	97.1 (94.4,98.6)	98.3 (96.9,99.1)	
Yes	3.1 (1.4,6.8)	2.3 (1.2,4.2)	2.9 (1.4,5.6)	1.7 (0.9,3.1)	
Hypertension					0.007
No	78.5 (74.9,81.8)	73.1 (67.5,78.1)	73.0 (68.9,76.7)	67.1 (61.2,72.5)	
Yes	21.5 (18.2,25.1)	26.9 (21.9,32.5)	27.0 (23.3,31.1)	32.9 (27.5,38.8)	
High cholesterol level					<0.001
No	81.6 (76.6,85.7)	76.0 (71.3,80.1)	66.5 (61.4,71.3)	55.9 (50.6,61.0)	
Yes	18.4 (14.3,23.4)	24.0 (19.9,28.7)	33.5 (28.7,38.6)	44.1 (39.0,49.4)	
Vigorous work activity					0.298
No	68.3 (63.2,73.1)	69.2 (65.7,72.4)	70.7 (65.1,75.7)	64.5 (58.1,70.4)	
Yes	31.7 (26.9,36.8)	30.8 (27.6,34.3)	29.3 (24.3,34.9)	35.5 (29.6,41.9)	

(Continued)

TABLE 1 Continued

ApoB (g/L) quartile	Q1 <0.7g/L	Q2 ≥0.7g/L to <0.9g/L	Q3 ≥0.9g/L to <1.1g/L	Q4 ≥1.1g/L	P-value
BMI (kg/m2)					<0.001
<25	45.6 (40.3,50.9)	30.1 (25.9,34.7)	22.0 (18.1,26.4)	16.2 (13.0,20.1)	
>=25	54.4 (49.1,59.7)	69.9 (65.3,74.1)	78.0 (73.6,81.9)	83.8 (79.9,87.0)	

Data in Table 1:
For continuous variables: survey-weighted mean (95% CI), P-value was by survey-weighted linear regression.
For categorical variables: survey-weighted percentage (95% CI), P-value was by survey-weighted Chi-square test.

TABLE 2 Univariate analysis for TT, Weighted.

Covariate	Mean/percentage (95% CI)	β (95%CI)	P-value
ApoB (g/L)	1.0 (0.9,1.0)	-113.4 (-146.6, -80.2)	<0.001
ApoB (g/L) quartile			
Q1	23.3 (21.2,25.6)	Ref	
Q2	26.1 (24.2,28.0)	-28.9 (-50.9, -7.0)	0.013
Q3	25.8 (23.2,28.6)	-58.4 (-85.8, -30.9)	<0.001
Q4	24.8 (22.2,27.6)	-74.0 (-97.3, 50.7)	<0.001
Ratio of family income to poverty	2.9 (2.8,3.0)	-6.2 (-11.7, -0.76)	0.031
Ratio of family income to poverty Tertile			
Low	23.0 (20.1,26.2)	Ref	
Middle	32.3 (29.9,34.8)	-24.8 (-43.0, -6.5)	0.010
High	44.7 (40.7,48.8)	-29.1(-52.0, -6.30)	0.016
Age(years)	41.1 (40.4,41.9)	-1.2 (-2.1, -0.35)	0.009
Age (years) Tertile			
Low	31.5 (28.9,34.2)	Ref	
Middle	35.1 (32.4,37.8)	-63.1 (-81.7, -44.4)	<0.001
High	33.4 (30.9,36.1)	-43.1 (-73.2, -13.0)	0.007
Race			
Mexican American	10.6 (8.2,13.6)	Ref	
Other Hispanic	7.2 (5.5,9.4)	-17.0 (-61.3, 27.3)	0.456
Non-Hispanic White	63.8 (58.8,68.5)	-9.6 (-32.9, 13.7)	0.424
Non-Hispanic Black	9.6 (7.7,11.9)	17.3 (-13.3, 47.8)	0.274
Other Race	8.8 (7.3,10.5)	1.4 (-25.2, 28.1)	0.916
Education level			
High School Grad and Less Than	40.2 (36.2,44.3)	Ref	
Above	59.8 (55.7,63.8)	-14.0 (-34.0, 6.0)	0.177
Marital status			
married or with partners	64.0 (61.1,66.9)	Ref	
widowed or divorced	10.4 (8.9,12.1)	-2.5 (-25.8, 20.8)	0.833
unmarried	23.8 (21.4,26.4)	53.3 (28.8, 77.8)	<0.001
separated	1.8 (1.3,2.5)	42.4 (-26.0, 110.9)	0.230

(Continued)

TABLE 2 Continued

Covariate	Mean/percentage (95% CI)	β (95%CI)	P-value
Hypertension			
No	72.9 (70.3,75.3)	Ref	
Yes	27.1 (24.7,29.7)	-56.7 (-78.9, -34.5)	<0.001
High cholesterol level			
No	69.8 (67.1,72.3)	Ref	
Yes	30.2 (27.7,32.9)	-42.5 (-57.8, -27.2)	<0.001
BMI(kg/m2)	28.8 (28.4,29.2)	-13.1 (-14.2, -11.9)	<0.001
BMI (kg/m2) dichotomous			
<25	28.2 (26.0,30.5)	Ref	
>=25	71.8 (69.5,74.0)	-151.6 (-171.6, -131.6)	<0.001
Diabetes			
No	92.2 (90.7,93.4)	Ref	
Yes	7.8 (6.6,9.3)	-72.5 (-116.1, -28.9)	0.002
Drinking			
No	97.5 (96.5,98.3)	Ref	
Yes	2.5 (1.7,3.5)	5.3 (-46.2, 56.9)	0.840
Smoke			
Not at all	50.9 (46.7,55.2)	Ref	
Every day	37.8 (33.4,42.4)	46.2 (14.0, 78.3)	0.007
Some days	11.3 (9.1,13.9)	51.3 (5.3, 97.2)	0.034
Vigorous work activity			
No	68.2 (65.3,71.0)	Ref	
Yes	31.8 (29.0,34.7)	7.8 (-9.6, 25.3)	0.384

between apoB and TT loses its linear correlation. The results were obtained after adjusting for Age, Race, Education level, Ratio of family income to poverty, Marital status, Hypertension, High cholesterol level, BMI, Diabetes, Drinking, and Vigorous work activity (Figure 2D).

In diabetic and non-diabetic patients, apoB was linearly correlated with TT, and TT decreased with the increase of apoB. The results were obtained after adjusting for Age, Race, Education level, Ratio of family income to poverty, Marital status, Hypertension, High cholesterol level, BMI, Drinking, Smoke, and Vigorous work activity (Figure 2E).

In the hypertensive and non-hypertensive population, apoB was linearly correlated with TT, and TT decreased with the increase of apoB. The results were obtained after adjusting for Age, Race, Education level, Ratio of family income to poverty, Marital status, High cholesterol level, BMI, Diabetes, Drinking, Smoke, and Vigorous work activity (Figure 2F).

In the high cholesterol level population, the change in TT is not significant with the increase of apoB; In the non-high cholesterol level population, apoB was linearly correlated with TT, and TT decreased with the increase of apoB. The results were obtained after adjusting for Age, Race, Education level, Ratio of family income to poverty, Marital status, Hypertension, BMI, Diabetes, Drinking, Smoke, and Vigorous work activity (Figure 2G).

Discussion

According to the findings of this study, in the population of adult males with an average age of 41.1 years and without cancer, there exists a negative linear correlation between serum apoB and TT, as illustrated in Figures 2A, B. This correlation remains consistent even in the presence of several cardiovascular risk factors such as smoking, diabetes, and hypertension, as depicted in Figures 2D-F. However, the relationship between apoB and TT takes a different pattern among individuals with alcohol consumption and high cholesterol levels. In the population with alcohol consumption, TT shows a declining trend as apoB increases, followed by a slower rate of decline (Figure 2C). On the other hand, in the population with high cholesterol levels, the change in TT is not significantly evident with increasing apoB (Figure 2G). Based on two crucial theoretical concepts: the strong correlation between apoB and LDL-C and the role of apoB as a

TABLE 3 Relationship between ApoB and TT, Weighted.

Outcome	Crude Model		Adjusted Model	
	βor OR (95%CI)	P-value	βor OR (95%CI)	P-value
ApoB (g/L)	-113.4 (-146.6, -80.2)	<0.001	-61.0 (-116.7, -5.2)	0.040
ApoB (g/L) quartile				
Q1 <0.7g/L	Ref		Ref	
Q2 ≥0.7g/L to <0.9g/L	-28.9 (-50.9, -7.0)	0.013	-26.7 (-61.8, 8.4)	0.148
Q3 ≥0.9g/L to <1.1g/L	-58.4 (-85.8, -30.9)	<0.001	-12.5 (-59.7, 34.6)	0.606
Q4 ≥1.1g/L	-74.0 (-97.3, 50.7)	<0.001	-47.2 (-91.2, -3.3)	0.045

Data in the table: β or OR (95%CI); P-value.
Result variable: TT (ng/dL).
Exposure variable: ApoB(g/L); ApoB(g/L) quartile.
The adjusted model adjusts for Age; Race (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, Other Race); Education level (High School Grad and Less Than and Above); Ratio of family income to poverty; Marital status (married or with partners, widowed or divorced, unmarried, and separated); Hypertension (No, Yes); High cholesterol level (No, Yes); BMI (<=25, >25); Diabetes (No, Yes); Drinking (No, Yes); Smoke (Not at all, Every day, and Some days); Vigorous work activity (No, Yes).

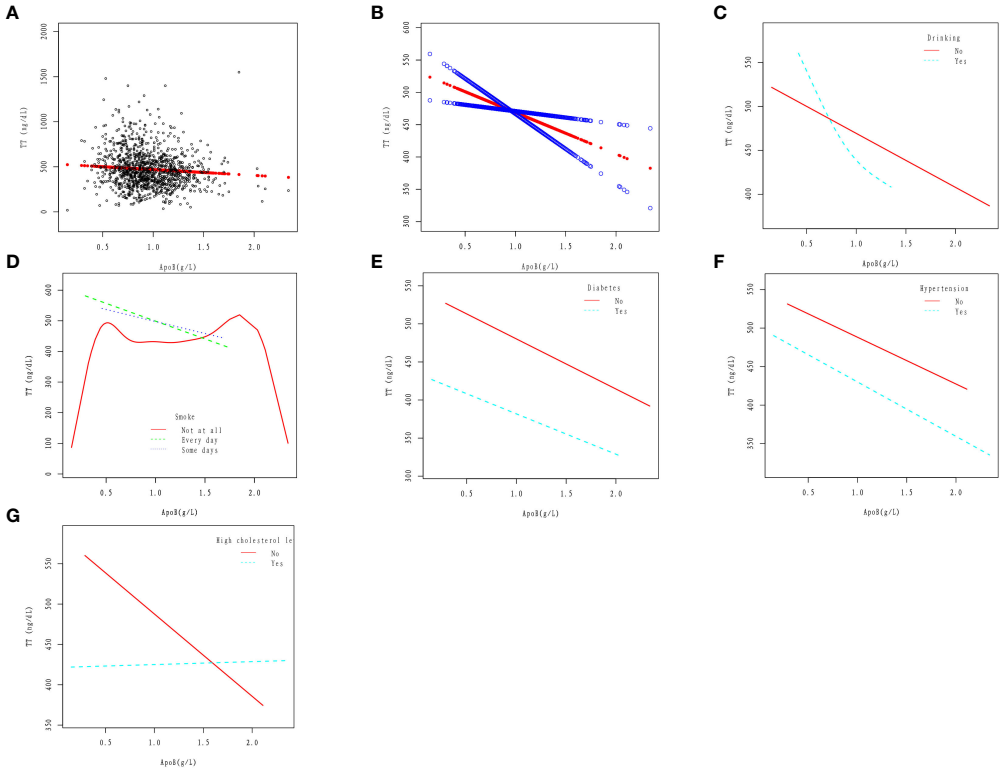

FIGURE 2
Association between apoB and TT. **(A)** Cross-combination modeling of each apoB and TT. A black dot represents a sample. **(B)** A linear association between apoB and TT was found in a generalized additive model (GAM). The Solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. The results were obtained after adjusting for Age, Race, Education level, Ratio of family income to poverty, Marital status, Hypertension, High cholesterol level, BMI, Diabetes, Drinking, Smoke, and Vigorous work activity. **(C)** Association between apoB and serum Klotho in Drinking (No, Yes) group. **(D)** Association between apoB and serum TT in Smoke (No, Yes) group. **(E)** Association between apoB and serum TT in Diabetes (No, Yes) group. **(F)** Association between apoB and serum TT in Hypertension (No, Yes) group. **(G)** Association between apoB and serum TT in High cholesterol level (No, Yes) group.

potential risk factor for CVD (26). The association between testosterone and CVD is easily conceivable. Some researchers have pointed out that testosterone and its synthetic derivatives are related to sudden cardiac death (27, 28). As demonstrated by epidemiological research, individuals who misuse steroids are more prone to engage in criminal activities, with steroids occasionally identified as an indirect cause of death (29, 30). Kintz et al., based on the increased risk of antisocial lifestyle associated with steroid hormones, explored methods for detecting steroid hormones through body hair analysis (31). Thus, elevated levels of apoB may serve as a significant predictor of CVD risk, particularly in individuals with normal or low LDL-C levels. Consequently, further investigation into the relationship between apoB and TT could prove valuable in identifying potential CVD patients within this population. Additionally, for the treatment of testosterone deficiency, apart from direct testosterone supplementation, maintaining apoB at a certain level may also hold promise as a potential adjunctive treatment.

Several studies have explored the potential connection between serum TT and blood lipids. These studies have revealed that testosterone deficiency can lead to elevated serum TG levels and increased white adipose tissue in male mice fed a high-fat diet (32). Additionally, the San Antonio Heart Research Center found negative correlations between testosterone and TG, TC, and LDL-C, and a positive correlation with HDL-C (33). Similar associations were observed in the Tromsa study, with testosterone being negatively correlated with TG and positively correlated with HDL-C (34). The Turku Male Aging Study reported positive correlations between testosterone and HDL-C, and negative correlations with TC and TG, although no statistically significant relationship was observed with LDL-C (35). These findings demonstrate the close relationship between testosterone and CVD-related factors such as HDL-C, LDL-C, TG, and TC. However, the investigation of the relationship between apoB and serum TT has received limited attention thus far. Previous evidence has demonstrated the beneficial effects of testosterone replacement therapy in male patients with hypogonadism, including improvements in certain cardiovascular risk factors (36). Elevated serum testosterone levels can lead to adverse outcomes. Coward et al. found that the administration of synthetic androgens may result in testicular dysfunction. Males under 50 years old, previously exposed to assimilative androgens, exhibit over 10 times the likelihood of testicular dysfunction compared to those aged 50 and above, and these findings are statistically significant (37). In a rat model, Shirpoor et al. observed cardiac and aortic remodeling induced by the synthetic androgenic steroid, Nandrolone. They documented an increase in systolic pressure, diastolic pressure, mean arterial pressure, and necrotic pressure (38). Esposito et al. summarized that the misuse of synthetic androgenic steroids can induce various side effects on organ systems, including the reproductive system (39).

In our study, we examined the association between serum apoB and TT, while considering various potential confounders including age, race, education level, ratio of family income to poverty, marital status, hypertension, high cholesterol level, BMI, diabetes, drinking, smoking, and vigorous work activity. Through the use of linear regression models and smooth curve fitting, we discovered a negative correlation between serum apoB concentration and TT concentration. Overall, our study sheds light on an underexplored area of research and provides valuable insights into the association between serum apoB and TT in the context of cardiovascular health in adult males without cancer. These findings contribute to our understanding of the complex interplay between lipoproteins, testosterone, and cardiovascular risk factors.

Lee et al. utilized the Framingham Risk Score (FRS) to assess the impact of testosterone on cardiovascular disease in patients with sexual dysfunction (40). The findings revealed an association between testosterone levels and FRS in the sexual dysfunction cohort, suggesting that elevated testosterone levels may reduce the risk of cardiovascular disease in these individuals. In another study, Grandys et al. found that reduced serum testosterone concentrations were associated with increased inflammation and worsening of blood lipids in men (41). Diverging from prior research (18, 40, 41), our study investigates the association between serum TT and apoB across diverse populations and exposure factors. Within individuals devoid of cancer, we explore the linear relationship between serum TT and apoB in various CVD. This study represents a pioneering effort to investigate the correlation between serum TT and the potential cardiovascular risk factor apoB using publicly available NHANES data. While this study offers notable strengths, it is crucial to recognize and address its limitations. Firstly, the cross-sectional design of the NHANES database restricts our ability to establish any causal relationship between human serum apoB and TT. Secondly, it is plausible that there are unidentified confounding variables that could influence serum TT and apoB levels, which were not accounted for in this study. It is important to consider these limitations when interpreting the findings and to encourage further research to explore these associations in more depth.

Conclusions

This nationally representative study indicates a negative linear correlation between serum ApoB and TT in adult American males without cancer. Upon adjustment, for every 1 g/L increase in ApoB, there is a significant decrease of 61.0 ng/dL in TT. This suggests that reducing ApoB concentration could be beneficial in preventing low testosterone occurrence. Furthermore, low testosterone levels can serve as predictive indicators for the development of CVD. These findings offer a promising avenue for the prevention and treatment of both low testosterone and cardiovascular disease. In general, investigating the relationship between apoB and serum testosterone levels can yield profound insights into preventing and managing testosterone deficiency, aiding in the development of more effective preventive and therapeutic strategies. This necessitates a comprehensive consideration of multiple factors, including lipid metabolism, cardiovascular health, and lifestyle factors.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.cdc.gov/nchs/nhanes/.

Ethics statement

The study was conducted by following the Declaration of Helsinki, and the National Center for Health Statistics institutional review board approved the overall NHANES. This study was approved by the Institutional Review Board and documented consent was obtained from participants. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

ZC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. EZ: Writing – original draft. LG: Writing – original draft. GJ: Writing – original draft. QD: Writing – original draft. MH: Conceptualization, Writing – original draft. HL: Conceptualization, Writing – original draft. GH: Conceptualization, Funding acquisition, Supervision, Writing – original draft.

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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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References

1. Zirkin BR, Papadopoulos V. Leydig cells: formation, function, and regulation. *Biol Reprod* (2018) 99(1):101–11. doi: 10.1093/biolre/ioy059

2. Miller WL. Molecular biology of steroid hormone synthesis. *Endocr Rev* (1988) 9 (3):295–318. doi: 10.1210/edrv-9-3-295

3. Sterling J, Bernie AM, Ramasamy R. Hypogonadism: Easy to define, hard to diagnose, and controversial to treat. *Can Urol Assoc J* (2015) 9(1-2):65–8. doi: 10.5489/cuaj.2416

4. Kumar P, Kumar N, Thakur DS, Patidar A. Male hypogonadism: Symptoms and treatment. *J Adv Pharm Technol Res* (2010) 1(3):297–301. doi: 10.4103/0110-5558.72420

5. Plant TM. 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-pituitary-gonadal axis. *J Endocrinol* (2015) 226(2):T41–54. doi: 10.1530/JOE-15-0113

6. Kaprara A, Huhtaniemi IT. The hypothalamus-pituitary-gonad axis: Tales of mice and men. *Metabolism* (2018) 86:3–17. doi: 10.1016/j.metabol.2017.11.018

7. Dufau ML, Catt KJ. Gonadotropin receptors and regulation of steroidogenesis in the testis and ovary. *Vitam Horm* (1978) 36:461–592. doi: 10.1016/s0083-6729(08) 60989-9

8. Lee JH, Jung HD, Choi JD, Kang JY, Yoo TK, Park YW. Non-linear association between testosterone and LDL concentrations in men. *Andrology* (2023) 11(6):1107–13. doi: 10.1111/andr.13393

9. Liu Q, Huang Y, Wang M, Jiang H, Zhang X. Association of lipid accumulation products with testosterone deficiency in adult American men: A cross-sectional study. *Andrology* (2023) 11(3):551–60. doi: 10.1111/andr.13355

10. Hurley BF, Seals DR, Hagberg JM, Goldberg AC, Ostrove SM, Holloszy JO, et al. High-density-lipoprotein cholesterol in bodybuilders v powerlifters. *Negative effects androgen use JAMA* (1984) 252(4):507–13. doi: 10.1001/jama.1984.03350040037019

11. Lamberts SW, van den Beld AW, van der Lely AJ. The endocrinology of aging. *Science* (1997) 278(5337):419–24. doi: 10.1126/science.278.5337.419

12. Mohr BA, Guay AT, O'Donnell AB, McKinlay JB. Normal, bound and nonbound testosterone levels in normally ageing men: results from the Massachusetts Male Ageing Study. *Clin Endocrinol (Oxf)* (2005) 62(1):64–73. doi: 10.1111/j.1365-2265.2004.02174.x

13. Barrett-Connor E, Khaw KT. Endogenous sex hormones and cardiovascular disease in men. A prospective population-based study. *Circulation* (1988) 78(3):539–45. doi: 10.1161/01.cir.78.3.539

14. Smith GD, Ben-Shlomo Y, Beswick A, Yarnell J, Lightman S, Elwood P. Cortisol, testosterone, and coronary heart disease: prospective evidence from the Caerphilly study. *Circulation* (2005) 112(3):332–40. doi: 10.1161/CIRCULATIONAHA.104.489088

15. Sinning D, Landmesser U. Is there a need to revise goals in the management of dyslipidemias? *Curr Cardiol Rep* (2019) 21(6):51. doi: 10.1007/s11886-019-1128-6

16. Boekholdt SM, Hovingh GK, Mora S, Arsenault BJ, Amarenco P, Pedersen TR, et al. Very low levels of atherogenic lipoproteins and the risk for cardiovascular events: a meta-analysis of statin trials. *J Am Coll Cardiol* (2014) 64(5):485–94. doi: 10.1016/j.jacc.2014.02.615

17. Sniderman AD, Williams K, Contois JH, Monroe HM, McQueen MJ, de Graaf J, et al. A meta-analysis of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B as markers of cardiovascular risk. *Circ Cardiovasc Qual Outcomes* (2011) 4(3):337–45. doi: 10.1161/CIRCOUTCOMES.110.959247

18. Stefanick ML, Williams PT, Krauss RM, Terry RB, Vranizan KM, Wood PD. Relationships of plasma estradiol, testosterone, and sex hormone-binding globulin with lipoproteins, apolipoproteins, and high density lipoprotein subfractions in men. *J Clin Endocrinol Metab* (1987) 64(4):723–9. doi: 10.1210/jcem-64-4-723

19. Packard CJ. Determinants of achieved LDL cholesterol and "Non-HDL" Cholesterol in the management of dyslipidemias. *Curr Cardiol Rep* (2018) 20(8):60. doi: 10.1007/s11886-018-1003-x

20. Kato Y, Arakawa E, Kinoshita S, Shirai A, Furuya A, Yamano K, et al. Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys. *Biochem Biophys Res Commun* (2000) 267(2):597–602. doi: 10.1006/bbrc.1999.2009

21. Khairullah A, Klein LC, Ingle SM, May MT, Whetzel CA, Susman EJ, et al. Testosterone trajectories and reference ranges in a large longitudinal sample of male adolescents. *PloS One* (2014) 9(9):e108838. doi: 10.1371/journal.pone.0108838

22. Kelsey TW, Li LQ, Mitchell RT, Whelan A, Anderson RA, Wallace WH. A validated age-related normative model for male total testosterone shows increasing variance but no decline after age 40 years. *PloS One* (2014) 9(10):e109346. doi: 10.1371/journal.pone.0109346

23. Yeap BB. Are declining testosterone levels a major risk factor for ill-health in aging men? *Int J Impot Res* (2009) 21(1):24–36. doi: 10.1038/ijir.2008.60

24. Chen X, Wei G, Jalili T, Metos J, Giri A, Cho ME, et al. The associations of plant protein intake with all-cause mortality in CKD. *Am J Kidney Dis* (2016) 67(3):423–30. doi: 10.1053/j.ajkd.2015.10.018

25. Zipf G, Chiappa M, Porter KS, Ostchega Y, Lewis BG, Dostal J. National health and nutrition examination survey: plan and operations, 1999-2010. *Vital Health Stat 1* (2013) (56):1–37.

26. Barter PJ, Ballantyne CM, Carmena R, Castro Cabezas M, Chapman MJ, Couture P, et al. Apo B versus cholesterol in estimating cardiovascular risk and in guiding therapy: report of the thirty-person/ten-country panel. *J Intern Med* (2006) 259 (3):247–58. doi: 10.1111/j.1365-2796.2006.01616.x

27. Esposito M, Licciardello G, Privitera F, Iannuzzi S, Liberto A, Sessa F, et al. Forensic post-mortem investigation in AAS abusers: investigative diagnostic protocol. A systematic review. *Diagnostics (Basel), Switzerland* (2021) 11(8):1307. doi: 10.3390/diagnostics11081307

28. Sculthorpe N, Grace F, Jones P, Davies B. Evidence of altered cardiac electrophysiology following prolonged androgenic anabolic steroid use. *Cardiovasc Toxicol* (2010) 10(4):239–43. doi: 10.1007/s12012-010-9090-y

29. Lehmann S, Thomas A, Schiwy-Bochat KH, Geyer H, Thevis M, Glenewinkel F, et al. Death after misuse of anabolic substances (clenbuterol, stanozolol and metandienone). *Forensic Sci Int* (2019) 303:109925. doi: 10.1016/j.forsciint.2019.109925

30. Klotz F, Garle M, Granath F, Thiblin I. Criminality among individuals testing positive for the presence of anabolic androgenic steroids. *Arch Gen Psychiatry* (2006) 63 (11):1274–9. doi: 10.1001/archpsyc.63.11.1274

31. Kintz P, Gheddar L, Raul JS. Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis. *Drug Test Anal* (2021) 13(7):1445–51. doi: 10.1002/dta.3020

32. Dubois V, Laurent MR, Jardi F, Antonio L, Lemaire K, Goyvaerts L, et al. Androgen deficiency exacerbates high-fat diet-induced metabolic alterations in male mice. *Endocrinology* (2016) 157(2):648–65. doi: 10.1210/en.2015-1713

33. Haffner SM, Mykkanen L, Valdez RA, Katz MS. Relationship of sex hormones to lipids and lipoproteins in nondiabetic men. *J Clin Endocrinol Metab* (1993) 77(6):1610–5. doi: 10.1210/jcem.77.6.8263149

34. Agledahl I, Skjaerpe PA, Hansen JB, Svartberg J. Low serum testosterone in men is inversely associated with non-fasting serum triglycerides: the Tromso study. *Nutr Metab Cardiovasc Dis* (2008) 18(4):256–62. doi: 10.1016/j.numecd.2007.01.014

35. Makinen JI, Perheentupa A, Irjala K, Pollanen P, Makinen J, Huhtaniemi I, et al. Endogenous testosterone and serum lipids in middle-aged men. *Atherosclerosis* (2008) 197(2):688–93. doi: 10.1016/j.atherosclerosis.2007.05.009

36. Allan CA, Strauss BJ, Burger HG, Forbes EA, McLachlan RI. Testosterone therapy prevents gain in visceral adipose tissue and loss of skeletal muscle in nonobese aging men. *J Clin Endocrinol Metab* (2008) 93(1):139–46. doi: 10.1210/jc.2007-1291

37. Coward RM, Rajanahally S, Kovac JR, Smith RP, Pastuszak AW, Lipshultz LI. Anabolic steroid induced hypogonadism in young men. *J Urol* (2013) 190(6):2200–5. doi: 10.1016/j.juro.2013.06.010

38. Shirpoor A, Heshmatian B, Tofighi A, Eliasabad SN, Kheradmand F, Zerehpoosh M. Nandrolone administration with or without strenuous exercise increases cardiac fatal genes overexpression, calcium/calmodulin-dependent protein kinaseiidelta, and monoamine oxidase activities and enhances blood pressure in adult wistar rats. *Gene* (2019) 697:131–37. doi: 10.1016/j.gene.2019.02.053

39. Esposito M, Salerno M, Calvano G, Agliozzo R, Ficarra V, Sessa F, et al. Impact of anabolic androgenic steroids on male sexual and reproductive function: a systematic review. *Panminerva Med* (2023) 65(1):43–50. doi: 10.23736/S0031-0808.22.04677-8

40. Lee WC, Kim MT, Ko KT, Lee WK, Kim SY, Kim HY, et al. Relationship between serum testosterone and cardiovascular disease risk determined using the framingham risk score in male patients with sexual dysfunction. *World J Mens Health* (2014) 32(3):139–44. doi: 10.5534/wjmh.2014.32.3.139

41. Grandys M, Majerczak J, Zapart-Bukowska J, Duda K, Kulpa JK, Zoladz JA. Lowered serum testosterone concentration is associated with enhanced inflammation and worsened lipid profile in men. *Front Endocrinol (Lausanne)* (2021) 12:735638. doi: 10.3389/fendo.2021.735638

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Prevalence and clinical correlates of hyperhomocysteinemia in Chinese urban population with hypertension

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Context: The coexistence of hypertension and elevated homocysteine (Hcy) levels has a mutually reinforcing impact on the susceptibility to cardio-cerebrovascular disease.

Objective: The aim was to assess the prevalence, clinical correlation, and demographic characteristics of hyperhomocysteinemia (HHcy) within the Chinese urban population with hypertension.

Methods: A cohort of 473 individuals with hypertension were selected from four communities in Shenzhen, China. Demographic attributes, clinical profiles, and lifestyle behaviors were gathered and compared between individuals with and without HHcy. A logistic regression model was employed to examine potential factors associated with the prevalence of HHcy. Correlation between Hcy levels and clinical characteristics was assessed through multiple linear regression analysis.

Results: The prevalence of HHcy in the population with hypertension was 31.3%. In comparison to individuals without HHcy, those with HHcy exhibited a higher proportion of males, a higher prevalence of smoking and alcohol consumption, and a higher proportion of cases with the homozygous (TT) genotype at the MTHFR C677T polymorphism. Moreover, individuals with HHcy had lower levels of folic acid (FA), and lower fruit and vitamin B12 intake. Furthermore, the risk factors for HHcy were male (B = 1.430, OR = 4.179) and MTHFR (TT) (B = 1.086, OR = 2.961). In addition, the multiple linear regression analysis revealed a significant association between Hcy levels and gender (B = -2.784, P = 0.004), MTHFR genotypes (B = 1.410, P = 0.005), and FA levels (B = -0.136, P = 0.030).

Conclusion: The high prevalence of HHcy among hypertensive patients in this Chinese urban population underscores the necessity for interventions targeting modifiable risk factors such as dietary choices and lifestyle practices.

KEYWORDS

hyperhomocysteinemia, hypertension, urban, prevalence, clinical correlates

Introduction

In the past three decades, there has been a persistent global rise in the prevalence of cardio-cerebrovascular disease. Specifically, cardiovascular disease (CVD) persists as the leading cause of mortality and morbidity on a global scale. Over the course of the last three decades, the burden of CVD has consistently escalated, with a notable 53.7% increase in the number of deaths (from 12.1 million in 1990 to 18.6 million in 2019) and a substantial 94.4% rise in years lived with disability (from 17.7 million in 1990 to 34.4 million in 2019) (1). Significantly, China experienced the highest number of CVD fatalities, with CVD ranking as the primary cause of death in both rural and urban regions, constituting 46.7% and 44.3% of total mortalities in 2019, respectively (2). Similarly, cerebrovascular disease ranks as the second most lethal ailment on a global scale, and acute cerebrovascular disease exhibits a higher incidence of disability compared to any other individual disease, thereby imposing substantial societal burdens (3). Hypertension has emerged as the primary risk factor for cardio-cerebrovascular disease (4). Due to the widespread adoption of unhealthy lifestyles resulting from rapid economic development and the accelerated aging process, hypertension has witnessed an increase in low- and middle-income countries, including China (5). According to recent survey data from China, the prevalence of hypertension among individuals aged 18 years from 2012 to 2015 reached a significant rate of 27.9% (6). Unfortunately, the existing measures for preventing and controlling hypertension are inadequate (7, 8). Consequently, determining the most effective approach to enhance hypertension management and alleviate the burden of cardio-cerebrovascular disease is a significant global concern.

Homocysteine (Hcy) is a sulfur-containing intermediary amino acid generated through the metabolic conversion of methionine to cysteine. The condition of having elevated plasma concentrations of Hcy above the established normal threshold of 15 μmol/l is referred to as hyperhomocysteinemia (HHcy) (9, 10). HHcy could potentially have detrimental effects on individuals with hypertension, as it collaboratively enhances the risk of cardio-cerebrovascular disease through a multiplicative effect (11, 12). At present, the specific mechanisms by which HHcy influences the susceptibility to hypertension and cardio-cerebrovascular disease remain uncertain. Several hypotheses have been proposed. HHcy has been suggested to potentially elevate the likelihood of developing hypertension and cardio-cerebrovascular disease through the subsequent mechanisms (13): (1) elevation of arterial blood pressure; (2) induction of endothelial dysfunction by augmenting oxidant stress and reducing nitric oxide release, thereby impairing vasodilation; (3) provoking oxidative damage to vascular endothelial cells and hindering the synthesis of nitric oxide, a potent vasodilator, by the endothelium; or (4) augmenting platelet adhesion to endothelial cells, thereby facilitating the proliferation of vascular smooth muscle cells. A nested case-control study conducted in China, involving a cohort of 39,165 Chinese participants who were followed for an average duration of 6.2 years, revealed that individuals diagnosed with hypertension and exhibiting elevated levels of Hcy faced a significantly heightened risk of stroke and stroke-related mortality (14). Compared to those with normal blood pressure and Hcy levels, the aforementioned group experienced an 11.7-fold increase in the likelihood of stroke and a 10.7-fold increase in the likelihood of stroke-related death (14). More recently, a retrospective cohort study conducted in China, involving a total of 1226 participants and a follow-up period of 17 years, presents compelling evidence indicating that individuals with hypertension and an Hcy level exceeding 15 μmol/L had a significantly higher risk of developing stroke and cardiovascular diseases compared to those with hypertension and an Hcy level below 10 μmol/L (15). The adjusted hazard ratios for stroke and cardiovascular diseases were found to be 2.12 and 2.24, respectively (15). Therefore, the identification of distinct risk factors associated with HHcy in hypertensive patients holds significant academic importance in mitigating the occurrence of cardio-cerebrovascular events.

In China, the prevalence of H-type hypertension, characterized by hypertension and HHcy, is observed to be higher compared to other populations (16). This can be attributed to inadequate dietary intake of folic acid (FA) and B vitamins, as well as a high mutation rate of the methylenetetrahydrofolate reductase (MTHFR) gene (the C677T single nucleotide polymorphism), leading to elevated levels of Hcy (17, 18). In the present study, we evaluated the prevalence, clinical association, and demographic attributes of HHcy among hypertensive individuals in the Chinese urban population, with the aim to identify specific risk factors of HHcy in hypertensive patients and provide intervention and management strategies for cardio-cerebrovascular disease.

Methods

Subjects

This study employed a cross-sectional observational design and involved the recruitment of 473 individuals diagnosed with hypertension from four communities in Shenzhen, China. Inclusion criteria for all participants encompassed: (1) being over 18 years of age; (2) belonging to the Han Chinese population; (3) adhering to the Chinese Guidelines for the Prevention and Treatment of Hypertension (2018 Revised Edition); (4) being local residents. Exclusion criteria for all participants encompassed: (1) the existence of a neurological diagnosis, a serious medical condition, or an unstable medical condition; (2) a diagnosis of major psychiatric disorders such as schizophrenia and major depressive disorder, severe somatic disease, or substance abuse. The study protocol received approval from the Clinical Research Ethics Committee of Shenzhen Second People's Hospital (ID number: 20210824001) and was conducted in adherence to the principles outlined in the Declaration of Helsinki. Prior to participation, all participants provided written informed consent subsequent to receiving a comprehensive explanation of the study.

Demographic and clinical data collection

Each participant completed a comprehensive questionnaire capturing general information, sociodemographic characteristics, and lifestyle behaviors. Additionally, a physical examination was conducted, encompassing assessments of height, weight, body mass

index (BMI), heart rate, and blood pressure measurements. Furthermore, fasting venous blood samples were collected from each subject between 8:00 and 9:00 AM. Subsequently, the blood samples were subjected to centrifugation at a speed of 2500 revolutions per minute for a duration of 20 minutes at a temperature of 4°C. The resulting serum was then collected and preserved at a temperature of -80°C until the time of analysis. Biochemical parameters pertaining to liver and kidney function, serum lipids, and glucose levels were quantified using an automated biochemical analyzer. Plasma Hcy, folic acid (FA), vitamin B12 and vitamin D levels, and methylenetetrahydrofolate reductase (MTHFR) C677T and reduced folate carrier (RFC) G80A genotypes were determined by the Shenzhen Tailored Medical Laboratory (Shenzhen, China). In terms of MTHFR C677T genotype and RFC G80A genotype, they were analyzed using a fluorescence PCR detection kit (PCR-fluorescence probe). Specifically, Fluorescence PCR detection was performed using a 4-μl whole genome DNA sample and a 10-μl PCR reaction system. The denaturation step was carried out at 95°C for 15 s, followed by annealing/extension at 60°C for 60 s, as recommended by the manufacturer. The reaction was subjected to 45 cycles. Following the completion of the PCR, the endpoint fluorescence in each well of the samples was quantified utilizing the ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). Subsequently, the genotyping outcomes were precisely ascertained employing the ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The data analysis was carried out utilizing SPSS (Version 17.0; SPSS, Inc, Chicago, IL, USA). The Kolmogorov-Smirnov one-sample test was utilized to identify normal distributions. Descriptive analyses were conducted, with normally distributed quantitative variables presented as means ± standard deviation (SD), and skewed quantitative variables presented as median and interquartile range (IQR; 25-75%). The Chi-square test was employed for categorical variables. To account for multiple testing, the Bonferroni correction was implemented. In order to investigate the risk factors associated with HHcy in individuals diagnosed with hypertension, the study employed univariate analyses to compare subjects with HHcy to those without HHcy. Variables with a significance level of $P < 0.1$ were further analyzed using logistic regression analyses. Additionally, Spearman correlation analysis and multiple linear regression analysis were conducted to examine the associations between Hcy levels and clinical factors. Statistical significance was determined at a P-value of less than 0.05 (two-tailed). Goodness-of-fit test based on the Chi-square was used to determine whether the genotype frequency distribution conformed to Hardy-Weinberg equilibrium (HWE). Sample size calculation was calculated using the G*Power 3.1 software, with power set at 0.99 to detect the medium-effect size (d = 0.50) and alpha = 0.01 with allocation ratio N2/N1 of 2. Considering a previous study involving 5935 hypertensive patients in China demonstrated a HHcy prevalence of 31.4% (13), the allocation ratio N2/N1 was set at 2. The required sample size was 436, with 145 subjects with HHcy and 291 subjects without HHcy. In the present study, a total of 473 individuals diagnosed with hypertension were enrolled, with 148 subjects with HHcy and 325 subjects without HHcy.

Results

Prevalence of HHcy in Chinese urban population with hypertension

HHcy was operationally defined as plasma Hcy levels exceeding 15 μmol/L. Consequently, a total of 473 hypertensive individuals were enrolled in this study, of which 148 (31.3%) fulfilled the diagnostic criteria for HHcy.

Differences in demographic characteristics, clinical profiles, distribution of MTHFR and RFC genotypes, and lifestyle behaviors of subjects with or without HHcy

According to the data presented in Table 1, there is a notable disparity in the gender distribution between individuals with and without HHcy. The proportion of males is significantly higher (χ^2 = 68.237, $P < 0.001$, OR = 7.110; 95% confidence interval (CL): 4.30-11.76) among those with HHcy (85.1%) compared to those without HHcy (44.6%). Moreover, individuals with HHcy exhibit significantly lower levels of FA (Z = -3.511, $P < 0.001$) in comparison to individuals without HHcy.

As shown in Table 2, the results of Goodness-of-fit test based on the Chi-square showed that the genotype frequency distribution of RFC G80A and MTHFR C677T conformed to HWE, and the difference was not statistically significant ($P > 0.05$), which demonstrated that the study objects were community representative. According to the data presented in Table 3, CT (OR = 1.611, 95% CI = 1.038-2.501, $P = 0.034$) and TT (OR = 3.054, 95% CI = 1.658-5.624, $P < 0.001$) genotypes of MTHFR C677T polymorphism increased the risk of HHcy compared to CC genotype. The RFC G80A variant was not associated with HHcy in any inheritance models tested (co-dominant, dominant and recessive).

In terms of lifestyle behaviors, individuals with HHcy exhibited a greater prevalence of tobacco smoking (χ^2 = 30.103, $P < 0.001$) and alcohol use (χ^2 = 33.922, $P < 0.001$), as well as a lower intake of fruit (χ^2 = 8.141, $P = 0.017$) and vitamin B12 (χ^2 = 5.298, $P = 0.021$), in comparison to those without HHcy (Table 4).

Risk factors for HHcy in Chinese urban population with hypertension

A binary logistic regression model was applied to detect risk factors for HHcy (subjects without HHcy = 0, subjects with HHcy = 1), including gender (assignment: male = 1, female = 2), heart rate, FA, MTHFR genotypes (assignment: CC = 1, CT = 2, TT = 3), fruit consumption (assignment: ≤ 2 days/week = 1, 3-4 days/week = 2, ≥

TABLE 1 Demographic characteristics and clinical profiles of subjects with or without HHcy.

	Subjects without HHcy n = 325	Subjects with HHcy n = 148	*Z*	*P*
Age (years)	53 (48, 59)	54 (49, 59.75)	-1.009	0.313
BMI (kg/m^2)	25.23 (23.38, 27.66)	25.68 (23.54, 28.02)	-0.536	0.592
Male, n (%)	145 (44.6%)	126 (85.1%)	68.237	<0.001
SBP (mmHg)	131.50 (125.00, 141.75)	133.50 (124.75, 143.25)	-0.948	0.343
DBP (mmHg)	83.00 (77.00, 89.50)	84.50 (78.25, 91.50)	-1.522	0.128
LSBP (mmHg)	131 (124, 141.5)	132 (125, 143)	-0.870	0.384
LDBP (mmHg)	83 (76, 89)	85 (78, 91)	-1.662	0.097
Heart rate (beats per minute)	75 (71, 81)	77 (71, 85)	-1.843	0.065
RSBP (mmHg)	132 (126, 142.5)	133 (127, 145.5)	-0.865	0.387
RDBP (mmHg)	83 (78, 90)	85 (78, 91)	-1.178	0.239
GLU (mmol/L)	5.22 (4.80, 6.10)	5.20 (4.80, 6.14)	-0.320	0.749
ALT (U/L)	21.5 (16.0, 35.0)	20.0 (14.0, 29.75)	-1.466	0.143
AST (U/L)	19.0 (16.0, 24.0)	19.0 (16.0, 22.0)	-1.357	0.175
ALB (g/L)	47.1 (45.6, 49.0)	47.65 (46.12, 49.1)	-1.254	0.210
TBIL (μmol/L)	11.0 (8.3, 14.3)	10.6 (8.9, 14.1)	-0.352	0.725
DBIL (μmol/L)	3.45 (2.60, 4.42)	3.3 (2.60, 4.35)	-0.449	0.653
BUN (μmol/L)	5.4 (4.4, 6.5)	5.6 (4.45, 6.9)	-0.593	0.553
CREA (μmol/L)	68 (54, 81)	69.5 (52.5, 85.25)	-0.907	0.364
TCHO (mmol/L)	4.62 (3.94, 5.43)	4.54 (3.83, 5.43)	-0.672	0.502
TG (mmol/L)	1.55 (1.04, 2.29)	1.37 (1.07, 1.49)	-0.066	0.947
HDL (mmol/L)	1.27 (1.05, 1.49)	1.21 (1.07, 1.49)	-0.257	0.797
LDL (mmol/L)	2.92 (2.16, 3.61)	2.71 (2.13, 3.50)	-0.685	0.493
FA (ng/ml)	10.43 (6.47, 15.15)	8.29 (4.52, 12.86)	-3.511	<0.001
Vitamin B12 (pg/ml)	339.4 (193.05, 433.37)	274.40 (191.07, 425.87	-0.817	0.414
Vitamin D (ng/ml)	16.61 (12.61, 21.39)	17.77 (13.74, 22.06)	-1.618	0.106
Hcy (μmol/L)	11.35 (9.41, 12.97)	18.25 (16.40, 20.63)	-17.448	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LSBP, left ventricular systolic pressure; LDBP, left diastolic blood pressure; RSBP, right systolic blood pressure; RDBP, right diastolic blood pressure; GLU, glucose; ALT, alanine transaminase; AST, aspartate aminotransferase; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; BUN, Blood urea nitrogen; CREA, creatinine; TCHO, Total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FA, folic acid; Hcy, homocysteine. Units of measurement are reported in Table 1 where applicable.

TABLE 2 Hardy-Weinberg equilibrium (HWE) results of RFC G80A and MTHFR C677T variants polymorphisms.

Gene	Genotype	Actual frequencies n (%)	Predicted frequencies n	χ^2	*P*
RFC G80A	GG	21.90 (99)	23.5	0.372	0.830
	GA	52.88 (239)	50.0		
	AA	25.22 (114)	26.6		
MTHFR C677T	CC	49.78 (225)	47.3	1.258	0.533
	CT	38.05 (172)	42.9		
	TT	12.17 (55)	9.7		

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TABLE 3 Genotypic and allelic frequencies of RFC and MTHFR variants polymorphisms in subjects with or without HHcy.

Polymorphism	Subjects without HHcy n = 325	Subjects with HHcy n = 148	OR (95% CI)	*P*
RFC G80A				
Codominant				
GG	66 (21.1%)	33 (23.7%)	1.00	–
GA	170 (54.3%)	69 (49.6%)	0.812 (0.491, 1.342)	0.416
AA	77 (24.6%)	37 (26.6%)	0.961 (0.542, 1.704)	0.892
Dominant				
GG	66 (21.1%)	33 (23.7%)	1.00	–
GA+AA	247 (78.9%)	106 (76.3%)	0.858 (0.533, 1.381)	0.529
Recessive				
GG+GA	236 (75.4%)	102 (73.4%)	1.00	–
AA	77 (24.6%)	37 (26.6%)	1.112 (0.705, 1.754)	0.649
Allele				
G	302 (48.2%)	135 (48.6%)	1.00	–
A	324 (51.8%)	143 (51.4%)	0.993 (0.717, 1.374)	0.966
MTHFR C677T				
Codominant				
CC	171 (54.6%)	54 (38.8%)	1.00	–
CT	114 (36.4%)	58 (41.7%)	1.611 (1.038-2.501)	0.034
TT	28 (8.9%)	27 (19.4%)	3.054 (1.658-5.624)	< 0.001
Dominant				
CC	171 (54.6%)	54 (38.8%)	1.00	–
CT+TT	142 (45.4%)	85 (61.2%)	1.896 (1.261-2.848)	0.002
Recessive				
CC+CT	285 (91.1%)	112 (80.6%)	1.00	–
TT	28 (8.9%)	27 (19.4%)	2.454 (1.385-4.348)	0.002
Allele				
C	456 (72.8%)	166 (59.7%)	1.00	–
T	170 (27.2%)	112 (40.3%)	1.523 (1.077-2.154)	0.017

–, not given.

5 days/week = 3), types of staple foods (assignment: mainly rice = 1, mainly pasta = 2), type of meat (assignment: no meat = 1, mainly lean meat = 2, eat both fat and lean meat = 3, mainly fat meat = 4), beverage consumption (assignment: basically not drinking = 1, 1-2 times/week = 2, 3-5 times/week = 3, ≥ 6 times/week = 4), vitamin B12 supplements (assignment: no = 0, yes = 1), smoking (assignment: never smoke = 1, quit smoking = 2, always smoke = 3), frequency of alcohol consumed (assignment: never = 1, occasionally = 2, frequently = 3, every day = 4), and alcohol drinking status (assignment: never drinks alcohol = 1, quit drinking = 2, drinking = 3). As shown in Table 5, the risk factors for HHcy were as follows: male (B = 1.430, P < 0.001, OR = 4.179, 95%CL: 2.005-8.707) and MTHFR (TT) (B = 1.086, P = 0.006, OR = 2.961, 95%CL: 1.357-6.464).

Factors influencing Hcy levels in Chinese urban population with hypertension

Spearman correlation analysis showed significant correlations between Hcy levels and the following parameters: gender (r = -0.462, P < 0.001), MTHFR genotypes (r = 0.231, P < 0.001), DBP (r = 0.098, P = 0.036), heart rate (r = 0.093, P = 0.047), ALB (r = 0.136, P = 0.025), FA (r = -0.203, P < 0.001), Vitamin D (r = 0.131, P = 0.010), fruit

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TABLE 4 Lifestyle behaviors of subjects with or without HHcy.

	Subjects without HHcy n = 325	Subjects with HHcy n = 148	χ^2	*P*
Exercise			3.229	0.358
no exercise	59 (18.8%)	32 (23.4%)		
occasional	41 (13.1%)	12 (8.6%)		
more than once a week	41 (13.1%)	14 (10.2%)		
daily	173 (55.1%)	79 (57.7%)		
Vegetable consumption			2.765	0.251
≤ 2 days/week	19 (6.4%)	11 (9.2%)		
3-4 days/week	15 (5.1%)	10 (8.3%)		
≥ 5 days/week	262 (88.5%)	99 (82.5%)		
Fruit consumption			8.141	0.017
≤ 2 days/week	96 (32.4%)	54 (45.4%)		
3-4 days/week	49 (16.6%)	22 (18.5%)		
≥ 5 days/week	151 (51.0%)	43 (36.1%)		
Types of staple foods			3.764	0.052
mainly rice	284 (96.3%)	110 (91.7%)		
mainly pasta	11 (3.7%)	10 (8.3%)		
Meat consumption			2.718	0.437
basically not eating	30 (10.2%)	11 (9.2%)		
1-2 times/week	25 (8.5%)	9 (7.5%)		
3-5 times/week	42 (14.3%)	25 (20.8%)		
6 times/week	197 (67.0%)	75 (62.5%)		
Type of meat			6.855	0.077
no meat	15 (5.1%)	5 (4.3%)		
mainly lean meat	212 (71.6%)	71 (60.7%)		
eat both fat and lean meat	60 (20.3%)	33 (28.2%)		
mainly fat meat	9 (3.0%)	8 (6.8%)		
Tofu consumption			1.717	0.633
basically not eating	175 (59.3%)	64 (54.2%)		
1-2 times/week	96 (32.5%)	45 (38.1%)		
3-5 times/week	19 (6.4%)	6 (5.1%)		
≥ 6 times/week	5 (1.7%)	3 (2.5%)		
Nut consumption			1.396	0.706
basically not eating	230 (78.0%)	93 (78.8%)		
1-2 times/week	37 (12.5%)	14 (11.9%)		
3-5 times/week	16 (5.4%)	4 (3.4%)		
≥ 6 times/week	12 (4.1%)	7 (5.9%)		
Beverage consumption			6.658	0.084
basically not drinking	261 (88.2%)	94 (79.7%)		

(Continued)

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TABLE 4 Continued

	Subjects without HHcy n = 325	Subjects with HHcy n = 148	χ^2	*P*
1-2 times/week	19 (6.4%)	13 (11.0%)		
3-5 times/week	8 (2.7%)	3 (2.5%)		
≥ 6 times/week	8 (2.7%)	8 (6.8%)		
Water consumption			3.294	0.349
basically not drinking	3 (1.0%)	0 (0.0%)		
1-2 times/week	4 (1.4%)	0 (0.0%)		
3-5 times/week	12 (5.4%)	3 (2.6%)		
≥ 6 times/week	276 (93.6%)	111 (97.4)		
Kitchen type			3.695	0.158
open	90 (30.6%)	33 (28.0%)		
semi-open	120 (40.8%)	40 (33.9%)		
closed	84 (28.6%)	45 (38.1%)		
Dietary habit			2.387	0.303
cooking at home	266 (90.8%)	102 (86.4%)		
takeout eating	1 (0.3%)	0 (0.0%)		
dining out	26 (8.9%)	16 (13.6%)		
Vitamin B12 supplements			5.298	0.021
no	238 (81.2%)	105 (90.5%)		
yes	55 (18.8%)	11 (9.5%)		
Smoking			30.103	<0.001
never smoke	220 (73.3%)	63 (47.0%)		
quit smoking	29 (9.7%)	19 (14.2%)		
always smoke	51 (17.0%)	52 (38.8%)		
Frequency of alcohol consumed			33.922	<0.001
never	240 (78.9%)	73 (54.9%)		
occasionally	53 (17.4%)	43 (32.3%)		
frequently	10 (3.3%)	9 (6.8%)		
every day	1 (0.3%)	8 (6.0%)		
Alcohol drinking status			27.504	<0.001
never drinks alcohol	240 (78.9%)	73 (54.9%)		
quit drinking	20 (6.6%)	14 (10.5%)		
drinking	44 (14.5%)	46 (34.6%)		
Use of antihypertensive drugs			0.277	0.599
no	32 (10.4%)	17 (12.1%)		
yes	276 (89.6%)	124 (87.9%)		

TABLE 5 Binary logistic regression analysis of potential risk factors related to HHcy in the hypertension population.

	B	*S.E.*	*P*	Exp (B)	*95% CL*
Gender (male)	1.430	0.375	< 0.001	4.179	2.005-8.707
LDBP	-0.007	0.013	0.569	0.993	0.968-1.018
Heart rate	0.020	0.012	0.088	1.020	0.997-1.043
FA	-0.019	0.024	0.415	0.981	0.936-1.028
MTHFR (CC)			0.017		
MTHFR (CT)	0.520	0.295	0.078	1.682	0.943-3.000
MTHFR (TT)	1.086	0.398	0.006	2.961	1.357-6.464
Fruit consumption	-0.089	0.153	0.561	0.915	0.677-1.235
Type of staple food	0.614	0.589	0.298	1.847	0.582-5.859
Type of meat	0.149	0.218	0.494	1.161	0.757-1.779
Beverage consumption	0.222	0.182	0.222	1.249	0.874-1.785
Vitamin B12 supplements	-0.884	0.476	0.064	0.413	0.162-1.051
Smoking	0.079	0.174	0.651	1.082	0.769-1.523
Frequency of alcohol consumed	0.684	0.352	0.052	1.981	0.993-3.951
Alcohol drinking status	-0.271	0.292	0.353	0.762	0.430-1.352

consumption (r = -0.179, P < 0.001), type of meat (r = 0.100, P = 0.044), vitamin B12 supplements (r = -0.123, P = 0.012), smoking (r = 0.304, P < 0.001), frequency of alcohol consumed (r = 0.276, P < 0.001), and alcohol drinking status (r = 0.270, P < 0.001). Figure 1 shows a scatter plot representing the correlation serum Hcy and FA levels. All variables except for DBP, ipulse, ALB, Vitamin D, type of meat, and vitamin B12 supplements passed Bonferroni correction (P < 0.05/30 = 0.0017). These significantly different variables from univariate analysis were included in multiple linear regression to detect correlates of Hcy levels (Table 6). Finally, the multiple linear regression revealed a significant association between Hcy levels and gender (B = -2.784, t = -2.931, P = 0.004), MTHFR genotypes (B = 1.410, t = 2.815, P = 0.005), and FA levels (B = -0.136, t = -2.192, P = 0.030).

Discussion

The objective of this study was to assess the prevalence, clinical correlation, and demographic characteristics of HHcy in the Chinese urban population with hypertension, with a focus on identifying risk factors for HHcy in hypertensive patients. The study yielded three key findings. Firstly, the prevalence of HHcy in the Chinese urban population with hypertension was determined to be 31.3%. Secondly, logistic regression analysis identified male gender and the presence of the MTHFR (TT) genotype as significant risk factors for HHcy. Lastly, the multiple linear regression revealed a significant association between Hcy levels and gender, MTHFR genotypes, and FA levels.

Multiple studies have examined the occurrence of HHcy in individuals with hypertension. One such study revealed that 73.3% of newly diagnosed hypertensive patients residing in seven urban communities in Nanjing, China, exhibited HHcy (19). Similarly, another study found a comparable prevalence of HHcy. Specifically, among 105 patients diagnosed with primary hypertension in Vietnam, 74.3% (78 patients) displayed elevated plasma total Hcy levels ≥ 15 μmol/L (20). The current investigation aimed to ascertain the prevalence of HHcy among the urban Chinese population with hypertension, revealing a rate of 31.3%. This finding closely aligns with the outcomes of a prior study conducted within China (13). Notably, a separate investigation involving 5935 hypertensive patients in China demonstrated a HHcy prevalence of 31.4% (13). Discrepancies in HHcy prevalence across studies may be attributed to various contextual factors, including population demographics, geographical location, and patient age. A meta-analysis conducted in China involving a sample size of 60,754 individuals aged 3-97 years revealed a positive correlation between age and the prevalence of HHcy, with the highest occurrence observed among individuals aged 65 years and above (21). Consequently, it is reasonable to hypothesize that the dissimilarity in HHcy prevalence between the present study and the study conducted in Nanjing (31.3% vs. 73.3%) could potentially be attributed to the variance in age distribution among the respective subject groups (54.1 vs. 63.3).

Previous evidence has indicated that gender plays a significant role in determining the levels of Hcy in humans. A study conducted on healthy individuals revealed that males tend to have higher serum Hcy levels compared to females (22). Moreover, a meta-analysis encompassing 36 studies and involving 60,754 subjects from 19 provinces and municipalities in China found that the prevalence of HHcy was notably higher in men than in women (21). Furthermore, elevated levels of serum homocysteine have been observed in male patients with hypertension (23), primary chronic venous disease (24), and psoriasis (25). Potential factors contributing to the observed sex-specific variation encompass disparities in muscle mass, estrogen

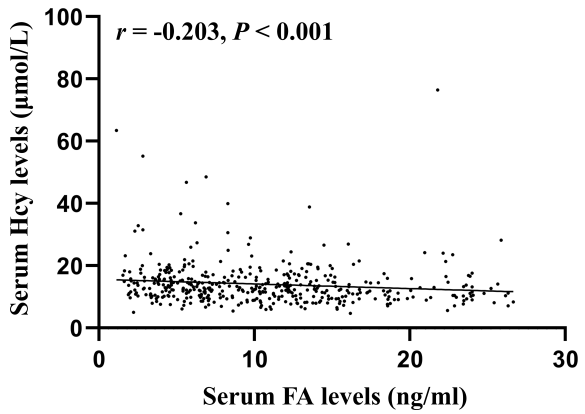

FIGURE 1
Correlation between serum Hcy and FA levels in Chinese urban population with hypertension.

levels, lifestyles, and vitamin levels. Specifically, Notably, the connection between Hcy production and creatine-creatinine synthesis is widely acknowledged. Given that males typically possess greater muscle mass, they exhibit an augmented requirement for creatine synthesis, consequently leading to increased Hcy production (26). Moreover, Chinese men exhibit a higher prevalence of alcohol consumption and cigarette smoking compared to women, both of which exhibit positive correlations with Hcy concentrations (27). Furthermore, the presence of hormonal disparities between males and females may also play a role in the observed sex-related discrepancy. Research has indicated that the administration of long-term hormone replacement therapy leads to decreased overall concentrations of Hcy in women who have undergone menopause (28). In the present study, the proportion of males was notably greater among individuals with HHcy compared to those without HHcy. Through the utilization of logistic regression analysis, male gender was identified as a significant risk factor for HHcy, while multiple linear regression analysis demonstrated a significant association between Hcy levels and gender. These findings offer additional empirical evidence establishing a correlation between gender and Hcy levels.

The enzyme MTHFR, which is dependent on folate, plays a significant role in the conversion of the amino acid homocysteine to methionine (29). The activity of MTHFR has a detrimental impact on the levels of Hcy in the serum (25). The MTHFR rs1801133 polymorphism involves a substitution of C to T at position 677, resulting in the conversion of alanine to valine. This missense mutation leads to a reduction of approximately 70% and 35% in the normal activity of the MTHFR enzyme in carriers with the TT and CT genotypes, respectively (30). Additionally, the TT allele is associated with increased levels of homocysteine in the serum (31). In this study, CT and TT genotypes of MTHFR C677T polymorphism increased the risk of HHcy compared to CC genotype. Additionally, logistic regression analysis revealed that the presence of the MTHFR (TT) genotype was a significant risk factor for HHcy. Furthermore, multiple linear regression analysis demonstrated a significant association between homocysteine levels and the MTHFR genotype. Given that approximately 25% of the Chinese population possesses the MTHFR C677T TT genotype (32), the assessment of MTHFR genotype within the Chinese populace, particularly among individuals with hypertension, holds significant clinical implications in terms of cardio-cerebrovascular disease prevention and treatment.

Presently, the utilization of FA and B vitamins is employed to attain desired Hcy levels, exhibiting evident efficacy in diminishing plasma Hcy concentrations (33). While not yet substantiated, the reduction of overall plasma Hcy levels via FA and/or vitamin B12 intake may potentially mitigate the susceptibility to vascular diseases among HHcy individuals (34). Another clinical trial investigation has documented that the addition of FA as a supplementary therapy,

TABLE 6 Associated factors for Hcy levels in Chinese urban population with hypertension based on multiple linear regression model.

Variables	Unstandardized B	Coefficient Std.error	Standardized coefficients Beta	*t*	*P*	VIF
Gender	-2.784	0.950	-0.239	-2.931	0.004	1.880
MTHFR genotypes	1.410	0.501	0.173	2.815	0.005	1.075
DBP	-0.067	0.035	-0.127	-1.903	0.059	1.257
Ipulse	0.053	0.029	0.124	1.863	0.064	1.251
ALB	0.023	0.052	0.027	0.433	0.658	1.023
FA	-0.136	0.062	-0.144	-2.192	0.030	1.231
Vitamin D	0.197	0.066	0.191	2.961	0.003	1.174
Fruit consumption	0.050	0.401	0.008	0.125	0.901	1.109
Type of meat	1.005	0.599	0.108	1.677	0.095	1.171
Vitamin B12 supplements	-1.411	0.976	-0.088	-1.445	0.150	1.050
Smoking	0.802	0.524	0.113	1.530	0.128	1.560
Frequency of alcohol consumed	1.583	0.939	0.193	1.686	0.094	3.729
Alcohol drinking status	-0.454	0.815	-0.065	-0.556	0.579	3.897

administered at a dosage of 5 mg per day, exhibits the potential to markedly decrease serum Hcy levels (35). This intervention may play a contributory role in the pathogenesis of vascular disorders, including Buerger's disease (35). In the current investigation, individuals diagnosed with HHcy exhibited notably diminished levels of FA in comparison to those without HHcy. Regarding lifestyle habits, individuals with HHcy demonstrated a reduced intake of vitamin B12 in comparison to those without HHcy. Furthermore, inverse associations were observed between Hcy levels and both FA levels and vitamin B12 supplementation. Additionally, fruits and vegetables are recognized as abundant sources of FA and B vitamins, which contribute to elevated plasma levels of these vitamins and decreased Hcy levels (36). In the present study, it was consistently observed that individuals with HHcy exhibited lower levels of fruit intake compared to those without HHcy. These findings imply that augmenting FA levels and incorporating vitamin B12 supplements could potentially decrease Hcy levels in hypertensive patients, consequently reducing the incidence of cardio-cerebrovascular disease.

Evidence consistently supports the notion that smoking, alcohol consumption, and physical inactivity have a significant impact on elevating Hcy levels (37, 38). Consequently, these lifestyle factors were incorporated into the current study to ascertain the specific risk factors associated with HHcy in hypertensive individuals. Correspondingly, our findings revealed a heightened prevalence of tobacco smoking and alcohol use among subjects with HHcy, thereby reinforcing the recommendation for hypertensive patients with HHcy to adopt lifestyle modifications such as reducing alcohol intake and quitting smoking.

Several limitations to this study need to be acknowledged. First, this is a cross-sectional study design and cannot show a causal relationship between the presence of HHcy and a given risk factor. Second, this study is limited by its observational design and small subset size, which reduces the power of the statistical analysis. Third, generalizability was limited due to the fact that the study was performed in only one region of China. Fourth, an additional limitation of this study pertains to the absence of validation for the questionnaire tool and the omission of a reliability assessment. Fifth, given that diuretics decrease the absorption of FA, the absence of precise categorizations pertaining to the types of antihypertensive drugs is regarded as a limitation of this research.

In conclusion, our study reveals a notable occurrence of HHcy among the Chinese urban population with hypertension, those at greatest risk are male, genotype MTHFR 677TT, and a decreased FA level. Consequently, it is imperative to implement future strategies for managing and intervening in these factors to mitigate the development of H type hypertension and minimize associated cardio-cerebrovascular events within this population. For example, to mitigate the occurrence of HHcy and cardio-cerebrovascular disease, particularly among male individuals with the MTHFR C677T TT genotype, it is recommended to implement pharmacotherapy and/or lifestyle adjustments encompassing physical activity, nutrition, and dietary supplementation, such as FA. Notably, HHcy has been considered a specific disease in the Chinese Han population (39). The population with the TT genotype accounts for 25% of the Chinese Han population, whereas the population with the TT genotype in North America and Europe accounts for only approximately 12% of the combined population (40). Thus, the risk factors for HHcy identified in the Chinese population in this study, specifically the MTHFR677TT genotype, may not be applicable to other populations, such as North Americans and Europeans.

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 author.

Ethics statement

The studies involving humans were approved by Clinical Research Ethics Committee of Shenzhen Second People's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YX: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft. HF: Investigation, Methodology, Validation, Writing – original draft. LZ: Software, Supervision, Writing – original draft. YL: Formal analysis, Resources, Software, Writing – original draft. FC: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. LR: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

Publisher's note

References

1. Roth G, Mensah G, Fuster V. The global burden of cardiovascular diseases and risks: A compass for global action. *J Am Coll Cardiol* (2020) 76:2980–1. doi: 10.1016/j.jacc.2020.11.021

2. Wang H, Zhang H, Zou Z. Changing profiles of cardiovascular disease and risk factors in China: A secondary analysis for the global burden of disease study 2019. *Chin Med J* (2023) 136:2431–41. doi: 10.1097/CM9.0000000000002741

3. Katan M, Luft A. Global burden of stroke. *Semin Neurol* (2018) 38:208–11. doi: 10.1055/s-0038-1649503

4. Fan F, David Spence J, Huo Y. Beyond hypertension: Hypertension with hyperhomocysteinemia. *Sci Bull* (2023) 68:1975–7. doi: 10.1016/j.scib.2023.08.018

5. Zheng W, Wang X, Xue X, Li W, Fan L, Zhang S, et al. Characteristics of hypertension in the last 16 years in high prevalence region of China and the attribute ratios for cardiovascular mortality. *BMC Public Health* (2023) 23:114. doi: 10.1186/s12889-022-14974-0

6. GBD 2019 Risk Factors Collaborators. Global burden of 87 risk factors in 204 countries and territories, 1990-2019: A systematic analysis for the global burden of disease study 2019. *Lancet (London England)* (2020) 396:1223–49. doi: 10.1016/S0140-6736(20)30752-2

7. Lauder L, Mahfoud F, Azizi M, Bhatt D, Ewen S, Kario K, et al. Hypertension management in patients with cardiovascular comorbidities. *Eur Heart J* (2023) 44:2066–77. doi: 10.1093/eurheartj/ehac395

8. Zhang M, Shi Y, Zhou B, Huang Z, Zhao Z, Li C, et al. Prevalence, awareness, treatment, and control of hypertension in China, 2004-18: Findings from six rounds of a national survey. *BMJ (Clinical Res ed.)* (2023) 380:e071952. doi: 10.1136/bmj-2022-071952

9. Wang Y, Xia H, Song Z, Zhou X, Zhang H. Plasma homocysteine as a potential marker of early renal function decline in iga nephropathy. *Front Med* (2022) 9:812552. doi: 10.3389/fmed.2022.812552

10. Liu W, Li Q, Wang Q, Ma S, Yang X, Zhang J, et al. Association between body fat composition and hyperhomocysteinemia in the analysis of the baseline data of the northwest China natural population cohort: Ningxia project (cnc-nx). *J Clin Hypertension (Greenwich Conn)* (2023) 25:573–81. doi: 10.1111/jch.14666

11. Yan J, Zhou J, Huang J, Zhang H, Deng Z, Du Y. The outcomes of acute myocardial infarction patients comorbidity with hypertension and hyperhomocysteinemia. *Sci Rep* (2021) 11:22936. doi: 10.1038/s41598-021-02340-w

12. Ganguly P, Alam S. Role of homocysteine in the development of cardiovascular disease. *Nutr J* (2015) 14:6. doi: 10.1186/1475-2891-14-6

13. Wang C, Chen Z, Zhang T, Liu J, Chen S, Liu S, et al. Elevated plasma homocysteine level is associated with ischemic stroke in chinese hypertensive patients. *Eur J Internal Med* (2014) 25:538–44. doi: 10.1016/j.ejim.2014.04.011

14. Li J, Jiang S, Zhang Y, Tang G, Wang Y, Mao G, et al. H-type hypertension and risk of stroke in chinese adults: A prospective, nested case-control study. *J Trans Internal Med* (2015) 3:171–8. doi: 10.1515/jtim-2015-0027

15. Feng Y, Kang K, Xue Q, Chen Y, Wang W, Cao J. Value of plasma homocysteine to predict stroke, cardiovascular diseases, and new-onset hypertension: A retrospective cohort study. *Medicine* (2020) 99:e21541. doi: 10.1097/MD.0000000000021541

16. Zhang P, Zhang Y. Association of homocysteine with acute stroke and its subtypes in the chinese population. *Neuropsychiatr Dis Treat* (2023) 19:1435–42. doi: 10.2147/NDT.S409591

17. Kataria N, Yadav P, Kumar R, Kumar N, Singh M, Kant R, et al. Effect of vitamin b6, b9, and b12 supplementation on homocysteine level and cardiovascular outcomes in stroke patients: A meta-analysis of randomized controlled trials. *Cureus* (2021) 13:e14958. doi: 10.7759/cureus.14958

18. Peng X, Zhou Y, Wu X, Wang X, Bai H, Li Y, et al. Association of methylenetetrahydrofolate reductase (mthfr) variant c677t and risk of carotid atherosclerosis: A cross-sectional analysis of 730 chinese han adults in chongqing. *BMC Cardiovasc Disord* (2020) 20:222. doi: 10.1186/s12872-020-01505-1

19. Wang W, Ji P, Wang Y, Guo H, Bian R, Xu J, et al. Prevalence of hyperhomocysteinemia and its associated factors in patients with primary hypertension in chinese urban communities: A cross-sectional study from nanjing. *Clin Exp Hypertension (New York NY: 1993)* (2018) 40:495–500. doi: 10.1080/10641963.2017.1403621

20. Tran S, Ngo T, Nguyen P, Truong A, Truong G, Tran K, et al. Hyperhomocysteinemia in patients with newly diagnosed primary hypertension in can tho city, Vietnam. *Healthc (Basel Switzerland)* (2023) 11:234. doi: 10.3390/healthcare11020234

21. Yang B, Fan S, Zhi X, Wang Y, Wang Y, Zheng Q, et al. Prevalence of hyperhomocysteinemia in China: A systematic review and meta-analysis. *Nutrients* (2014) 7:74–90. doi: 10.3390/nu7010074

22. Moon H, Whang D, Ko Y, Joo S, Yun Y, Hur M, et al. Reference interval and determinants of the serum homocysteine level in a korean population. *J Clin Lab Anal* (2011) 25:317–23. doi: 10.1002/jcla.20476

23. Pang H, Han B, Fu Q, Hao L, Zong Z. Association between homocysteine and conventional predisposing factors on risk of stroke in patients with hypertension. *Sci Rep* (2018) 8:3900. doi: 10.1038/s41598-018-22260-6

24. Smith R, Quigley F, Tosenovsky P, Velu R, Bradshaw B, Buettner P, et al. Serum homocysteine is associated with the severity of primary chronic venous disease. *Phlebology* (2016) 31:409–15. doi: 10.1177/0268355515592076

25. Zhang Q, Lin J, Zhang Z, Han L, Huang Q, Zhu J, et al. Mthfr polymorphism and folic acid supplementation influence serum homocysteine levels in psoriatic patients treated with methotrexate. *J Clin Med* (2022) 11:4580. doi: 10.3390/jcm11154580

26. Rauh M, Verwied S, Knerr I, Dörr H, Sönnichsen A, Koletzko B. Homocysteine concentrations in a german cohort of 500 individuals: Reference ranges and determinants of plasma levels in healthy children and their parents. *Amino Acids* (2001) 20:409–18. doi: 10.1007/s007260170037

27. de Bree A, Verschuren W, Blom H, Kromhout D. Lifestyle factors and plasma homocysteine concentrations in a general population sample. *Am J Epidemiol* (2001) 154:150–4. doi: 10.1093/aje/154.2.150

28. Madsen J, Kristensen S, Klitgaard N, Bladbjerg E, Abrahamsen B, Stilgren L, et al. Effect of long-term hormone replacement therapy on plasma homocysteine in postmenopausal women: A randomized controlled study. *Am J Obstet Gynecol* (2002) 187:33–9. doi: 10.1067/mob.2002.123030

29. Zhao L, Li T, Dang M, Li Y, Fan H, Hao Q, et al. Association of methylenetetrahydrofolate reductase (mthfr) rs1801133 (677c>t) gene polymorphism with ischemic stroke risk in different populations: An updated meta-analysis. *Front Genet* (2022) 13:1021423. doi: 10.3389/fgene.2022.1021423

30. Kong Y, Zheng J, Li L, Lu L, Wang J. Association of mthfr polymorphisms with h-type hypertension: A systemic review and network meta-analysis of diagnostic test accuracy. *Int J Hypertension* (2022) 2022:2861444. doi: 10.1155/2022/2861444

31. Chen L, Wu C, Dong Z, Cao S, Ren N, Yan X. Methylenetetrahydrofolate reductase polymorphisms and elevated plasma homocysteine levels in small vessel disease. *Brain Behav* (2023) 13:e2960. doi: 10.1002/brb3.2960

32. Wu D, Wu Y, Deng J. Changes in homocysteine levels affect serum lipid response to atorvastatin in patients with acute coronary syndrome: A retrospective observational study. *Clin Appl Thromb/Hemost* (2020) 26:1076029620920369. doi: 10.1177/1076029620920369

33. Zhou Y, Tang J, Wu M, Lu J, Wei X, Qin Y, et al. Effect of folic acid supplementation on cardiovascular outcomes: A systematic review and meta-analysis. *PloS One* (2011) 6:e25142. doi: 10.1371/journal.pone.0025142

34. Boushey C, Beresford S, Omenn G, Motulsky A. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* (1995) 274:1049–57. doi: 10.1001/jama.274.13.1049

35. Modaghegh M, Ravari H, Haghighi M, Rajabnejad A. Effect of folic acid therapy on homocysteine level in patients with atherosclerosis or buerger's disease and in healthy individuals: A clinical trial. *Electronic Physician* (2016) 8:3138–43. doi: 10.19082/3138

36. Khan M, Saeedullah A, Andrews S, Iqbal K, Qadir S, Shahzad B, et al. Adolescent afghan refugees display a high prevalence of hyperhomocysteinemia and associated micronutrients deficiencies indicating an enhanced risk of cardiovascular disease in later life. *Nutrients* (2022) 14:1751. doi: 10.3390/nu14091751

37. Kowluru R, Mohammad G, Sahajpal N. Faulty homocysteine recycling in diabetic retinopathy. *Eye Vision (London England)* (2020) 7:4. doi: 10.1186/s40662-019-0167-9

38. Losso J. The potential of dietary bioactive compounds against sars-cov-2 and covid-19-induced endothelial dysfunction. *Mol (Basel Switzerland)* (2022) 27:1623. doi: 10.3390/molecules27051623

39. Hou Y, Yun L, Zhang L, Lin J, Xu R. A risk factor-based predictive model for new-onset hypertension during pregnancy in chinese han women. *BMC Cardiovasc Disord* (2020) 20:155. doi: 10.1186/s12872-020-01428-x

40. Zhao M, Wang X, He M, Qin X, Tang G, Huo Y, et al. Homocysteine and stroke risk: Modifying effect of methylenetetrahydrofolate reductase c677t polymorphism and folic acid intervention. *Stroke* (2017) 48:1183–90. doi: 10.1161/STROKEAHA.116.015324

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Serum fetuin-a and risk of thoracic aortic aneurysms: a two-sample mendelian randomization study

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Background: Recent studies have revealed a significant decrease in serum fetuin-A levels in atherosclerotic aneurysms, indicating that fetuin-A may play a protective role in the progression of arterial calcification. However, the specific mechanism behind this phenomenon remains unclear. We aimed to examine the association between fetuin-A levels in thoracic aortic aneurysms (TAAs) and risk of TAAs and to evaluate whether this association was causal.

Methods: A total of 26 SNPs were selected as instrumental variables for fetuin-A in 9,055 participants of European ancestry from the CHARGE consortium, and their effects on thoracic aortic aneurysm and decreased descending thoracic aortic diameter were separately estimated in 353,049 and 39,688 individuals from FinnGen consortium. We used two-sample Mendelian randomization (MR) analysis to examine the causal association. At the same time, we employed various methods, including random-effects inverse variance weighting, weighted median, MR Egger regression, and MR PRESSO, to ensure the robustness of causal effects. We assessed heterogeneity using Cochran's Q value and examined horizontal pleiotropy through MR Egger regression and retention analysis.

Results: Fetuin-A level was associated with a significantly decreasing risk of thoracic aortic aneurysm (odds ratio (OR) 0.64, 95% CI 0.47 - 0.87, $P = 0.0044$). Genetically predicted fetuin-A was also correlated with the decreased descending thoracic aortic diameter ($\beta = -0.086$, standard error (SE) 0.036, $P = 0.017$).

Conclusions: Serum fetuin-A level was negatively associated with risk of TTAs and correlated with the decreased descending thoracic aortic diameter. Mendelian randomization provides support for the potential causal relationship between fetuin-A and thoracic aortic aneurysm.

KEYWORDS

fetuin-A, thoracic aortic aneurysm, thoracic aortic diameter, mendelian randomization analysis, mechanisms

1 Introduction

Aortic aneurysm is a critical cardiovascular disease characterized by exceptionally high mortality rates. It is a complex multifactorial condition influenced by both genetic and environmental risk factors. Thoracic aortic aneurysms (TAAs) refer to the dilation of the ascending part of the aorta, accounting for one-third of the incidence of aortic aneurysm, which may lead to dissection or aortic rupture (1). It is related to age, male, smoking, hypertension, family history and genetic susceptibility. Atherosclerosis is an infrequent underlying cause in most cases. The precise etiology, however, remains incompletely elucidated, underscoring the practical significance of early biomarker identification and detection.

Fetuin-A, also referred to as α2-Heremans-Schmid glycoprotein or α2-HS glycoprotein, is a heterodimeric plasma glycoprotein composed of 282 amino acids in the A chain and 27 amino acids in the B chain (2). It is primarily secreted by the liver and can also be found widely expressed in various tissues (3). Fetuin-A has also been shown to play a vital role in the development of several disorders. It plays a role in regulating calcium metabolism, osteogenesis, and insulin signaling pathways. Additionally, it functions as a heterotopic calcification inhibitor, protease inhibitor, inflammatory mediator, anti-inflammatory mediator, and atherogenic factor (4). Recent studies (5, 6) have demonstrated that elevated levels of plasma fetuin-A are linked to an elevated risk of myocardial infarction and stroke. This suggests that fetuin-A plays a role in the pathophysiology of cardiovascular diseases. In an observational study (7) comprising 30 cases of atherosclerotic aortic aneurysms, 15 cases of Marfan syndrome, 30 cases of peripheral arterial diseases, and healthy controls, Szeberin et al. discovered a significant lower in the serum level of fetuin-A in the atherosclerotic aortic aneurysm group. This finding lends support to the notion that fetuin-A plays a protective role in the process of arterial calcification. The plasma samples from six patients with TAA before and after surgery, as well as from six healthy controls, were analyzed by Kazamia et al. (8). The findings revealed a significant decrease in the concentration of alpha-2-HS glycoprotein (AHSG), also known as fetuin-A, in preoperative plasma samples from TAA patients compared to healthy controls. These results suggest that AHSG (fetuin-A) holds promise as a potential biomarker for TAA. However, the involvement of fetuiu-A in the occurrence and progression of TAAs remains elusive.

Mendelian Randomization (MR) is a method that utilizes genetic variations, particularly single nucleotide polymorphisms (SNPs), as instrumental variables (IVs) to establish causal relationships between diseases (outcomes) and risk factors (exposures) (9). To date, no Mendelian Randomization (MR) analysis has assessed the association between fetuin-A and thoracic aortic aneurysm. Nevertheless, clinical data indicates a significant reduction in serum fetuin-A levels within the aortic aneurysm group. Hence, it is imperative to further investigate the potential causal link between fetuin-A levels and thoracic aortic aneurysm.

Our hypothesis posits that serum fetuin-A levels are inversely correlated with the risk of thoracic aortic aneurysms. Given the limited evidence regarding the causal relationship between fetuin-A and the risk of thoracic aortic aneurysm, our objective is to employ Genome-Wide Single Nucleotide Polymorphism Array(GWAS) data to examine the causal between serum fetuin-A levels and the risk of thoracic aortic aneurysms. Additionally, we will explore potential causal associations through MR analysis, utilizing two distinct datasets sourced from GWSA data.

2 Methods

2.1 Study population and instrumental variables selection

The summary-level variants associated with fetuin-A expression were obtained from the CHARGE Consortium, which included 9,055 participants of European ancestry (10). This study further derived 26 SNPs fetuin-A using a criterion of $P < 5 \times 10^{-8}$ and LD $r^2 < 0.4$. The F-statistic indicates the strength of the relationship between SNPs and exposures. A higher F-statistic, typically greater than 10, suggests a lower likelihood of causing weak instrument bias. The genetic information of selected SNPs in detail is shown in Supplementary Table 1.

The summary datasets of thoracic aortic aneurysms were obtained from FinnGen release 9. We chose genetic association data from a total of 353,049 European participants. Descending

thoracic aortic diameter were derived from Pirruccello et al., which trained deep learning model to identify genetic variants associated with thoracic aortic diameter in 39,688 individuals (11). The maximum size in the elliptical minor axis during the cardiac cycle was defined as the aortic diameter, and its association with genetic variants was analyzed in UK biobank patients. All MR Analyses satisfy three basic assumptions: 1) instrumental variables are strongly correlated with exposure factors; 2) Instrumental variables are not correlated with confounding factors; 3) Instrumental variables are not directly related to the results, and their impact on the results can only be reflected through exposure.

2.2 Mendelian randomization analysis

We conducted MR analysis using the 'TwoSampleMR' package in R. The inverse variance weighted (IVW) meta-analysis method was chosen as the primary MR analysis, as it provides optimum accuracy with decent IV quality (12). Different supplementary methods, namely MR-Egger, weighted median, simple mode, weighted mode, and maximum likelihood were utilized to evaluate the causal association. The intercept of the MR-Egger regression can represent horizontal pleiotropy, and it can be applied in the presence of unbalanced pleiotropy (13). The weighted median method continues to offer a consistent estimate of the causal effect, even when over 50% of the instrumental variables are deemed invalid (14). The robust adjusted profile score (RAPS) method can better address the bias caused by potential weak instruments and pleiotropy.

2.3 Sensitivity analyses

To further ensure IV quality, extensive sensitivity analyses were performed. We used Cochran's Q test to evaluate heterogeneity between each IV. MR-Egger regression was employed to assess pleiotropy, with an MR-Egger intercept indicating its existence. MR pleiotropy residual sum and outlier (MR-PRESSO) method is also a powerful complement for testing pleiotropy in MR, with the global test detecting horizontal pleiotropy, and the outlier test can correct the estimate by removing outliers if necessary (15). Furthermore, we utilized the PhenoScanner database (http://www.phenoscanner.medschl.cam.ac.uk/) to evaluate the potential associations between the selected IVs and any confounding factors that could potentially impact outcomes (16). A leave-one-out analysis was conducted to identify potentially influential SNPs with significant effects on remaining IVW results.

2.4 Statistical analysis

All analyses were performed using R software (version 4.1.2). The 'MRPRESSO' package (version 1.0) was used for the MR-PRESSO method, and 'mr.raps' package (version 0.2) for the RAPS method. An online web tool (https://sb452.shinyapps.io/power/) was utilized for power analysis. A $P < 0.05$ was considered statistically significant in the primary analyses and Sensitivity analyses.

3 Results

3.1 The causal effect of fetuin-A level on thoracic aorta outcomes

Complete MR results are shown in the Supplementary Table 1. Among four pollution exposures, IVW estimates that fetuin-A expression was associated with a significantly decreasing risk of thoracic aortic aneurysm (OR = 0.64, 95% CI: 0.47 - 0.87, P = 0.0044). Genetically predicted fetuin-A was also correlated with the decreased descending thoracic aortic diameter (β = -0.086, SE = 0.036, P = 0.017) (Figure 1, Table 1). The ORs in all seven models (fixed effects IVW, multiplicative random effects IVW, MR-Egger, weighted median, simple mode, weighted mode, and maximum likelihood) demonstrated the same direction, as evidenced in the negative slopes of all the lines between fetuin-A levels and aortic outcomes (Figure 2). The direction and significance of MR-PRESSO and RAPS analyses are consistent with IVW for both thoracic aortic aneurysm risks (MR-PRESSO: OR = 0.64, 95% CI: 0.50 - 0.83, P = 0.0024; RAPS: OR = 0.62, 95% CI: 0.47 - 0.83, P = 0.0014) and descending thoracic aortic diameter (MR-PRESSO: β = -0.097, SE = 0.028, P = 0.0021; RAPS: β = -0.099, SE = 0.034, P = 0.0033).

3.2 Sensitivity analysis

The Cochran's Q test detected no heterogeneity in our study. MR-Egger regression indicated the existence of pleiotropy in the relationship between fetuin-A level and the risk of thoracic aortic aneurysm (P = 0.046). However, no pleiotropy was detected using the MR-PRESSO global tests and no SNP was removed. The leave-one-out analysis also determined that no IV substantially influenced overall MR analysis results for all significant exposure-outcome estimates (Supplementary Figure 1). All SNPs passed the correlation strength threshold, as the minimum F-statistic was 33.18, indicating unlikely weak instrument bias for MR analysis (Supplementary Table 2). The PhenoScanner search results revealed that no IVs were associated with confounding traits of thoracic aortic aneurysm risk or descending thoracic aortic diameter (Supplementary Table 3). The MR results in our study were relatively robust, indicating that high fetuin-A level is a protective factor for thoracic aortic aneurysm and enlargement of the descending thoracic aorta.

4 Discussion

This study employed a bidirectional two-sample Mendelian randomization (MR) design to investigate the associations between fetuin-A and thoracic aortic aneurysm, marking the first instance of such analysis. Two-sample Mendelian randomization analyses demonstrated that higher fetuin-A levels were associated with a significantly reduced risk of thoracic aortic aneurysm (odds ratio (OR) = 0.64, 95% confidence interval (CI): 0.47 - 0.87, P = 0.0044) and a decrease in the diameter of thoracic aortic aneurysms (β = -0.086, standard error (SE) = 0.036, P = 0.017).

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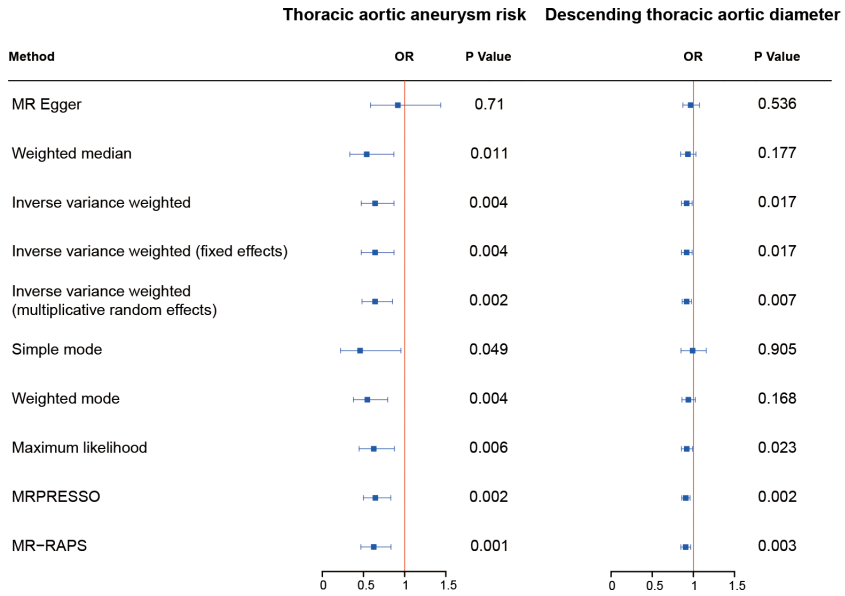

FIGURE 1
The causal effect of fetuin-A level on thoracic aorta outcomes. The odds ratios (ORs) are adjusted to genetically predict serum fetuin-A levels for assessing the risk of thoracic aortic aneurysm. OR, odds ratio.

The presence of aortic aneurysms pose a significant risk to cardiovascular health and can potentially be life-threatening. It can be categorized into two types: thoracic aortic aneurysm and abdominal aortic aneurysm, each with distinct underlying causes. The development of thoracic aortic aneurysm primarily revolves around changes in the extracellular matrix (17). As research progresses, an increasing body of evidence has confirmed the correlation between calcification of aneurysm walls and the mortality rate associated with aneurysms (18, 19).

Kazamia et al (8). utilized liquid chromatography-tandem mass spectrometry to examine proteins extracted from 14 thoracic aortic aneurysms (TAAs) tissue samples and 12 non-aneurysmal thoracic aortic tissue samples. They also analyzed plasma samples from 6 TAA patients before and after surgery and 6 healthy control individuals. The findings revealed a notable reduction in the concentration of Alpha-2-HS-glycoprotein (AHSG) (Also known as fetuin-A) in the preoperative plasma samples compared to those from the healthy controls, indicating that AHSG might serve as a promising biomarker for TAA. This study represented the first observation of the relationship between Fetuin-A and patients with aortic aneurysms of various causes. Szeberin et al (7). conducted a single-center cross-sectional observational study involving 105 patients, including 30 with atherosclerotic aortic aneurysm, 15 with Marfan syndrome, 30 with peripheral arterial disease, and 30 healthy controls. They analyzed the serum levels of fetuin-A. The findings revealed a significant reduction in serum fetuin-A levels in the atherosclerotic aortic aneurysm group, supporting the notion that fetuin-A plays a protective role in the development of arterial calcification. These results further substantiate the hypothesis that fetuin-A may serve as an inhibitor of arterial calcification in atherosclerotic patients, independent of diabetes and in the absence of obvious renal dysfunction. This discovery has the potential to enhance diagnostic capabilities for this condition. This is the initial proposition suggesting that fetuin-A plays a role

TABLE 1 Causal effects assessed by IVW, RAPS, and MR-PRESSO, and sensitivity analyses for fetuin-A level on aortic outcomes.

Outcome	Methods	Beta	SE	P-value	MR-Egger's P-value	Cochran Q test's P-value	MR-PRESSO Global Test P-value
Thoracic aortic aneurysm	IVW	-0.44	0.15	0.0044*	0.046#	0.631	0.537
	RAPS	-0.47	0.15	0.001*			
	MR-PRESSO	-0.44	0.13	0.002*			
Descending thoracic aortic diameter	IVW	-0.09	0.04	0.017*	0.192	0.765	0.814
	RAPS	-0.10	0.03	0.003*			
	MR-PRESSO	-0.10	0.03	0.002*			

*P-value < 0.05 for mendelian randomization analysis.
#P-value < 0.05 for sensitivity analysis.
IVW, inverse variance weighted. RAPS, robust adjusted profile score. MR-PRESSO, mendelian randomization pleiotropy residual sum and outlier.

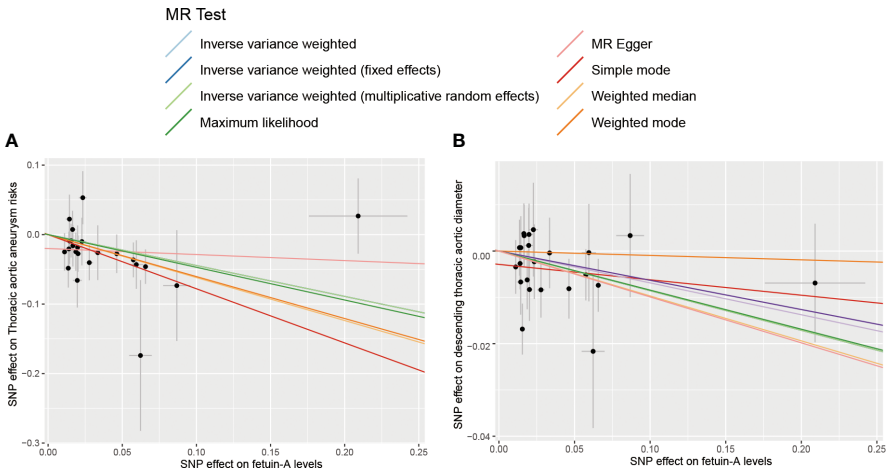

FIGURE 2
The scatter plot for MR analyses of fetuin-A levels and the risk of thoracic aortic aneurysm using different MR methods. **(A)** The effect of SNPs on the risk of thoracic aortic aneurysm; **(B)** The effect of SNPs on the risk of descending thoracic aortic diameter.

in the development of thoracic aortic aneurysms (TAAs) and may potentially offer a protective effect.

Currently, the potential connection between fetuin-A and TAAs remains uncertain. We employed a two-sample Mendelian randomization study to explore whether a causal link exists between them, and to assess the causal association between fetuin-A and aortic diameter. The findings demonstrated a significant correlation between fetuin-A expression and a reduced risk of thoracic aortic aneurysms (OR=0.64, 95% CI: 0.47-0.87, P=0.00044). Additionally, gene-predicted fetuin-A was also linked to a reduction in the diameter of thoracic aortic aneurysms (β = -0.086, SE = 0.036, P = 0.017).

Previous studies have indicated that the size of thoracic aortic aneurysms is a critical predictor of rupture. Aneurysms with a diameter of 5 to 6 centimeters tend to grow more rapidly and are at a higher risk of rupturing compared to smaller aneurysms (20). In recent years, an increasing number of studies have demonstrated that aortic aneurysms often coincide with vascular calcification. The calcification of arteries reduces their elasticity, which worsens the occurrence and progression of aneurysms (21). As is widely known, the primary pathophysiological mechanisms of thoracic aortic aneurysms (TAAs) involve inflammation, apoptosis of smooth muscle cells, and the secretion of proteases, which result in the degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, are activated and increased in expression in thoracic aortic aneurysm (TAA) (22, 23). The action of MMPs, which damage ECM, can result in a weakened medial layer of the aorta, potentially leading to an aneurysm (24). Our findings demonstrate a significant association between the level of fetuin-A and a reduced risk of thoracic aortic aneurysm, along with a decrease in the diameter of the thoracic aortic aneurysms. Meanwhile, fetuin-A, acting as an inhibitor of ectopic calcification and proteases, is suggested to play a protective role in the development of arterial calcification, according to the relevant literature. Consequently, we propose that the mechanism by which fetuin-A protects against thoracic aortic aneurysms appears to involve the inhibition of arterial calcification, an influence on arterial diameter, and the suppression of MMPs.

Nonetheless, our study has several limitations that warrant consideration. Firstly, future updated Mendelian randomization analyses using summary statistics from larger genetic studies should be conducted to confirm the findings of our current MR study. A significant constraint in our study is the inability to directly assess the associations of individual genetic variants with potential confounding factors related to the link between fetuin-A levels and TAA. This limitation stems from our lack of knowledge regarding potential confounders and the unavailability of individual-level data. Notably, potential confounding variables, such as disease type, disease state, age, sex, and others, may introduce biases into the causal inferences drawn. A more comprehensive exploration of the functions of selected instrumental variables and the refinement of algorithms within the MR framework could potentially help mitigate the impact of confounding factors. Thirdly, the measured fetuin-A level at a specific timepoint may be influenced by transient factors, such as age and inflammation, which might not accurately represent the lifelong fetuin-A levels determined by the encoding gene. Fourth, it is worth noting that the GWAS data used primarily comprised samples of European ancestry, which may limit the generalizability of our findings to other racial or ethnic populations. Finally, although our study suggests a causal association between CSF fetuin-A levels and TAA, it's important to recognize that mendelian randomization analysis provides a predictive result that requires further validation. Therefore, the establishment of a causal relationship and the underlying pathological mechanisms must be explored. Although clinical trials have suggested the prioritization of fetuin-A detection in thoracic aortic aneurysms, further evidence-based confirmation is still required through large-scale clinical randomized controlled trials.

5 Conclusion

In conclusion, Mendelian randomization analysis has revealed a negative correlation between serum fetuin-A levels and the risk of TAA, accompanied by a lower in the diameter of TAA. The findings of our study provide persuasive evidence for the early detection and diagnosis of thoracic aortic aneurysms. Nevertheless, the underlying mechanism remains unclear at present and warrants further validation through animal experiments and large-scale clinical trials.

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 author.

Ethics statement

The present study exclusively utilized published or publicly accessible data. Ethical approval for each included study can be found in the primary publications, which also encompassed informed consent from all participants.

Author contributions

YC: Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. DP: Funding acquisition, Project administration, Supervision, Writing – review & editing. JZ: Investigation, Methodology, Validation, Writing – review & editing. XG: Investigation, Methodology, Validation, Writing – review & editing. CC: Investigation, Methodology, Validation, Writing – review & editing. XX: Investigation, Methodology, Validation, Writing – review & editing. BZ: Investigation, Methodology, Validation, Writing – review & editing. SW: Investigation, Methodology, Validation, Writing – review & editing. DH: Conceptualization, Investigation, Methodology, Software, Writing – review & editing. YL: Conceptualization, Investigation, Methodology, Software, Writing – review & editing. SW: Conceptualization, Investigation, Methodology, Software, Writing – review & editing. JH: Conceptualization, Investigation, Methodology, Software, Writing – review & editing. YY: Conceptualization, Investigation, Methodology, Software, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1361416/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The leave-one-out analysis plot and funnel plot of fetuin-A levels and the risk of thoracic aortic aneurysm. **(A)** Leave-one-out plot of fetuin-A and thoracic aortic aneurysm; **(B)** Leave-one-out plot of fetuin-A and thoracic aortic diameter; **(C)** Funnel plot of fetuin-A and thoracic aortic aneurysm; **(D)** Funnel plot of fetuin-A and thoracic aortic diameter.

References

1. Kuivaniemi H, Platsoucas CD, Tilson MD 3rd. Aortic aneurysms: an immune disease with a strong genetic component. *Circulation*. (2008) 117:242–52. doi: 10.1161/CIRCULATIONAHA.107.690982.

2. Osawa M, Umetsu K, Sato M, Ohki T, Yukawa N, Suzuki T, et al. Structure of the gene encoding human alpha 2-HS glycoprotein (AHSG). *Gene*. (1997) 196:121–5. doi: 10.1016/S0378-1119(97)00216-3.

3. Gerst F, Kemter E, Lorza-Gil E, Kaiser G, Fritz AK, Nano R, et al. The hepatokine fetuin-A disrupts functional maturation of pancreatic beta cells. *Diabetologia*. (2021) 64:1358–74. doi: 10.1007/s00125-021-05435-1.

4. Chekol Abebe E, Tilahun Muche Z, Behaile TMA, Mengie Ayele T, Mekonnen Agidew M, Teshome Azezew M, et al. The structure, biosynthesis, and biological roles of fetuin-A: A review. *Front Cell Dev Biol*. (2022) 10:945287. doi: 10.3389/fcell.2022.945287.

5. Ketteler M, Bongartz P, Westenfeld R, Wildberger JE, Mahnken AH, Böhm R, et al. Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. *Lancet*. (2003) 361:827–33. doi: 10.1016/S0140-6736(03)12710-9.

6. Weikert C, Stefan N, Schulze MB, Pischon T, Berger K, Joost HG, et al. Plasma fetuin-a levels and the risk of myocardial infarction and ischemic stroke. *Circulation*. (2008) 118:2555–62. doi: 10.1161/CIRCULATIONAHA.108.814418.

7. Szeberin Z, Fehervari M, Krepuska M, Apor A, Rimely E, Sarkadi H, et al. Fetuin-A serum levels in patients with aortic aneurysms of Marfan syndrome and atherosclerosis. *Eur J Clin Invest*. (2011) 41:176–82. doi: 10.1111/eci.2010.41.issue-2.

8. Kazamia R, Keravnou A, Moushi A, Sokratous K, Michailidou K, Yiangou K, et al. Tissue and plasma proteomic profiling indicates AHSG as a potential biomarker for ascending thoracic aortic aneurysms. *BMC Cardiovasc Disord*. (2023) 23:138. doi: 10.1186/s12872-023-03154-6.

9. Skrivankova VW, Richmond RC, Woolf BAR, Yarmolinsky J, Davies NM, Swanson SA, et al. Strengthening the reporting of observational studies in epidemiology using mendelian randomization: the STROBE-MR statement. *JAMA*. (2021) 326:1614–21. doi: 10.1001/jama.2021.18236.

10. Wu P, Wang Y, Ye Y, Yang X, Lu Q, Yuan J, et al. Serum fetuin-A and risk of gestational diabetes mellitus: an observational study and mendelian randomization analysis. *J Clin Endocrinol Metab*. (2022) 107:e3841–e9. doi: 10.1210/clinem/dgac335.

11. Pirruccello JP, Chaffin MD, Chou EL, et al. Deep learning enables genetic analysis of the human thoracic aorta. *Nat Genet*. (2022) 54:40–51. doi: 10.1038/s41588-021-00962-4.

12. Yavorska OO, Burgess S. MendelianRandomization: an R package for performing Mendelian randomization analyses using summarized data. *Int J Epidemiol*. (2017) 46:1734–9. doi: 10.1093/ije/dyx034.

13. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol*. (2015) 44:512–25. doi: 10.1093/ije/dyv080.

14. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet Epidemiol*. (2016) 40:304–14. doi: 10.1002/gepi.21965.

15. Verbanck M, Chen CY, Neale B, Do R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat Genet*. (2018) 50:693–8. doi: 10.1038/s41588-018-0099-7.

16. Staley JR, Blackshaw J, Kamat MA, Ellis S, Surendran P, Sun BB, et al. PhenoScanner: a database of human genotype-phenotype associations. *Bioinformatics*. (2016) 32:3207–9. doi: 10.1093/bioinformatics/btw373.

17. Quintana RA, Taylor WR. Cellular mechanisms of aortic aneurysm formation. *Circ Res*. (2019) 124:607–18. doi: 10.1161/CIRCRESAHA.118.313187.

18. Chowdhury MM, Zielinski LP, Sun JJ, Lambracos S, Boyle JR, Harrison SC, et al. Editor's choice - calcification of thoracic and abdominal aneurysms is associated with mortality and morbidity. *Eur J Vasc Endovasc Surg*. (2018) 55:101–8. doi: 10.1016/j.ejvs.2017.11.007.

19. Bartstra JW, Mali W, Spiering W, de Jong PA. Abdominal aortic calcification: from ancient friend to modern foe. *Eur J Prev Cardiol*. (2021) 28:1386–91. doi: 10.1177/2047487320919895.

20. Kouchoukos NT, Dougenis D. Surgery of the thoracic aorta. *N Engl J Med*. (1997) 336:1876–88. doi: 10.1056/NEJM199706263362606.

21. Rusanescu G, Weissleder R, Aikawa E. Notch signaling in cardiovascular disease and calcification. *Curr Cardiol Rev*. (2008) 4:148–56. doi: 10.2174/157340308785160552.

22. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest*. (2002) 110:625–32. doi: 10.1172/JCI0215334.

23. Airhart N, Brownstein BH, Cobb JP, Schierding W, Arif B, Ennis TL, et al. Smooth muscle cells from abdominal aortic aneurysms are unique and can independently and synergistically degrade insoluble elastin. *J Vasc Surg*. (2014) 60:1033–41; discussion 41-2. doi: 10.1016/j.jvs.2013.07.097.

24. Ailawadi G, Eliason JL, Upchurch GR Jr. Current concepts in the pathogenesis of abdominal aortic aneurysm. *J Vasc Surg*. (2003) 38:584–8. doi: 10.1016/S0741-5214(03)00324-0.

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Non-causal relationship of polycystic ovarian syndrome with homocysteine and B vitamins: evidence from a two-sample Mendelian randomization

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Objective: Previous observational studies have identified a correlation between elevated plasma homocysteine (Hcy) levels and polycystic ovary syndrome (PCOS). This study aimed to determine whether a causal relationship exists between Hcy and PCOS at the genetic level.

Methods: A two-sample Mendelian Randomization (TSMR) study was implemented to assess the genetic impact of plasma levels of Hcy, folate, vitamin B12, and vitamin B6 on PCOS in individuals of European ancestry. Independent single nucleotide polymorphisms (SNPs) associated with Hcy (n=12), folate (n=2), vitamin B12 (n=10), and vitamin B6 (n=1) at genome-wide significance levels ($P<5\times10^{-8}$) were selected as instrumental variables (IVs). Data concerning PCOS were obtained from the Apollo database. The primary method of causal estimation was inverse variance weighting (IVW), complemented by sensitivity analyses to validate the results.

Results: The study found no genetic evidence to suggest a causal association between plasma levels of Hcy, folate, vitamin B12, vitamin B6, and PCOS. The effect sizes, determined through random-effect IVW, were as follows: Hcy per standard deviation increase, OR = 1.117, 95%CI: (0.842, 1.483), P = 0.442; folate per standard deviation increase, OR = 1.008, CI: (0.546, 1.860), P = 0.981; vitamin B12 per standard deviation increase, OR = 0.978, CI: (0.808, 1.185), P = 0.823; and vitamin

B6 per standard deviation increase, OR = 0.967, CI: (0.925, 1.012), P = 0.145. The fixed-effect IVW results for each nutrient exposure and PCOS were consistent with the random-effect IVW findings, with additional sensitivity analyses reinforcing these outcomes.

Conclusion: Our findings indicate no causal link between Hcy, folate, vitamin B12, vitamin B6 levels, and PCOS.

KEYWORDS

polycystic ovary syndrome, homocysteine, B vitamins, Mendelian randomization, instrumental variables

1 Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine metabolic disorder characterized by hyperandrogenemia, oligo- or anovulation, and polycystic ovarian morphology. According to the Rotterdam criteria, approximately 8%-13% of women globally are diagnosed with PCOS (1, 2). Beyond infertility, individuals with PCOS frequently experience long-term health issues, including obesity, insulin resistance, cardiovascular diseases, and other metabolic dysfunctions (3, 4). The precise origins of PCOS remain elusive; however, pathophysiological research indicates that it is a heterogeneous condition influenced by genetic predispositions, environmental factors, and hereditary components (4, 5). Recent studies have highlighted a notably increased risk of cardiovascular conditions such as coronary heart disease and stroke among those with PCOS (6–8) and have observed alterations in vascular endothelia associated with the syndrome (9). Two extensive cohort studies in Denmark have established that the risk of cardiovascular disease in PCOS is elevated, independent of body mass index (BMI) (10, 11). Some researchers have proposed viewing the cardiovascular risks associated with PCOS and its sequelae as a "risk enhancer" (12). Nevertheless, findings from recent Mendelian randomization (MR) studies challenge earlier clinical observations (13) by refuting a direct causal link between PCOS and major cardiovascular events like coronary heart disease and stroke (14), indicating that comorbid conditions of PCOS may significantly contribute to its long-term adverse effects.

Homocysteine (Hcy), a sulfur-containing amino acid, is an intermediate product in the metabolic conversion of methionine. Hyperhomocysteinemia (HHcy, defined as a plasma Hcy level ≥15 mmol/L) can result from low dietary intake of folate or vitamin B12, or from mutations in the MTHFR and CBS genes (15). A recent meta-analysis illustrated that the pooled prevalence of HHcy among Chinese females is 28%, indicating an upward trend (16). Furthermore, studies have uncovered a causal link between reduced vitamin B12 levels and an increased risk of PCOS (17), as vitamin B12 deficiency contributes to elevated Hcy levels. Various investigations have confirmed the association between higher Hcy levels and PCOS, demonstrating significant increases in both circulating plasma and follicular fluid Hcy levels in PCOS patients (18, 19). Research involving PCOS patients who experienced recurrent pregnancy loss (RPL) has shown that high levels of Hcy in serum and follicular fluid induce apoptosis in granulosa cells and impair villous angiogenesis, which may lead to defects in embryo implantation and early miscarriage (20). However, folate supplementation has been shown to mitigate the effects of Hcy (21). Elevated Hcy levels in PCOS patients have also been linked to poor oocyte maturation, reduced fertilization rates, and decreased embryo quality, thereby adversely affecting fertility (22, 23). Moreover, high Hcy levels show linkage with obesity, insulin resistance, and elevated androgen levels (24), contrasting with previous meta-analysis results (25). High Hcy levels are also related to insulin resistance and exacerbate hyperandrogenism, a key feature of PCOS (25). After adjusting for age, BMI, insulin resistance, and other variables, multivariable logistic regression analysis revealed that serum Hcy significantly increases the risk of PCOS [OR=1.172, CI: (1.032, 1.330)] (26). Consequently, Saadeh N et al. have noted that serum Hcy strongly correlates with PCOS and serves as an effective predictor for diagnosing PCOS [AUC=0.855, CI: (0.811, 0.898)] (27).

The relationship between elevated Hcy levels and the incidence of cardiovascular disease (CVD) has been substantiated by previous research (28). Additionally, the interplay between HHcy and biochemical HHcy may exacerbate cardiovascular risks in women with PCOS (29). Insulin resistance and HHcy are prominent features of PCOS, with insulin resistance prompting compensatory HHcy. It has also been suggested that endogenous opiates may contribute to HHcy in PCOS patients (30). Despite adjustments for age, BMI, insulin resistance, and other factors, serum Hcy levels remain significantly higher in PCOS patients, potentially increasing the risk of developing PCOS (26). While elevated Hcy levels are implicated in linking PCOS to cardiovascular incidents, the direct association between PCOS

and cardiovascular outcomes continues to be a subject of debate, largely due to varying diagnostic approaches for PCOS and definitions of CVD (31). Thus, further research is imperative to elucidate the relationship between elevated Hcy levels and PCOS.

MR studies, through the natural grouping of instrumental variables (IVs), offer a robust method for addressing potential confounders and biases inherent in observational studies, establishing a reliable causal relationship between Hcy and PCOS at the genetic level, and also testing for reverse causality. This study aimed to determine whether there is a causal relationship between Hcy, folate, vitamin B12, and vitamin B6 and PCOS using MR analysis. This investigation is crucial for identifying potential causes of elevated Hcy levels in women with PCOS and the role of folate supplementation in managing elevated serum Hcy levels in this population.

2 Materials and methods

2.1 Data sources

Genome-wide association study (GWAS) data sources for Hcy, vitamin B6, vitamin B12, folate, and PCOS are readily accessible online (Figure 1). Single nucleotide polymorphisms (SNPs) associated with these variables were employed as IVs in this study. Ethical approval is not required for this study since it utilizes data collected from published studies and public databases. Detailed information regarding the ethical approval and informed consent for each subject can be found in the original publications where these data were first reported.

2.1.1 Exposed data sources

SNPs associated with serum Hcy concentrations were selected from the largest genome-wide association meta-analysis conducted to date (44,147 individuals of European ancestry) (32). SNPs related to folate and vitamin B12 were derived from previous GWAS studies, incorporating 37,465 and 45,576 individuals of European descent, respectively (33). SNPs associated with vitamin B6 were obtained from earlier GWAS research involving 1,864 individuals of European descent (34, 35).

2.1.2 Data source of outcome

SNPs associated with PCOS were extracted from the current largest GWAS, which included 10,074 cases and 103,164 controls from seven European cohorts involved in the 1000 Genomes Project or HapMap2 (36). The diagnostic criteria for PCOS were defined as follows: NIH criteria (2,540 cases/15,020 controls), Rotterdam criteria (2,669 cases/17,035 controls), and self-reported cases (5,284 cases/82,759 controls). SNPs related to insulin resistance and total testosterone levels were sourced from the IEU Open GWAS project. Additionally, SNPs related to obesity were obtained from FinnGen release 8 (http://r8.finngen.fi). The FinnGen project is a pioneering research initiative that combines genetic data with digital healthcare records from over 500,000 participants in Finnish biobanks (35).

2.2 Screening of IVs

Utilizing the 1000 European Genomic Reference Panels, we applied the PLINK clustering method to evaluate the linkage

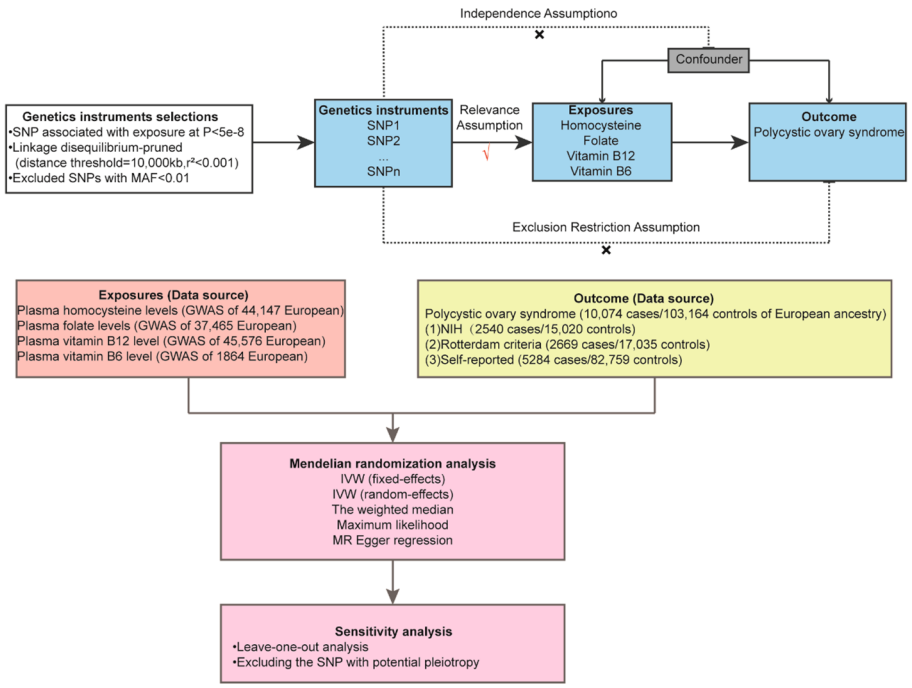

FIGURE 1
Overview Mendelian randomization analysis of plasma homocysteine, folate, vitamin B12, and vitamin B6 level with polycystic ovary syndrome.

disequilibrium of SNPs and selected independent SNPs with no linkage disequilibrium as IVs. The selection criteria for these SNPs were as follows: [1] They must satisfy the independence hypothesis, with an r^2<0.001, window size=10000kb and *P* value<5E-0^8; [2] A minor allele frequency (MAF) of ≥0.01; [3] The absence of correlation with potential confounding factors, as verified through the PhenoScanner database (http://www.phenoscanner.medschl.cam.ac.uk) (37); [4] In cases where specific PCOS GWAS data for an SNP are unavailable, no proxy SNP is sought; [5] SNP harmonization was carried out to correct allelic orientation, and palindromic SNPs were excluded; [6] The strength of selected IVs was calculated utilizing the F-statistic and R^2, where $R^2 = 2 \times EAF \times (1 - EAF) \times \beta^2/(2 \times EAF \times (1 - (EAF) \times \beta^{2+} 2 \times EAF \times (1 - EAF) \times N \times SE \times \beta^2]$ (EAF: effect allele frequency, β: beta, N: sample size, SE, standard error). The F-statistic was calculated as = $(N-2) \times R^2/(1-R^2)$ (N: sample size) (38). An F-statistic below 10 was considered indicative of a weak IV (39). The final selected SNPs are presented in Supplementary Table S3.

2.3 MR analysis

To investigate the causal relationship between Hcy levels and PCOS, we conducted a two-sample MR (TSMR) analysis. The foundational assumptions of MR include [1] a strong correlation between the IVs and the exposure, [2] no correlation between IVs and potential confounding variables, and [3] IVs influence the outcome exclusively through the exposure. The inverse variance weighting method (IVW) served as the primary analytical approach for MR analysis (40). Cochrane's Q value was employed to evaluate the heterogeneity of SNP estimates. In the absence of significant heterogeneity ($P < 0.05$), a fixed-effect model was utilized. If heterogeneity was detected, a random-effects model was adopted (41). Several important sensitivity analyses were then implemented. The weighted median method provided an estimate consistent with IVW when the effective IV proportion exceeded 50% (42). MR-Egger, which uses the P-value of its intercept to assess horizontal pleiotropy, often yields a broad confidence interval due to its limited statistical efficiency (43). Furthermore, MR-PRESSO, grounded in the InSIDE hypothesis, was used to detect and correct for bias potentially introduced by pleiotropic outliers through a global test and outlier removal (44). The robustness of the primary results was confirmed through leave-one-out analysis, systematically excluding one SNP at a time. To mitigate potential confounding effects related to PCOS, SNPs were screened for confounding factors employing the Phenoscanner V2 database. Power analyses were performed using the mRnd network calculation tool (https://shiny.cnsgenomics.com/mRnd/). Additional MR analyses were implemented to further substantiate the relationship between Hcy, B vitamins, and PCOS symptoms. All statistical analyses were executed utilizing R software (version 4.3.1) with the "TwoSampleMR" and "MR-PRESSO" packages. A *P*-value of <0.05 was deemed statistically significant.

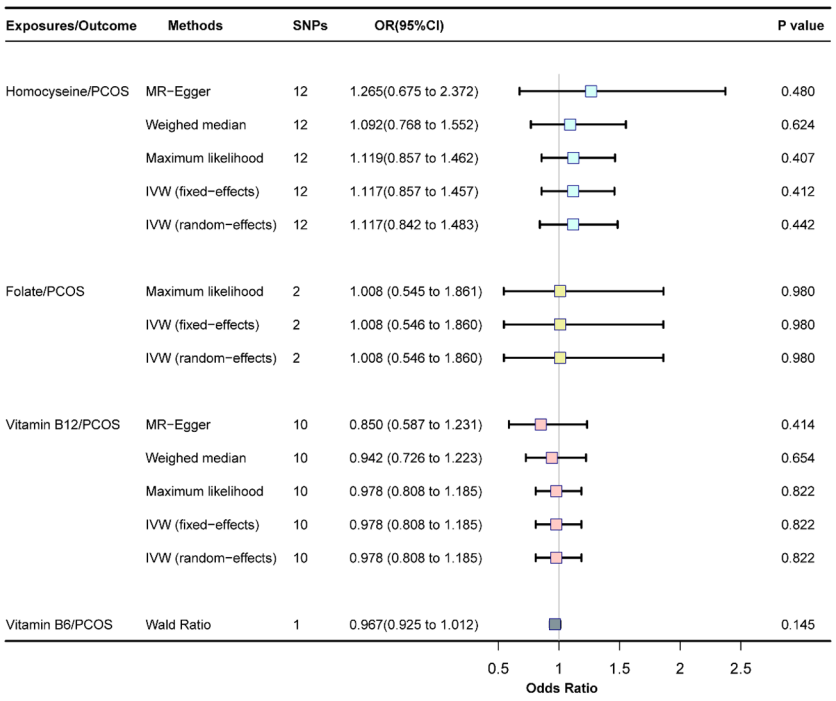

FIGURE 2
Association of homocysteine, folate, vitamin B12, and vitamin B6 with PCOS, SNP, single nucleotide polymorphism; OR, odds ratio; CI, confience interval; PCOS, polycystic ovary syndrome.

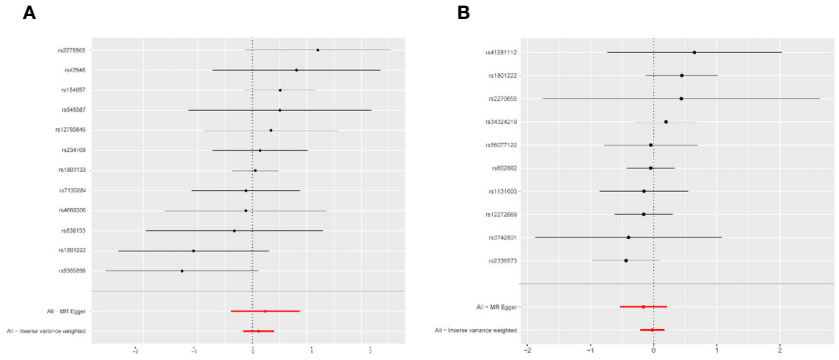

FIGURE 3
Forest plot of the potential effects of homocysteine associated SNPs and vitamin B12-associated SNPs on PCOS. **(A)** Homocysteine-associated SNPs on PCOS. **(B)** Vitamin B12 associated SNPs on PCOS. PCOS, polycystic ovary syndrome; MR, mendelian randomization; All-Inverse variance weighted, random effects inverse variance weighted analysis.

3 Results

3.1 Causal effects of Hcy on PCOS

Fixed-effects models analyzed through IVW revealed that genetically predicted Hcy levels were not significantly associated with PCOS [odds ratio (OR) = 1.117, CI: (0.857, 1.457), $P = 0.413$]. These results were consistent with those obtained from the random-effects model IVW analysis [OR = 1.117, CI (0.842, 1.483), $P = 0.442$]. Further analyses using MR-Egger, the weighted median method, and maximum likelihood estimation supported these findings (Figure 2). The potential causal effects of SNPs associated with Hcy on PCOS were further explored and are depicted in Figure 3. A funnel plot analysis indicated that the effects of Hcy were symmetrically distributed, suggesting the absence of directional pleiotropy (Supplementary Figure S1). Additionally, Cochran's Q test for heterogeneity was not significant (Q = 12.501, $P = 0.327$), indicating a lack of variance across studies. No evidence of horizontal pleiotropy was detected (P for intercept = 0.670), and MR-PRESSO analysis confirmed the absence of outliers (Global test P-value = 0.406) (Supplementary Table S5).

3.2 Causal effect of folate on PCOS

The fixed-effects model of IVW analysis revealed that genetically predicted folate levels were not significantly associated with PCOS [OR = 1.008, CI: (0.546, 1.860), P = 0.981]. This result was corroborated by the random-effects model IVW analysis, with consistent findings reported across all methods (Figure 2).

3.3 Causal effects of vitamin B12 on PCOS

The fixed-effects model of IVW analysis indicated that genetically predicted vitamin B12 levels were not associated with PCOS [OR = 0.978, CI:(0.808,1.185), $P = 0.823$]. These findings were consistent with the random-effects model IVW analysis. Further analyses using

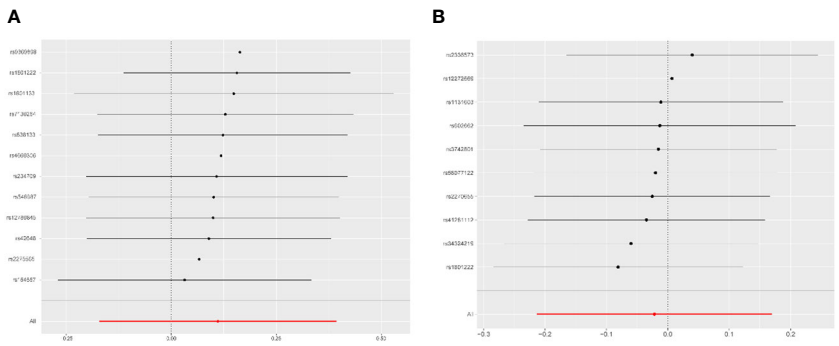

FIGURE 4
Leave-one-out analysis for the association of homocysteine and vitamin B12 with PCOS. **(A)** Homocysteine on PCOS. **(B)** Vitamin B12 on PCOS. PCOS, polycystic ovary syndromme.

MR-Egger, the weighted median, and maximum likelihood confirmed these results, as shown in Figure 2. The potential causal effect of SNPs associated with vitamin B12 on PCOS was assessed and detailed in Figure 3. The funnel plot analysis demonstrated symmetrically distributed effects of vitamin B12, with no evidence of directional pleiotropy (Supplementary Figure S1). Moreover, Cochran's Q test revealed no significant heterogeneity (Q = 7.470, $P = 0.588$), and there was no detection of horizontal pleiotropy (P for intercept = 0.410) or outliers in the MR-PRESSO analysis (global test $P = 0.583$) (Supplementary Table S5).

3.4 Causal effects of vitamin B6 on PCOS

The Wald Ratio analysis for genetically predicted vitamin B6 levels found no significant correlation with PCOS [OR=0.967, CI: (0.925,1.012), $P = 0.145$], as displayed in Figure 2. Due to the use of only one SNP in the IV set for vitamin B6, further pleiotropic tests and sensitivity analyses were not feasible.

3.5 Sensitivity analysis

Leave-one-out analysis demonstrated that the effects of Hcy and vitamin B12 on PCOS remained unchanged with the sequential exclusion of any single SNP (Figure 4). The power assessment results are detailed in Supplementary Table S4. Furthermore, a thorough review of each SNP's pleiotropy using the Phenoscanner V2 database revealed a previously identified Hcy SNP (rs548987) associated with BMI, which showed a significant association with PCOS (Supplementary Table S2). Reevaluation of the effect sizes, after excluding this SNP, yielded consistent results (Supplementary Figure S2). Additionally, we identified a causal association between vitamin B12 and obesity, evidenced by an OR of 0.938 (95% CI: 0.891–0.988, $P = 0.013$). No causal relationships were found between Hcy, B vitamins, and other metabolic traits such as insulin resistance, obesity, or total testosterone levels (Supplementary Figure S3). A reporting checklist for this TSMR study is provided in Supplementary Table S1.

4 Discussion

In this study, we investigated the potential causal associations between plasma levels of Hcy, folate, vitamin B12, and vitamin B6 and the risk of PCOS. Our findings indicated that there was no substantial evidence to suggest that genetically predicted levels of Hcy, folate, vitamin B12, and vitamin B6 were causal factors for PCOS.

Our investigation found no genetic causal connection between elevated genetically predicted plasma Hcy levels and the incidence of PCOS. This conclusion contrasts with several observational studies that have reported increased plasma Hcy levels in women with PCOS (18, 27, 45). Homocysteine thiolactone, an active metabolite of Hcy, has been demonstrated to disrupt tyrosine phosphorylation in the insulin receptor β-subunit and related substrates, hindering phosphatidylinositol 3-kinase activity and subsequently reducing insulin-mediated glycogen synthesis, a key factor in the development of insulin resistance (46). Experimental studies in a PCOS mouse model indicate that HHcy could intensify insulin resistance and inflammation in adipose tissue by altering macrophage M2 polarization via estrogen inhibition (19). Common metabolic disturbances associated with PCOS, such as insulin resistance and hyperinsulinemia, have also been associated with elevated Hcy levels, which are further linked to heightened risks of hyperinsulinemia and atherosclerosis (47). Biochemical hyperandrogenism, another hallmark of PCOS, significantly correlates with increased HHcy risks, showing an effect size of 2.24 (95% CI: 1.26–4.01) (29). Ting Li et al. found that androgens may escalate Hcy levels by inhibiting the mammalian target of rapamycin pathway in granulosa cells from PCOS-affected mice (48). It is hypothesized that the higher incidence of HHcy observed in the PCOS population might reflect an increased mutation rate of the MTHFR gene, particularly in Asian populations, where polymorphisms such as MTHFR rs1801131 and MTHFR rs1801133 could be contributing factors to elevated Hcy levels in PCOS (49). Despite these associations, our sensitivity analysis found no evidence of a causal link between Hcy levels and the risk of PCOS or its related symptoms, including insulin resistance, obesity, and total testosterone levels.

The relationship between vitamin B12 supplementation and the risk of PCOS remains a topic of debate. Vitamin B12 acts as a methyl donor in conjunction with folate in the methylation process. A deficiency in vitamin B12 can impede the Hcy remethylation pathway, leading to increased levels of circulating Hcy (50). Previous randomized controlled trials (RCTs) have demonstrated that supplementation with vitamin B12 and folate effectively reduces blood Hcy levels in PCOS patients (51). However, our results indicated no significant causal relationship between genetically predicted vitamin B12 levels and the risk of PCOS. Contrastingly, another recent MR study (17) reported findings that suggest genetically predicted vitamin B12 may reduce the risk of PCOS (IVW-MR: OR = 0.753, CI = [0.5688–0.998], $P = 0.048$) and obesity (IVW-MR: OR = 0.917, CI = [0.843–0.995], $P = 0.037$). These discrepancies between studies could be attributed to several factors: First, Shen JY et al. did not apply Bonferroni or False Discovery Rate (FDR) corrections for P values, which could increase the risk of false positives due to multiple testing. Second, the inconsistency in GWAS data sources for PCOS may also influence outcomes. Shen JY et al. utilized data from the FinnGen database, which included 642 cases and 118,228 controls, whereas our study used data from the largest PCOS GWAS to date, Apollo, with 10,074 cases and 103,164 controls. Despite these differences, our sensitivity analysis revealed that vitamin B12 deficiency was associated with an increased risk of obesity, aligning with previous findings (17).

Previous research has established that a deficiency of vitamin B6 in the general population leads to an elevated Hcy levels (52). Vitamin B6 is crucial for the catabolic pathway of Hcy, catalyzing the conversion to cysteine. However, in this study, only one effective IV for vitamin B6 was identified, which may limit the statistical power of our findings and affect the reliability of the observed negative causal relationship between vitamin B6 and PCOS (42).

folate supplementation is a recognized therapeutic strategy to reduce elevated HHcy levels. As a vital component in Hcy metabolism, a decrease in serum folate is one of the primary causes of HHcy. Recent meta-analyses and RCTs have shown that folate supplementation can improve insulin resistance and glucose metabolism in individuals (53). Specifically, two prior RCTs demonstrated that daily supplementation of 5 mg of folate for eight weeks significantly reduced Hcy levels, inflammatory markers, and HOMA-IR scores in PCOS patients (54, 55). The underlying mechanism is likely related to folate's role in enhancing the DNA methylation of genes involved in metabolic regulation (56), which helps to mitigate cellular and protein damage caused by oxidative stress and maintains endothelial function through single-carbon metabolism (57). Moreover, a recent systematic review involving eight RCTs highlighted that folate supplementation not only improves BMI in women with HHcy but also in women with PCOS (58); Folate supplementation could be particularly beneficial for obese PCOS patients with HHcy.

This study's primary strength lies in its utilization of the largest available GWAS data on Hcy-SNPs and PCOS-SNPs, employing a MR design. This approach enhances the causal inference of the relationship between Hcy, B vitamins, and PCOS by reducing residual confounding and other biases. However, the study is not without limitations. Firstly, the number of genome-wide association studies and single nucleotide polymorphisms available for analysis in PCOS is relatively limited. Azziz R has indicated that the identified loci might account for less than 20% of the heritability of PCOS, which raises concerns about the comprehensiveness of the results obtained from MR analysis (59). Secondly, the current GWAS data for PCOS do not include subtype classification based on the Rotterdam recommendations, which restricts our ability to discern potential associations between Hcy exposure and specific PCOS subtypes. Thirdly, this study is based primarily on data from European populations, limiting its applicability to other ethnic groups. Such geographical and genetic specificity might hinder the generalization of the findings to broader, more diverse populations. Fourthly, while we have elucidated the relationship between Hcy and PCOS from a genetic standpoint, it is important to recognize that PCOS is a complex endocrine and metabolic disorder influenced by genetic, metabolic, and environmental factors. Consequently, our results, focused solely on genetic contributions, may present certain limitations in fully capturing the multifaceted nature of PCOS.

5 Conclusions

The findings from our MR analysis currently provide no evidence to support a causal relationship between genetically predicted Hcy levels and PCOS. Additionally, supplementation with folate and vitamin B12 does not appear to reduce the risk of PCOS. Given these results, further research is essential to explore the impact of Hcy on various subtypes of PCOS, as defined by the Rotterdam criteria. Such investigations are critical for enhancing our understanding of PCOS and could potentially lead to more effective treatment strategies for PCOS patients exhibiting clinical HHcy.

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

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

NS: Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. JL: Writing – review & editing, Writing – original draft, Formal Analysis, Conceptualization. YX: Visualization, Writing – original draft. CH: Writing – review & editing. LC: Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1393847/full#supplementary-material

References

1. Bozdag G, Mumusoglu S, Zengin D, Karabulut E, Yildiz BO. The prevalence and phenotypic features of polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod*. (2016) 31:2841–55. doi: 10.1093/humrep/dew218

2. The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod*. (2004) 19:41–7. doi: 10.1093/humrep/deh098

3. Anagnostis P, Tarlatzis BC, Kauffman RP. Polycystic ovarian syndrome (PCOS): Long-term metabolic consequences. *Metabolism*. (2018) 86:33–43. doi: 10.1016/j.metabol.2017.09.016

4. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. *Nat Rev Endocrinol*. (2018) 14:270–84. doi: 10.1038/nrendo.2018.24

5. Spritzer PM, Lecke SB, Satler F, Morsch DM. Adipose tissue dysfunction, adipokines, and low-grade chronic inflammation in polycystic ovary syndrome. *Reproduction*. (2015) 149:R219–27. doi: 10.1530/REP-14–0435

6. Ollila MM, Arffman RK, Korhonen E, Morin-Papunen L, Franks S, Junttila J, et al. Women with PCOS have an increased risk for cardiovascular disease regardless of diagnostic criteria-a prospective population-based cohort study. *Eur J Endocrinol*. (2023) 189:96–105. doi: 10.1093/ejendo/lvad077

7. Okoth K, Chandan JS, Marshall T, Thangaratinam S, Thomas GN, Nirantharakumar K, et al. Association between the reproductive health of young women and cardiovascular disease in later life: umbrella review. *BMJ*. (2020) 371: m3502. doi: 10.1136/bmj.m3502

8. Berni TR, Morgan CL, Rees DA. Women with polycystic ovary syndrome have an increased risk of major cardiovascular events: a population study. *J Clin Endocrinol Metab*. (2021) 106:e3369–80. doi: 10.1210/clinem/dgab392

9. Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. *Hum Reprod Update*. (2007) 13:225–38. doi: 10.1093/humupd/dml063

10. Glintborg D, Rubin KH, Nybo M, Abrahamsen B, Andersen M. Cardiovascular disease in a nationwide population of Danish women with polycystic ovary syndrome. *Cardiovasc Diabetol*. (2018) 17:37. doi: 10.1186/s12933–018-0680–5

11. Oliver-Williams C, Vassard D, Pinborg A, Schmidt L. Risk of cardiovascular disease for women with polycystic ovary syndrome: results from a national Danish registry cohort study. *Eur J Prev Cardiol*. (2021) 28:e39–41. doi: 10.1177/2047487320939674

12. Daan NM, Louwers YV, Koster MP, Eijkemans MJ, de Rijke YB, Lentjes EW, et al. Cardiovascular and metabolic profiles amongst different polycystic ovary syndrome phenotypes: who is really at risk? *Fertil Steril*. (2014) 102:1444–51. doi: 10.1016/j.fertnstert.2014.08.001

13. Zhu T, Goodarzi MO. Causes and consequences of polycystic ovary syndrome: insights from mendelian randomization. *J Clin Endocrinol Metab*. (2022) 107:e899–911. doi: 10.1210/clinem/dgab757

14. Zhu T, Cui J, Goodarzi MO. Polycystic ovary syndrome and risk of type 2 diabetes, coronary heart disease, and stroke. *Diabetes*. (2021) 70:627–37. doi: 10.2337/db20–0800

15. Jakubowski H, Glowacki R. Chemical biology of homocysteine thiolactone and related metabolites. *Adv Clin Chem*. (2011) 55:81–103. doi: 10.1016/b978–0-12–387042–1.00005–8

16. Zeng Y, Li FF, Yuan SQ, Tang HK, Zhou JH, He QY, et al. Prevalence of hyperhomocysteinemia in China: an updated meta-analysis. *Biol (Basel)*. (2021) 10:959. doi: 10.3390/biology10100959

17. Shen JY, Xu L, Ding Y, Wu XY. Effect of vitamin supplementation on polycystic ovary syndrome and key pathways implicated in its development: A Mendelian randomization study. *World J Clin cases*. (2023) 11:5468–78. doi: 10.12998/wjcc.v11.i23.5468

18. Eskandari Z, Sadrkhanlou RA, Nejati V, Tizro G. PCOS women show significantly higher homocysteine level, independent to glucose and E2 level. *Int J Reprod BioMed*. (2016) 14:495–500. doi: 10.29252/ijrm.14.8.495

19. Qi X, Zhang B, Zhao Y, Li R, Chang HM, Pang Y, et al. Hyperhomocysteinemia promotes insulin resistance and adipose tissue inflammation in PCOS mice through modulating M2 macrophage polarization via estrogen suppression. *Endocrinology*. (2017) 158:1181–93. doi: 10.1210/en.2017–00039

20. Nelen WL, Bulten J, Steegers EA, Blom HJ, Hanselaar AG, Eskes TK. Maternal homocysteine and chorionic vascularization in recurrent early pregnancy loss. *Hum Reprod*. (2000) 15:954–60. doi: 10.1093/humrep/15.4.954

21. Di Simone N, Riccardi P, Maggiano N, Piacentani A, D'Asta M, Capelli A, et al. Effect of folic acid on homocysteine-induced trophoblast apoptosis. *Mol Hum Reprod*. (2004) 10:665–69. doi: 10.1093/molehr/gah091

22. Nafiye Y, Sevtap K, Muammer D, Emre O, Senol K, Leyla M. The effect of serum and intrafollicular insulin resistance parameters and homocysteine levels of nonobese, nonhyperandrogenemic polycystic ovary syndrome patients on in *vitro* fertilization outcome. *Fertil Steril*. (2010) 93:1864–69. doi: 10.1016/j.fertnstert.2008.12.024

23. Berker B, Kaya C, Aytac R, Satiroglu H. Homocysteine concentrations in follicular fluid are associated with poor oocyte and embryo qualities in polycystic ovary syndrome patients undergoing assisted reproduction. *Hum Reprod*. (2009) 24:2293–302. doi: 10.1093/humrep/dep069

24. Asanidze E, Kristesashvili J, Andguladze S. Correlation between levels of homocysteine, anti-mullerian hormone and insulin resistance in pcos patients with recurrent miscarriage. *Georgian Med News*. (2019), 25–9.

25. Meng Y, Chen X, Peng Z, Liu X, Sun Y, Dai S. Association between high serum homocysteine levels and biochemical characteristics in women with polycystic ovarian syndrome: A systematic review and meta-analysis. *PloS One*. (2016) 11:e0157389. doi: 10.1371/journal.pone.0157389

26. Bhushan R, Sinha P. Correlation of serum homocysteine levels and hyperinsulinaemia with body mass index in polycystic ovarian syndrome. *J Hum Reprod Sci*. (2022) 15:34–41. doi: 10.4103/jhrs.jhrs_147_21

27. Saadeh N, Alfaqih MA, Mansour H, Khader YS, Saadeh R, Al-Dwairi A, et al. Serum homocysteine is associated with polycystic ovarian syndrome in Jordan. *BioMed Rep*. (2018) 9:439–45. doi: 10.3892/br.2018.1149

28. Ji Y, Tan S, Xu Y, Chandra A, Shi C, Song B, et al. Vitamin B supplementation, homocysteine levels, and the risk of cerebrovascular disease: a meta-analysis. *Neurology*. (2013) 81:1298–307. doi: 10.1212/WNL.0b013e3182a823cc

29. Lin YH, Huang SY, Hsu MI, Chang YC, Cheng CY, Hsu CS, et al. Hyperhomocysteinaemia is associated with biochemical hyperandrogenaemia in women with reproductive age. *Eur J Obstet Gynecol Reprod Biol*. (2013) 171:314–18. doi: 10.1016/j.ejogrb.2013.09.041

30. Lanzone A, Fulghesu AM, Fortini A, Cutillo G, Cucinelli F, Di Simone N, et al. Effect of opiate receptor blockade on the insulin response to oral glucose load in polycystic ovarian disease. *Hum Reprod*. (1991) 6:1043–49. doi: 10.1093/oxfordjournals.humrep.a137482

31. Huddleston HG, Quinn MM, Kao CN, Lenhart N, Rosen MP, Cedars MI. Women with polycystic ovary syndrome demonstrate worsening markers of cardiovascular risk over the short-term despite declining hyperandrogenaemia: Results of a longitudinal study with community controls. *Clin Endocrinol (Oxf)*. (2017) 87:775–82. doi: 10.1111/cen.13497

32. van Meurs JB, Pare G, Schwartz SM, Hazra A, Tanaka T, Vermeulen SH, et al. Common genetic loci influencing plasma homocysteine concentrations and their effect on risk of coronary artery disease. *Am J Clin Nutr*. (2013) 98:668–76. doi: 10.3945/ajcn.112.044545

33. Grarup N, Sulem P, Sandholt CH, Thorleifsson G, Ahluwalia TS, Steinthorsdottir V, et al. Genetic architecture of vitamin B12 and folate levels

uncovered applying deeply sequenced large datasets. *PloS Genet*. (2013) 9:e1003530. doi: 10.1371/journal.pgen.1003530

34. Tanaka T, Scheet P, Giusti B, Bandinelli S, Piras MG, Usala G, et al. Genome-wide association study of vitamin B6, vitamin B12, folate, and homocysteine blood concentrations. *Am J Hum Genet*. (2009) 84:477–82. doi: 10.1016/j.ajhg.2009.02.011

35. Locke AE, Steinberg KM, Chiang C, Service SK, Havulinna AS, Stell L, et al. Exome sequencing of Finnish isolates enhances rare-variant association power. *Nature*. (2019) 572:323–28. doi: 10.1038/s41586–019-1457-z

36. Day F, Karaderi T, Jones MR, Meun C, He C, Drong A, et al. Large-scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria. *PloS Genet*. (2018) 14:e1007813. doi: 10.1371/journal.pgen.1007813

37. Kamat MA, Blackshaw JA, Young R, Surendran P, Burgess S, Danesh J, et al. PhenoScanner V2: an expanded tool for searching human genotype-phenotype associations. *Bioinformatics*. (2019) 35:4851–53. doi: 10.1093/bioinformatics/btz469

38. Papadimitriou N, Dimou N, Tsilidis KK, Banbury B, Martin RM, Lewis SJ, et al. Physical activity and risks of breast and colorectal cancer: a Mendelian randomisation analysis. *Nat Commun*. (2020) 11:597. doi: 10.1038/s41467–020-14389–8

39. Palmer TM, Lawlor DA, Harbord RM, Sheehan NA, Tobias JH, Timpson NJ, et al. Using multiple genetic variants as instrumental variables for modifiable risk factors. *Stat Methods Med Res*. (2012) 21:223–42. doi: 10.1177/0962280210394459

40. Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. *Genet Epidemiol*. (2013) 37:658–65. doi: 10.1002/gepi.21758

41. Hemani G, Bowden J, Davey SG. Evaluating the potential role of pleiotropy in Mendelian randomization studies. *Hum Mol Genet*. (2018) 27:R195–208. doi: 10.1093/hmg/ddy163

42. Bowden J, Davey SG, Haycock PC, Burgess S. Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet Epidemiol*. (2016) 40:304–14. doi: 10.1002/gepi.21965

43. Bowden J, Davey SG, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol*. (2015) 44:512–25. doi: 10.1093/ije/dyv080

44. Verbanck M, Chen CY, Neale B, Do R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat Genet*. (2018) 50:693–98. doi: 10.1038/s41588–018-0099–7

45. Gozukucuk M, Gursoy AY, Destegul E, Taskin S, Satiroglu H. Homocysteine and C-reactive protein levels in women with polycystic ovary syndrome. *Gynecol Minim Invasive Ther*. (2021) 10:210–14. doi: 10.4103/GMIT.GMIT_30_20

46. Najib S, Sanchez-Margalet V. Homocysteine thiolactone inhibits insulin signaling, and glutathione has a protective effect. *J Mol Endocrinol*. (2001) 27:85–91. doi: 10.1677/jme.0.0270085

47. Grodnitskaya EE, Kurtser MA. Homocysteine metabolism in polycystic ovary syndrome. *Gynecol Endocrinol*. (2012) 28:186–89. doi: 10.3109/09513590.2011.589927

48. Li T, Dong G, Kang Y, Zhang M, Sheng X, Wang Z, et al. Increased homocysteine regulated by androgen activates autophagy by suppressing the mammalian target of rapamycin pathway in the granulosa cells of polycystic ovary syndrome mice. *Bioengineered*. (2022) 13:10875–88. doi: 10.1080/21655979.2022.2066608

49. Wang X, Wang K, Yan J, Wu M. A meta-analysis on associations of FTO, MTHFR and TCF7L2 polymorphisms with polycystic ovary syndrome. *Genomics*. (2020) 112:1516–21. doi: 10.1016/j.ygeno.2019.08.023

50. Sukumar N, Saravanan P. Investigating vitamin B12 deficiency. *BMJ*. (2019) 365:l1865. doi: 10.1136/bmj.l1865

51. Kilicdag EB, Bagis T, Tarim E, Aslan E, Erkanli S, Simsek E, et al. Administration of B-group vitamins reduces circulating homocysteine in polycystic ovarian syndrome patients treated with metformin: a randomized trial. *Hum Reprod*. (2005) 20:1521–28. doi: 10.1093/humrep/deh825

52. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *Jama*. (1993) 270:2693–98. doi: 10.1001/jama.1993.03510220049033

53. Asbaghi O, Ashtary-Larky D, Bagheri R, Moosavian SP, Olyaei HP, Nazarian B, et al. Folic acid supplementation improves glycemic control for diabetes prevention and management: A systematic review and dose-response meta-analysis of randomized controlled trials. *Nutrients*. (2021) 13:2355. doi: 10.3390/nu13072355

54. Bahmani F, Karamali M, Shakeri H, Asemi Z. The effects of folate supplementation on inflammatory factors and biomarkers of oxidative stress in overweight and obese women with polycystic ovary syndrome: a randomized, double-blind, placebo-controlled clinical trial. *Clin Endocrinol (Oxf)*. (2014) 81:582–87. doi: 10.1111/cen.12451

55. Asemi Z, Karamali M, Esmaillzadeh A. Metabolic response to folate supplementation in overweight women with polycystic ovary syndrome: a randomized double-blind placebo-controlled clinical trial. *Mol Nutr Food Res*. (2014) 58:1465–73. doi: 10.1002/mnfr.201400033

56. Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. *Cell Metab*. (2017) 25:27–42. doi: 10.1016/j.cmet.2016.08.009

57. Friso S, Udali S, De Santis D, Choi SW. One-carbon metabolism and epigenetics. *Mol Aspects Med*. (2017) 54:28–36. doi: 10.1016/j.mam.2016.11.007

58. Jafari A, Gholizadeh E, Sadrmanesh O, Tajpour S, Yarizadeh H, Zamani B, et al. The effect of folic acid supplementation on body weight and body mass index: A systematic review and meta-analysis of randomized controlled trials. *Clin Nutr Espen*. (2023) 53:206–13. doi: 10.1016/j.clnesp.2022.11.020

59. Azziz R. PCOS and mendelian randomization: too soon? *J Clin Endocrinol Metab*. (2022) 107:e2195–96. doi: 10.1210/clinem/dgab827

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Common ground on immune infiltration landscape and diagnostic biomarkers in diabetes-complicated atherosclerosis: an integrated bioinformatics analysis

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Introduction: Type 2 diabetes mellitus (T2DM) is a major cause of atherosclerosis (AS). However, definitive evidence regarding the common molecular mechanisms underlying these two diseases are lacking. This study aimed to investigate the mechanisms underlying the association between T2DM and AS.

Methods: The gene expression profiles of T2DM (GSE159984) and AS (GSE100927) were obtained from the Gene Expression Omnibus, after which overlapping differentially expressed gene identification, bioinformatics enrichment analyses, protein–protein interaction network construction, and core genes identification were performed. We confirmed the discriminatory capacity of core genes using receiver operating curve analysis. We further identified transcription factors using TRRUST database to build a transcription factor–mRNA regulatory network. Finally, the immune infiltration and the correlation between core genes and differential infiltrating immune cells were analyzed.

Results: A total of 27 overlapping differentially expressed genes were identified under the two-stress conditions. Functional analyses revealed that immune responses and transcriptional regulation may be involved in the potential pathogenesis. After protein–protein interaction network deconstruction, external datasets, and qRT-PCR experimental validation, four core genes (IL1B, C1QA, CCR5, and MSR1) were identified. ROC analysis further showed the reliable value of these core genes. Four common differential infiltrating immune cells (B cells,

Abbreviations: T2DM, type 2 diabetes mellitus; AS, atherosclerosis; DEGS, differentially expressed genes; PPI, protein–protein interaction; ROC, receiver operating curve; Tregs, regulatory T cells; TF, transcription factors; TRRUST, transcriptional regulatory relationship unraveled by sentence-based text mining; IICs, infiltrating immune cells; AUC, area under the ROC curve; LAPTM5, lysosomal-associated protein transmembrane 5; Ig, immunoglobulin; MSRI, macrophage scavenger receptor 1; SPI1, SPI-1 proto-oncogene.

CD4+ T cells, regulatory T cells, and M2 macrophages) between T2DM and AS datasets were selected based on immune cell infiltration. A significant correlation between core genes and common differential immune cells. Additionally, five transcription factors (RELA, NFκB1, JUN, YY1, and SPI1) regulating the transcription of core genes were mined using upstream gene regulator analysis.

Discussion: In this study, common target genes and co-immune infiltration landscapes were identified between T2DM and AS. The relationship among five transcription factors, four core genes, and four immune cells profiles may be crucial to understanding T2DM complicated with AS pathogenesis and therapeutic direction.

KEYWORDS

atherosclerosis, type 2 diabetes mellitus, bioinformatics, immune infiltration, molecular mechanisms

1 Introduction

Atherosclerosis (AS) is a fatal complication of diabetes mellitus and the leading cause of death for patients worldwide (1). In 2021, there were approximately 537 million people with diabetes worldwide, and patients with type 2 diabetes mellitus (T2DM) represent over 90% of this population. Recent data have suggested that patients with T2DM have advanced coronary plaques with larger necrotic core areas and higher arterial media calcification (2). Severe and extensive AS develops almost two decades earlier than people without T2DM (3). Therefore, to reduce cardiovascular events, improving the diagnosis and treatment of high-risk plaques in susceptible populations is essential (4).

T2DM and AS are chronic inflammatory diseases primarily caused by metabolic disorders. Hyperinsulinemia increases the circulating fat levels of pro-inflammatory and pro-atherogenic factors (5). Similarly, glucose overload induces oxidative stress and activates pro-inflammatory signaling pathways (6). In addition, metabolic disorders are associated with an altered immune response. Autoimmunity is crucial in the coronary artery formation process, such as fat streak formation, plaque calcification, plaque rupture, and thrombosis.

Although these factors contribute to the modification of microvascular and macrovascular structures and plaque formation (7), the systematic pathological mechanism of T2DM complicated with AS in a genetic and cellular level is still unclear. Therefore, it restricts the research and development of targeted drugs. Bioinformatics analysis based on high-throughput data and gene microarray technology have provided new strategies for discovering therapeutic targets in recent years. We obtain high-throughput data and microarray datasets from GEO to investigate overlapping differentially expressed gene (DEGs) between T2DM and AS. Then, the network deconstruction method was used to dimension reduction to obtain the core genes. The biological function of the core genes were determined by enrichment analysis, and the correlation between core genes and the differential infiltrated immune cells (IICs), which was screened by immune infiltration analysis, was confirmed by Spearman test. To sum up, IL1B, C1QA, CCR5, and MSR1 were identified as core genes that might serve as biomarkers for T2DM complicated with AS. They were very informative for diagnosis and may become new therapeutic targets for therapy.

2 Materials and methods

2.1 Data collection

We searched for related gene expression or high-throughput sequencing datasets using 1) "atherosclerotic" and "type 2 diabetes" as keywords, 2) the test specimens in datasets derived from human tissues, and 3) the largest possible sample size. Finally, two high-throughput sequencing datasets (GSE159984 and GSE164416) and two microarray datasets (GSE100927 and GSE28829) were obtained from the National Center for Biotechnological Information (NCBI) Gene Expression Omnibus https://www.ncbi.nlm.nih.gov/geo/) database. GSE159984 (including 28 patients with T2DM and 58 controls) and GSE100927 (comprising 29 patients with AS and 12 controls) were used to screen for DEGs, while GSE164416 (including 39 patients with T2DM and 18 controls) and GSE28829 (comprising 16 patients with advanced carotid plaque and 13 with early carotid plaque) were used as external validation datasets. Table 1 summarizes the information for the datasets selected.

2.2 Overlapping DEG identfication and enrichment analyses

DEG analysis was filtered using the "edgeR" or "limma" package (8, 9) in R (version 4.3.1), and the results were visualized using the "ggplot2" package. We obtained DEGs with |log2fold change≥1 and

TABLE 1 The information of GEO datasets.

GEO dataset	Type	Platform	Disease samples	Sample type in patients	Control samples	Sample type in controls
GSE159984	high throughput sequencing	GPL16791	28	human islets from type 2 diabetic donor	58	human islets from non-diabetic donor
GSE100927	array	GPL17077	29	human atherosclerotic carotid artery from donor	12	human carotid artery arteries without atherosclerotic lesions from control donor
GSE164416	high throughput sequencing	GPL16791	39	human islets from type 2 diabetic donor	18	human islets from non-diabetic donor
GSE28829	array	GPL570	16	advanced atherosclerotic plaque	13	early atherosclerotic plaque

p.adj<0.05 in T2DM and AS diseases, respectively. Overlapping DEGs in the same direction between T2DM and AS were identified using the online Venn diagram tool (https://bioinfogp.cnb.csic.es/tools/venny/). KEGG and GO enrichment analyses of the overlapping DEGs were performed using the "clusterProfiler" package (10).

2.3 Protein–protein interaction network construction and hub gene screening

The overlapping genes were imported into the STRING database (http://string-db.org) (11) to construct a protein–protein interaction (PPI) network with complex relationships (interactions combined score >0.4), and this network was visualized in Cytoscape 3.82 (version 3.8.1). Four algorithms (MCC, MNC, Degree, and Closeness) take intersection to identify hub genes using the cytoHubba plug-in (12). Enrichment analysis and co-expression networks of hub genes were performed using "clusterProfiler" package and GeneMANIA (http://www.genemania.org/) (13), respectively. The molecular complex detection technology (MCODE), a plugin in Cytoscape, was used to deconstruct the functional modules. The selection criteria were set as degree cutoff=2, K-core=2, node score cutoff=0.2, and maximum depth=100.

2.4 External database verification and qRT-PCR experiments analysis

The GSE164416 and GSE28829 datasets were used for external verification. Significance was calculated using the Student's t-test, and $p < 0.05$ was considered statistically significant. Then, statistically significant genes were selected for further quantitative real-time PCR (qRT-PCR) analysis in animal models. Male C57BL/6J and ApoE$^{-/-}$ mice were purchased from Weitonglihua Corporation (Beijing, China). To induce diabetic atherosclerosis models, ApoE$^{-/-}$ mice were given streptozocin (50 mg/kg/day) by intraperitoneal injection for 5 days consecutively and fed on high-fat diet for 16 weeks (14). C57BL/6J mice were fed on normal-chow diet for 16 weeks after injected with vehicle used in the control group, pool of two groups, five mice per group. The total RNA of thoracic aortas was isolated with RNA extraction reagent (G3013, Servicebio, China) and reverse transcribed with SweScript All-in-one RT SuperMix (G3337, Servicebio, China). Reactions were run using CFX Connect (Bio-Rad) with 2× Universal Blue SYBR Green qPCR Master Mix (G3326, Servicebio, China). GAPDH was deemed as an internal control, and the results were determined with the $2^{-\Delta\Delta Ct}$ method.

Similarly, the level of significance used was 0.05.

Primers sequences are listed in Supplementary Table S1. All animal care and experimental procedures were approved by the animal ethics committee of the Ethics Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences.

2.5 Core gene identification and diagnosing

Based on the results of qRT-PCR analysis, genes with significant statistical differences ($p < 0.05$) were regarded as core genes. Thus, core genes were identified successfully through multiple bioinformatics mining, external datasets validation, and qRT-PCR experimental verification. To determine the value of each core genes and multiple genes in diagnosis of T2DM complicated with AS, we executed receiver operating characteristic (ROC) curve analysis, respectively. The diagnostic capacity of core genes was quantified using the area under ROC curve (AUC) in GSE164416 and GSE28829 datasets. The "pROC" R package was used to generate ROC curves (15). The greater the AUC value, the more superior the discriminatory ability of the model. An AUC closer to 1 indicates better prediction, and an AUC>0.7 indicates good diagnostic efficacy.

2.6 Transcription factor prediction

Transcriptional regulatory relationships unraveled by sentence-based text mining (TRRUST) were used to obtain candidate transcription factors (TFs) that regulate core genes (16). This database contains abundant information about TFs associated with target genes and their regulatory relationships with TFs. Statistical significance was defined as an adjusted $p<0.05$. We constructed a TF mRNA regulatory network and visualized it using Cytoscape.

2.7 Immune cell infiltration calculation and correlation analysis

We performed immune infiltration analysis to reveal the underlying immune pathogenesis of T2DM complicated with AS. QuanTIseq is a validated deconvolution-based algorithm that estimates the absolute proportions of relevant immune cell types. Thus, we used quanTIseq algorithm to obtain the immune cell infiltration differences between normal group and disease group. Based on "IOBR" packages, relative percentage, different immune cell types were analyzed using GSE164416 and GSE100927 datasets. The results were output as bar graphs and violon plots, respectively. Subsequently, the correlations between core genes and IICs were conducted using Spearman analysis, and the results were visualized using "ggplot2" package.

3 Results

3.1 Identification and functional analysis of overlapping DEGs

The study's flow chart is shown in Figure 1. In total, 116 and 803 DEGs were obtained from the GSE159984 and GSE100927 datasets, respectively (Figures 2A, B). After taking the intersection between the two datasets, 27 overlapping DEGs with the same expression trends (26 upregulated and 1 downregulated) were identified from the two datasets (Figures 2C, D). The list of the differential gene expression is included as Supplementary Data. The DEGs lists have been included as Supplementary Tables 2–4.

To explore the potential biological function, GO enrichment and KEGG pathway for the overlapping DEGs were performed using R. According to the GO analysis findings, positive regulation of cytokine production, activation of immune response, and negative regulation of leukocyte activation were significantly enriched in the biological process (BP) entries, specific granule, collagen trimer, and specific granule membrane were significantly enriched in the cellular component (CC) entries, and oxidoreductase activity, growth factor receptor binding, and phosphotyrosine residue binding were significantly enriched in the molecular function (MF) entries (Figure 3A; Supplementary Table S5). In addition, complement and coagulation cascades, chemokine signaling pathway, and IL-17 signaling pathway were significantly enriched in the KEGG entries (Figure 3B; Supplementary Table S6). These results indicate that immune-related processes and chemokines may be crucial in the development of T2DM complicated with AS.

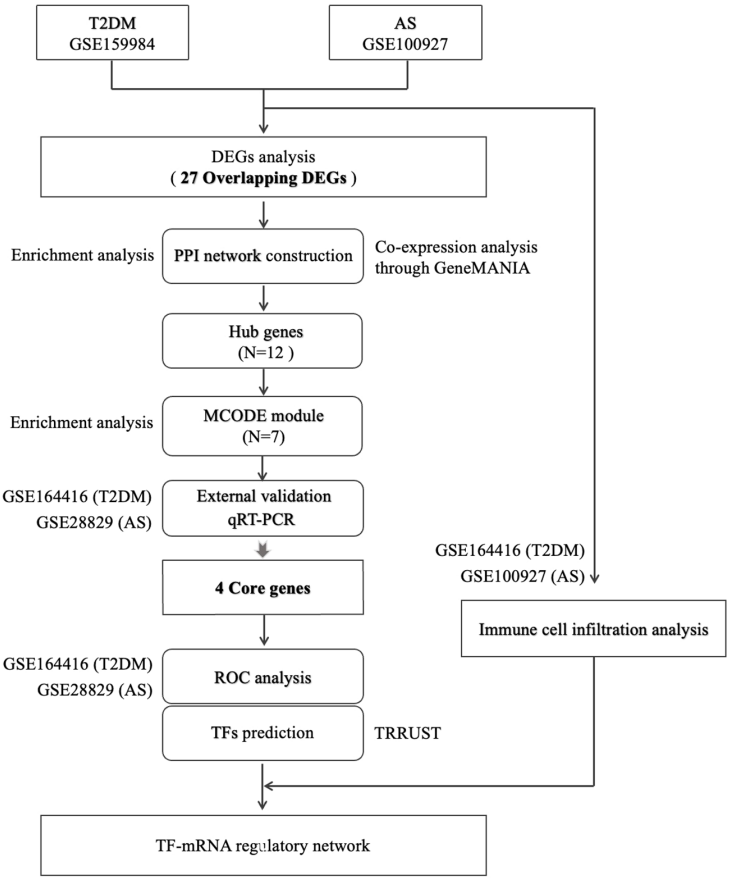

FIGURE 1
Flow diagram for this study.

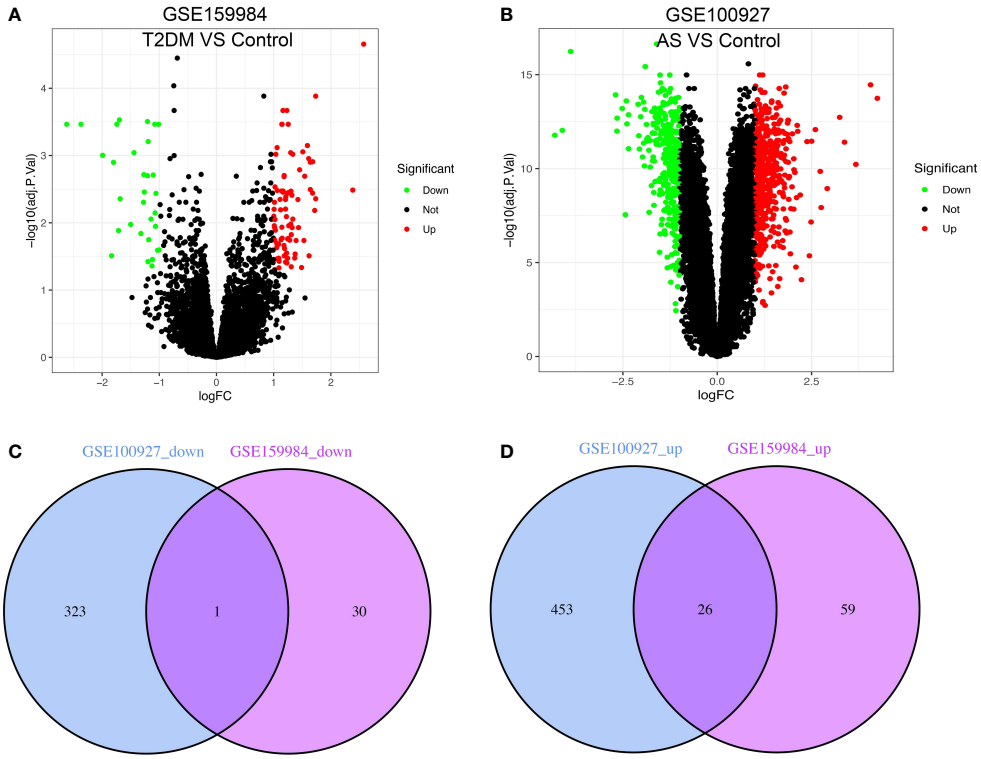

FIGURE 2
Venn diagram shows the intersection of differentially expressed genes (DEGs) between type 2 diabetes mellitus (T2DM) and atherosclerosis (AS). The volcano plot of the DEGs in GSE159984 and GSE100927 is shown in panels **(A, B)**. Red indicates gene upregulation, green indicates gene downregulation, and gray indicates that the genes had no significant changes. **(C)** Venn diagram shows the downregulated genes in GSE159984 and GSE100927. **(D)** Venn diagram shows the upregulated genes in GSE159984 and GSE100927.

3.2 PPI network construction and hub genes selection

The PPI network of overlapping DEGs contained 26 nodes and 77 interaction pairs. The topological parameters of nodes in this network were are in Supplementary Table S7. Four algorithms (MCC, MNC, Degree, and Closeness) in the CytoHubba plugin were used to identify the hub genes (Supplementary Table S8). The middle part of Figure 3C represents the intersection of the four algorithms. The schematic diagram of PPI network deconstruction and the network constructed with hub genes is shown in Figure 3D. A total of 12 hub genes were identified, and these genes were TYROBP, IL1B, C1QA, C3AR1, FGR, CCR5, LAPTM5, C1QC, CD52, MSR1, HAVCR2, and ALOX5AP. To further investigate the biological characteristics of these genes, we analyzed the related functions of hub genes' co-expression network using GeneMANIA database. The biological functions are associated with immune and inflammatory-related processes, such as the regulation of humoral immune response, complement activation, humoral immune response, interleukin-2 production, and negative regulation of immune system process (Supplementary Figure S1).

3.3 MCODE module partition and analysis

Molecular complex detection (MCODE) plugin was used to screen out an important subnetwork in hub genes network, and this subnetwork included 7 nodes and 18 pairs. The seven nodes were TYROBP, IL1B, C1QA, C3AR1, CCR5, C1QC, and MSR1, and the scores are shown in Supplementary Table S9. Then, the enrichment analysis revealed that the nodes in MCODE network were significantly enriched in inflammatory response and cytokine transcript regulation, specifically, the results of BP enrichment in activation of immune response, leukocyte-mediated immunity, and positive regulation of cytokine production, and the KEGG pathway enrichment in complement and coagulation cascades, efferocytosis, cytokine–cytokine receptor interaction, and type I diabetes mellitus (Figure 4; Supplementary Tables S10, S11). Together with the preceding results, we suggest that changes in the immune microenvironment affected by cytokines and inflammatory responses may be a common mechanism of the T2DM complicated with AS.

3.4 Diagnostic efficacy of core genes

To identify reliable core genes, we carried out both external validation and animal experiments according to genes in MCODE network. The expression levels of seven genes, except for C3AR1, in the T2DM dataset (GSE164416) were significantly higher than that in the control samples (Figure 5A). Similarly, the expression levels of the seven genes were upregulated in atherosclerotic samples compared with control (GSE28829, Figure 5B), and $p<0.05$ was considered statistically significant. Then, we detected the expression

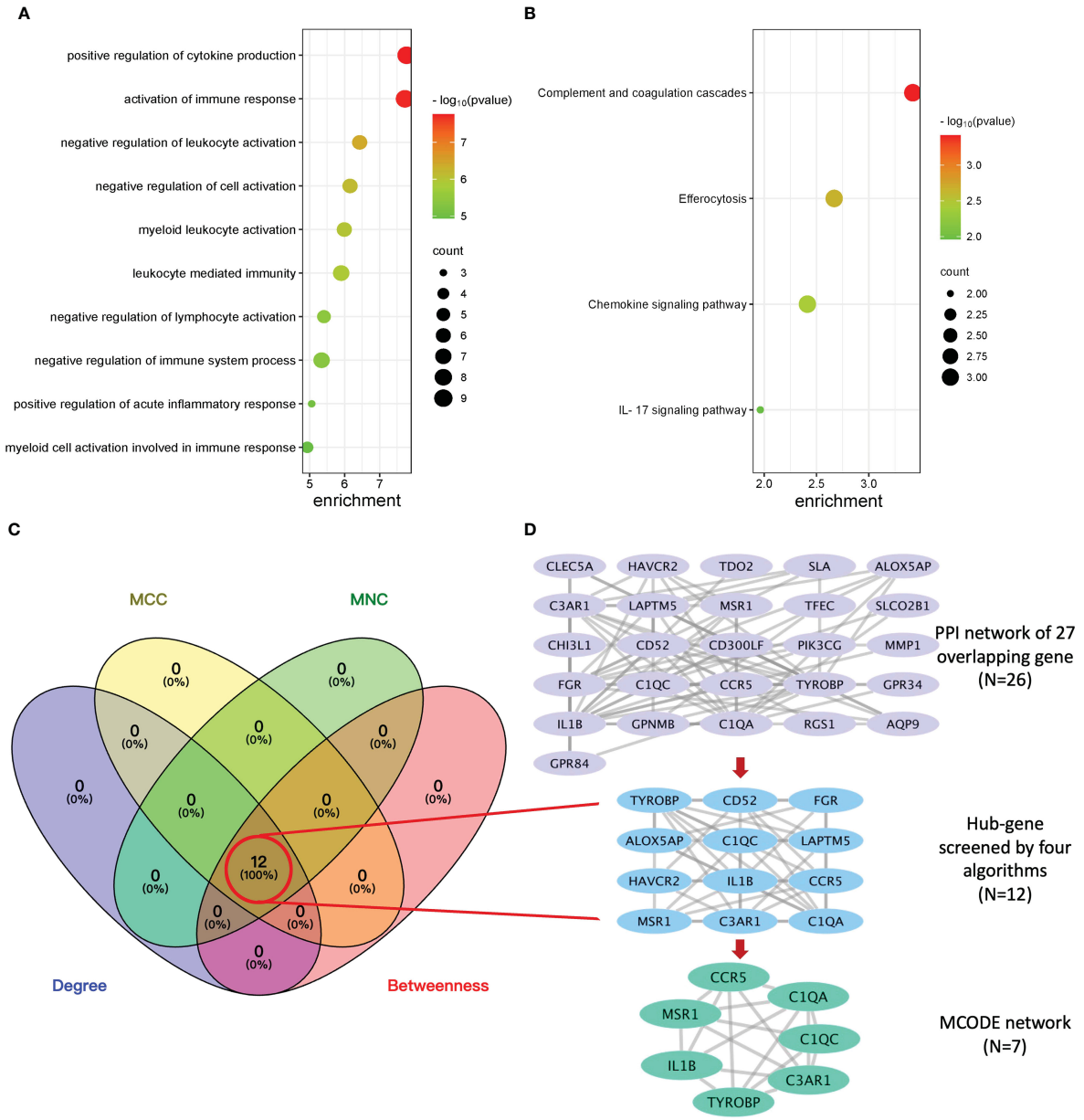

FIGURE 3
Enrichment analysis, protein–protein interaction (PPI) network, and sub-network construction of overlapping DEGs between T2DM and AS. **(A)** The GO enrichment analysis of the overlapping DEGs. **(B)** The pathway enrichment analysis of the overlapping DEGs. **(C)** The number of screened genes from four algorithms (MCC, MNC, Degree, and Closeness) in the PPI network was indicated as Venn diagrams. **(D)** The results of the PPI network of overlapping genes and sub-network obtained from it. From top to bottom is the overlapping genes' PPI network, hub genes' network, and MCODE module network, respectively.

levels of TYROBP, IL1B, C1QA, CCR5, C1QC, and MSR1 in thoracic aortas of mice. The results demonstrated that the expression of IL1B, C1QA, CCR5, and MSR1 was increased ($p<0.05$) in the model mice of T2DM complicated with AS compared with control groups (Figure 5C). Therefore, combining the above results, we identified four core genes, which are IL1B, C1QA, CCR5, and MSR1.

Subsequently, ROC curves were generated to further evaluate the diagnostic value of the validated core genes. The AUCs of IL1B, C1QA, CCR5, and MSR1 were 0.722, 0.778, 0.668, and 0.749, respectively, in the T2DM-related validation dataset (Figure 5D). Moreover, the AUC values of all validated core genes were >0.7 in the AS-related validation dataset, with AUC of 0.721, 0.938, 0.962, and 0.856 for IL1B, C1QA, CCR5, and MSR1, respectively (Figure 5E). At the multigene expression level, after linear fitting of all validated core gene expression models, the AUC value of the multigene combined diagnosis of T2DM and AS was 0.802 and 1.0, respectively (Figures 5F, G). These results reveal that core genes possess good discriminatory ability, and multigene combined diagnosis has a significantly higher predictive power than single gene.

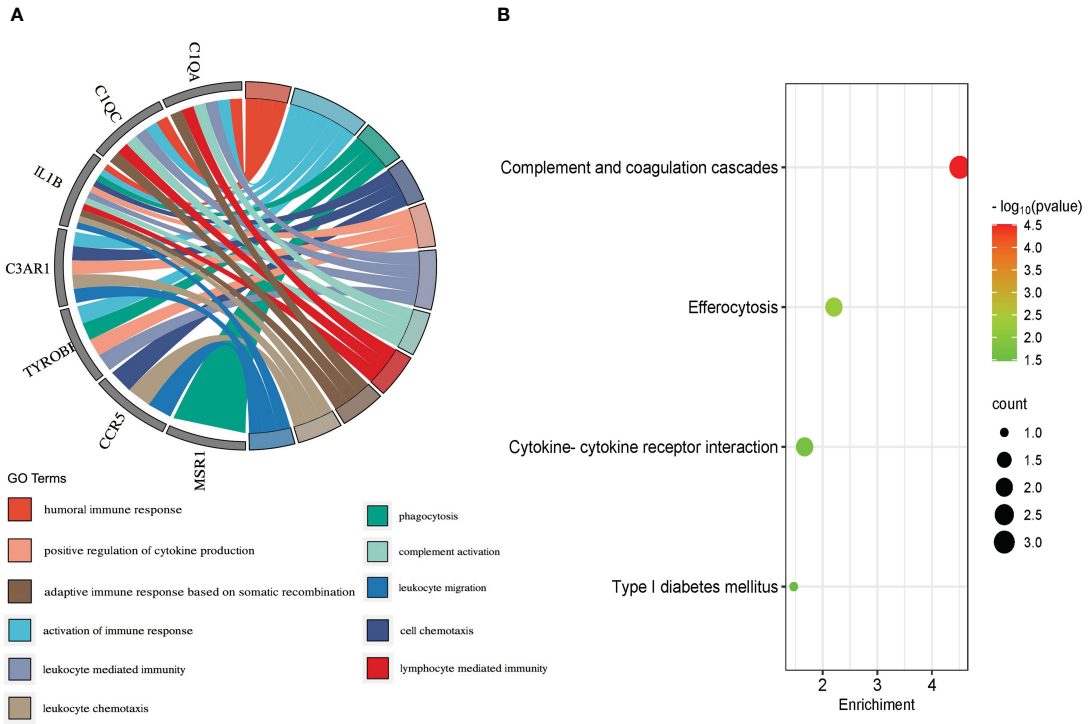

FIGURE 4
Biological process **(A)** and KEGG pathway analysis **(B)** of the genes in MCODE module.

3.5 Immune cell infiltration and correlation analysis

As described above, the result of the enrichment analysis suggested that the immune response might play a crucial role in the course of T2DM complicated with AS. We examine how the immune system works by immune infiltration analyses. This analysis revealed that T2DM diseases were infiltrated by several immune cells, of which regulatory T cells (Tregs), myeloid dendritic cells, and M2 macrophages occupied the top 3 most counts of immune cell subpopulations (Figure 6A). Subsequently, we further analyzed differences in immune cell subgroups between the T2DM samples and the control pools (Figure 6B). The number of M1 and M2 macrophages and CD4+ T cells in the T2DM group was significantly higher than that in the control group ($p<0.05$ or $p<0.01$), while the number of B cells and Tregs was lower ($p<0.01$).

For the case of AS, the immune cell compositions of the AS and control group are shown in Figure 6C. M1 and M2 macrophages and myeloid dendritic cells occupied the top 3 most counts of immune cell subpopulations. Patients with AS had significantly higher numbers of M2 macrophages and CD4+ T cells than the controls ($p<0.001$), and there were fewer B cells, monocytes, and Tregs than those in the control group ($p<0.01$) (Figure 6D).

In order to investigate whether or not core genes were linked to IICs, the correlations were conducted using Spearman analysis. In T2DM, there is a positive relationship between C1QA, CCR5, MSR1, and M1 macrophages, and a negative relationship between C1QA, MSR1, and CD8+ T cells, Tregs, and myeloid dendritic cells ($p<0.05$ or $p<0.01$). In AS, there have a positive relationship between IL1B, C1QA, CCR5, MSR1, and M1 macrophages, M2 macrophages, CD4+ T cells, CD8+ T cells, and myeloid dendritic cells, and a negative relationship between MSR1 and B cells and Tregs ($p<0.05$ or $p<0.01$) (Figure 6E). The details of differential IICs selection results and correlation analysis are shown in Supplementary Tables S12, S13, respectively.

3.6 Integrated TF-mRNA network

TRRUST is a TF–target interaction database that shows regulatory regulation between TF and target genes. According to TF binding site information provided in TRRUST, potential key regulators for core genes were selected, a total of 10 associations between five TFs (SPI1, RELA, NFKB1, YY1, and JUN) and four core genes (IL1B, C1QA, CCR5, and MSR1). As shown in Supplementary Table S14, SPI1 regulated two genes (IL1B and MSR1), YY1 regulated two genes (IL1B and CCR5), JUN regulated two genes (IL1B and MSR1), RELA regulated two genes (IL1B and CCR5), and NFKB1 regulated two genes (CCR5 and IL1B). Based on this result, we constructed a regulatory TF-mRNAs network using Cytoscape software (Figure 7). We use different shapes or colors to distinguish different types of mRNA and TFs. This figure shows the potential pathological regulation process found in this study.

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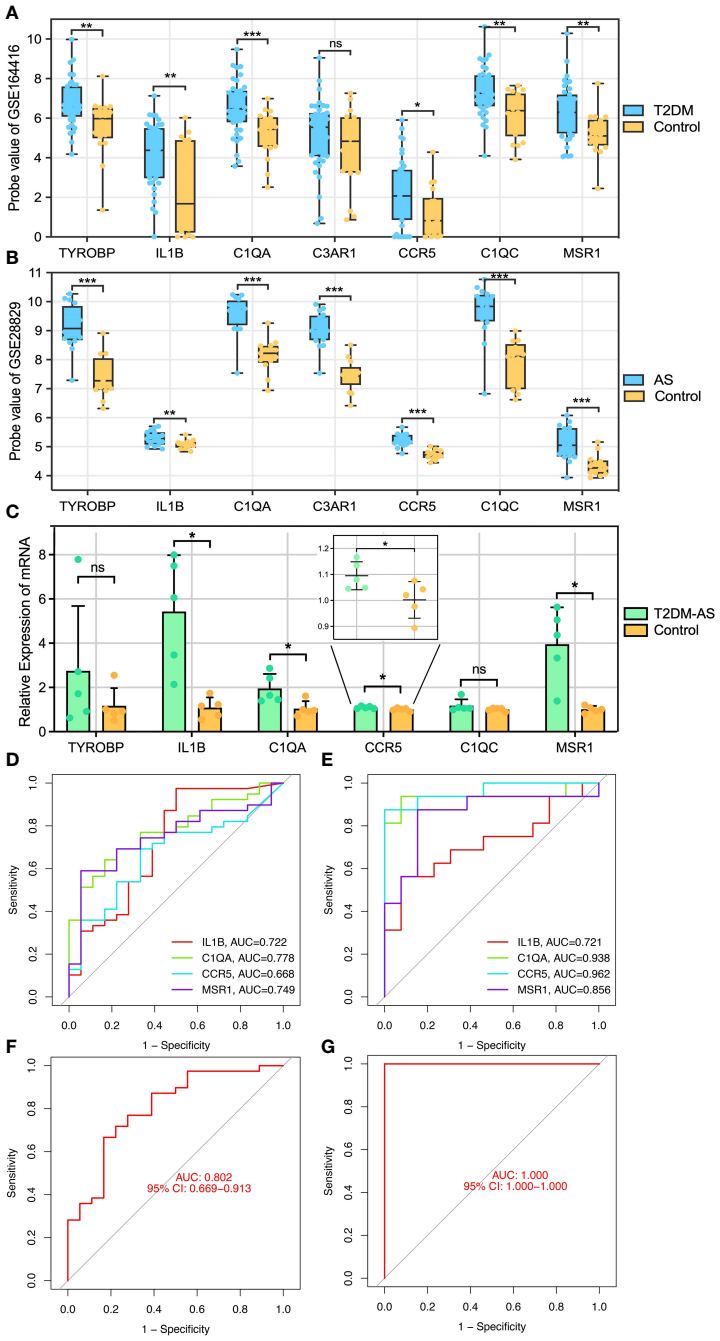

FIGURE 5
Core genes validation and diagnosis. **(A, B)** External validation: the genes expression in GSE164416 and GSE28829. **(C)** qRT-PCR experimental validation: the genes expression in model mice and control group. Receiver operating curve (ROC) for core gene in **(D)** GSE164416 and **(E)** GSE28829, respectively. Multi-index (all core genes) combined diagnosis in **(F)** GSE164416 and **(G)** GSE28829, respectively. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not statistically significant.

4 Discussion

Bioinformatics can help us better understand the complex biological processes. We identified 116 and 803 DEGs from the T2DM and AS datasets, respectively. Among these, 27 communal DEGs were identified between the two diseases. Based on enrichment analysis, these genes were significantly enriched in positive regulation of cytokine production, activation of immune response and negative regulation of leukocyte activation. Subsequently, 12 hub genes (TYROBP, IL1B, C1QA, C3AR1, FGR, CCR5, LAPTM5, C1QC, CD52, MSR1, HAVCR2, and ALOX5AP) were identified from four algorithms using the CytoHubba plugin. Then, MCODE was used to deconstruct the network for further dimension reduction. After external dataset and qRT-PCR experimental verification to these genes, a total of four core genes (IL1B, C1QA, CCR5, and MSR1) were obtained. ROC analysis indicated that those core genes owned higher diagnostic value both in T2DM and AS. Based on functional annotation

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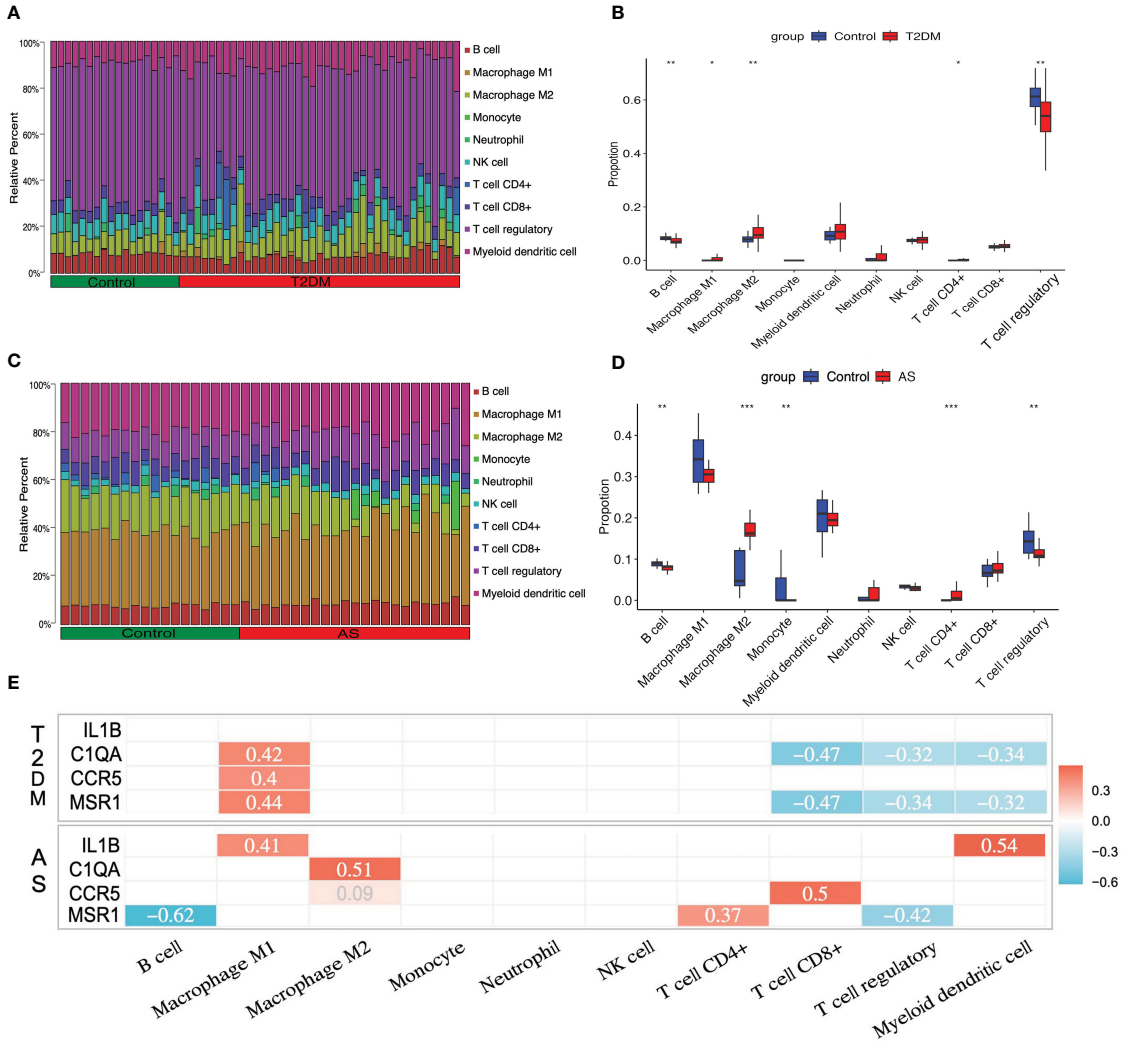

FIGURE 6
Immune cell infiltration analysis. The relative proportion of infiltrating immune cells (IICs) in **(A)** T2DM and **(C)** AS. Violin plot of distinct immune cell subtype compositions in T2DM vs. control **(B)** and AS vs. control **(D)**. **(E)** Correlation between core genes and immune infiltrating cells. Only statistically significant differences are reported in this heatmap. Red represents positive correlation, and blue represents negative correlation. *P < 0.05, **P < 0.01, ***P < 0.001.

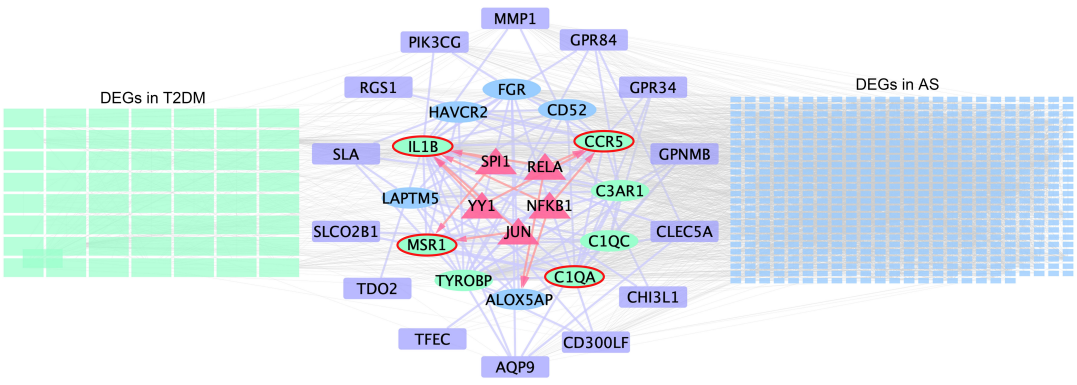

FIGURE 7
Schematic presentation of the TF-mRNA network. The green and blue rectangles on the left- and right-hand sides indicate the DEGs in T2DM and AS, respectively, and the common DEG analyses are shown in the middle. Ovals represent hub genes. Among them, genes included in the MCODE module are highlighted in green and core genes highlighted by red elliptical borders. Purple boxes indicate remaining genes in overlapping DEGs. Trigonal nodes in red represent key transcription factor genes regulating core gene expression.

analysis results, the immune system was emphasized as a critical component of T2DM complicated with AS. To further elucidate the potential biological roles of the core genes, we predicted their TFs in upstream and constructed a TF-mRNA regulatory network. Finally, based on CIBERSORT analysis, the correlation between key hub genes and IICs was evaluated to reveal the immune mechanism of T2DM and AS.

According to the immune infiltrating analysis, four differential immune cells (M2 macrophages, CD4+ T cells, B cells, and Tregs) regulated by C1QA, CCR5, and MSR1 may participate in the pathological process of T2DM complicated with AS. T2DM is a metabolic inflammatory disease mediated by a variety of immune cells and cytokines (17). In addition to innate immunity, such as macrophage and monocyte, adaptive immunity has also been confirmed to involved in this pathological process. CD4+T cells are an important subset of T cells, which are involved in the metabolic inflammation progressing through autocrine or paracrine. According to the functional characteristics of CD4+T cells, they can be divided into pro-inflammatory subsets (Th1, Th17, and Th22) and anti-inflammatory subsets (Tregs) (18). A proper balance between pro-inflammatory and anti-inflammatory subsets of CD4+T cells is essential for maintaining immune homeostasis and avoiding inflammatory response. The changes in the number and frequency of CD4+ Th subsets and the inflammatory response produced by cytokines are related to T2DM (19). Instead, Treg is a protective subtype of CD4 cells, which is related to its effect on macrophage exocytosis promoting and plaque remodeling (20, 21). The results of this study showed that CD4+T cells and Tregs showed an upward and downward trend in both two datasets, respectively.

B cells contribute significantly to innate and adaptive immunity by producing antibodies and cytokines (22, 23). B1 cell is an important subtype of B cell. It can synthesize and release IgM, a natural antioxidant low-density lipoprotein, which inhibits the uptake of ox-LDL by macrophages and ultimately inhibits the production of foam cells. In addition, the IgM can also inhibit the formation of necrotic core in AS plaque (24). However, the related research on this cell is rarely reported in the field of diabetes. In this study, the relative proportions of B cells showed significant decrease in two diseases, respectively. In addition to B cells and T cells, the changes in the number of macrophages show multiplicity changes. Different from the single view that M1 macrophages are involved in pro-inflammatory responses and M2 macrophages are involved in anti-inflammatory responses (25), the results of this study show that the relative proportion of M1 and M2 macrophages in T2DM is increased, while that of M2 macrophages in AS is significantly decreased. This exhibits that the macrophages have a high degree of plasticity in response to microenvironmental stimulus.

Complement protein C1q is a complex glycoprotein component of the classical complement pathway with 18 polypeptide chains. C1QA is among the three genes encoding C1q and is crucial in the innate immune response (26). C1q is significantly higher in advanced atherosclerotic plaques and those in patients with acute coronary syndrome than in early lesions and those with stable angina pectoris (27). In T2DM, C1QA protein abundance is altered in patient serum (28). Conversely, C1q has pro- and atheroprotective effects; however, few studies have focused on its role in T2DM (29). These results indicate that C1q is involved in the progression of AS and T2DM.

Some studies have shown that MSR1 polymorphisms are associated with AS and plasma fatty acid distribution (30). It is a scavenger receptor and can promote macrophage inflammation (31, 32). MSR1 was distributed in the macrophages and smooth muscle cells (33, 34), and its induction of atherosclerotic lesion formation facilitates phagocytosis (35, 36). Previous studies have demonstrated that high MSR1 expression causes cholesterol to feed into the vessel wall, whereas its deficiency causes a reduction in spontaneously developed AS (37). Advanced glycation end products are crucial in diabetes. Under high-advanced glycation end-product intake, MSR1 expression showed a tendency towards with insulin levels and may promote endocrine-related diseases (38).

Our enrichment results suggest that positive regulation of cytokine production and signaling in the immune system may regulate diseases. RELA (p65) and NFκB1 (p50) belong to the NFκB family. Both contain a Rel homologous domain at the N-terminal, which can mediate the specific binding, dimerization, and binding of NFκB to DNA. RELA and NFκB1 can also combine and form homologous or heterodimers. The binding of different dimers to DNA has different effects on inflammation regulation. The binding of p50/p50 and p65/p65 homodimers to DNA suppresses the expression of inflammatory genes, while p50/p65 heterodimer promotes the expression of pro-inflammatory factors related to NFκB (39, 40). Activating NFκB finally induces the synthesis and release of cytokines, such as TNF-α and IL-1β, and affects ROS levels, which can directly stimulate islet β-cell apoptosis and cause damage by activating macrophage and T-cell attack on islet β cells (41). In addition, NFκB can cause vascular endothelial injury, vascular smooth muscle proliferation, and foam cell formation (42).

The JUN family includes c-Jun, Jun B, and Jun D, which are the downstream proteins of the primary functions of the JNK signaling pathway. C-Jun exacerbates atherogenesis by decreasing cholesterol efflux from macrophages in atherosclerotic plaques (43). c-Jun is a novel regulator of T-cell lineage development and decision-making (44). In T2DM, the activation of JNK directly phosphorylates insulin receptor substrate 1, producing ROS and impairing insulin signaling. The active ASK1 induces pancreatic β-cell death (45). Emerging evidence suggests that JNK is involved in regulating cellular senescence by downregulating hypoxia-inducible factor-1α to accelerate hypoxia (46). This may be associated with the progression of T2DM. The SPI-1 proto-oncogene (SPI1) is crucial in the hematopoietic system, normal and pathogenic (47). SPI1 upregulation reportedly stimulated the TLR4/NFκB axis and aggravated myocardial infarction (48). Further findings suggested that SPI1 regulates copper homeostasis in diabetic cardiomyopathy (49). However, experimental evidence of SPI1 expression is lacking.

Various innate and adaptive immune cells promote the formation of an inflammatory microenvironment and are crucial in the progression of AS. Low-grade inflammation, essential for AS development, is an important feature of diabetes (50). The development of diabetic AS induces an immune microenvironment that shifts the normal balance toward a pro-inflammatory state. Although the immune microenvironment remains investigated, its exact role remains unknown. We hypothesized that AS and T2DM

share a common pathogenesis correlated with B cells, CD4+ T cells, Tregs, and M2 macrophages. Our results reveal that the relationship among five TFs, four core genes, and four immune cells profile may be crucial in understanding the pathogenesis therapeutic direction of T2DM complicated with AS. This represents a promising avenue to treat and prevent diseases. This study focuses on the common mechanisms and identification of hub genes and immune infiltration profiles in patients with AS and T2DM.

However, there are a few limitations to this study. First, despite the large sample size and experimental verification, it is a retrospective study that requires validation through a prospective study. Second, although validated in animal models, these core genes have not been evaluated in humans. Third, specific molecular mechanisms of immune responses regulated by core genes in T2DM complicated with AS remain poorly determined. These will be the focus of our future studies.

5 Conclusion

We identified four core genes (IL1B, C1QA, CCR5, and MSR1) and four diff-IICs (B cells, CD4+ T cells, Tregs, and M2 macrophages). The evidence of common pathogenesis points toward the immune microenvironment after core genes modulation, which might be modulated by five TFs (RELA, NFκB1, JUN, YY1, and SPI1). These results provide a direction for future studies on the potential key genes in patients with T2DM complicated with AS.

Data availability statement

The datasets presented in our study are available from the online repositories. Detailed information about it can be found in the article.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements. The animal study was approved by animal ethics committee of the Ethics Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YQ: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. YaZ: Writing – original draft, Software, Investigation, Data curation. SG: Writing – original draft, Methodology, Formal analysis, Data curation. LL: Writing – original draft, Methodology, Formal analysis, Data curation. HW: Writing – original draft, Visualization, Validation, Formal analysis. YC: Writing – original draft, Visualization, Validation, Formal analysis. QZ: Writing – original draft, Visualization, Validation, Methodology, Formal analysis. YiZ: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. FX: Writing – review & editing, Supervision, Resources, Project administration.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1381229/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The hub genes and their co-expression genes analyzed using GeneMANIA.

References

1. Mannucci E, Targher G, Nreu B, Pintaudi B, Candido R, Giaccari A, et al. Effects of insulin on cardiovascular events and all-cause mortality in patients with type 2 diabetes: a meta-analysis of randomized controlled trials. *Nutr Metab Cardiovasc Dis*. (2022) 32:1353–60. doi: 10.1016/j.numecd.2022.03.007

2. Ferket BS, Hunink MGM, Masharani U, Max W, Yeboah J, Burke GL, et al. Lifetime cardiovascular disease risk by coronary artery calcium score in individuals with and without diabetes: an analysis from the multi-ethnic study of atherosclerosis. *Diabetes Care*. (2022) 45:975–82. doi: 10.2337/dc21-1607

3. Kanter JE, Bornfeldt KE. Inflammation and diabetes-accelerated atherosclerosis: myeloid cell mediators. *Trends Endocrinol Metab*. (2013) 24:137–44. doi: 10.1016/j.tem.2012.10.002

4. Tomaniak M, Katagiri Y, Modolo R, Silva RD, Khamis RY, Bourantas CV, et al. Vulnerable plaques and patients: state-of-The-art. *Eur Heart J*. (2020) 41:2997–3004. doi: 10.1093/eurheartj/ehaa227

5. Nordestgaard BG. Triglyceride-rich lipoproteins and atherosclerotic cardiovascular disease: new insights from epidemiology, genetics, and biology. *Circ Res*. (2016) 118:547–63. doi: 10.1161/CIRCRESAHA.115.306249

6. Libby P, Plutzky J. Diabetic macrovascular disease: the glucose paradox? *Circulation*. (2002) 106:2760–3. doi: 10.1161/01.CIR.0000037282.92395.AE

7. McMaster WG, Kirabo A, Madhur MS, Harrison DG. Inflammation, immunity, and hypertensive end-organ damage. *Circ Res*. (2015) 116:1022–33. doi: 10.1161/CIRCRESAHA.116.303697

8. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616

9. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. (2015) 43:e47. doi: 10.1093/nar/gkv007

10. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. (2012) 16:284–7. doi: 10.1089/omi.2011.0118

11. Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, et al. String V9.1: Protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res*. (2013) 41:D808–15. doi: 10.1093/nar/gks1094

12. Chin CH, Chen SH, Wu HH, Ho CW, Ko MT, Lin CY. Cytohubba: Identifying hub objects and Sub-networks from complex interactome. *BMC Syst Biol*. (2014) 8 Suppl 4:S11. doi: 10.1186/1752-0509-8-S4-S11

13. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, et al. The genemania prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res*. (2010) 38:W214–20. doi: 10.1093/nar/gkq537

14. Nakamura T, Terajima T, Ogata T, Ueno K, Hashimoto N, Ono K, et al. Establishment and pathophysiological characterization of type 2 diabetic mouse model produced by streptozotocin and nicotinamide. *Biol Pharm Bull*. (2006) 29:1167–74. doi: 10.1248/bpb.29.1167

15. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinf*. (2011) 12:77. doi: 10.1186/1471-2105-12-77

16. Han H, Cho JW, Lee S, Yun A, Kim H, Bae D, et al. Trrust V2: An expanded reference database of human and mouse transcriptional regulatory interactions. *Nucleic Acids Res*. (2018) 46:D380–d6. doi: 10.1093/nar/gkx1013

17. Conti P, Ronconi G, Kritas SK, Caraffa A, Theoharides TC. Activated mast cells mediate low-grade inflammation in type 2 diabetes: interleukin-37could be beneficial. *Can J Diabetes*. (2018) 42:568–73. doi: 10.1016/j.jcjd.2018.01.008

18. Kan AKC, Tang WT, Li PH. Helper T cell subsets: Development, function and clinical role in hypersensitivity reactions in the modern perspective. *Heliyon*. (2024) 10: e30553. doi: 10.1016/j.heliyon.2024.e30553

19. Valentine Y, Nikolajczyk BS. T cells in obesity-associated inflammation: The devil is in the details. *Immunol Rev*. (2024). doi: 10.1111/imr.13354

20. Proto JD, Doran AC, Gusarova G, Yurdagul A Jr, Sozen E, Subramanian M, et al. Regulatory T cells promote macrophage efferocytosis during inflammation resolution. *Immunity*. (2018) 49:e666. doi: 10.1016/j.immuni.2018.07.015

21. Sharma M, Schlegel MP, Afonso MS, Brown EJ, Rahman K, Weinstock A, et al. Regulatory T cells license macrophage pro-resolving functions during atherosclerosis regression. *Circ Res*. (2020) 127:335–53. doi: 10.1161/CIRCRESAHA.119.316461

22. Pattarabanjird T, Li C, McNamara CB. Cells in atherosclerosis: mechanisms and potential clinical applications. *JACC Basic Transl Sci*. (2021) 6:546–63. doi: 10.1016/j.jacbts.2021.01.006

23. Syeda MZ, Hong T, Huang C, Huang W, Mu Q. B cell memory: from generation to reactivation: a multipronged defense wall against pathogens. *Cell Death Discov*. (2024) 10:117–32. doi: 10.1038/s41420-024-01889-5

24. Harrison J, Newland SA, Jiang W, Giakomidi D, Zhao X, Clement M, et al. Marginal zone B cells produce 'natural' atheroprotective IgM antibodies in a T cell-dependent manner. *Cardiovasc Res*. (2024) 120:318–28. doi: 10.1093/cvr/cvae027

25. Yang B, Hang S, Xu S, Gao Y, Yu W, Zang G, et al. Macrophage polarisation and inflammatory mechanisms in atherosclerosis: Implications for prevention and treatment. *Heliyon*. (2024) 10:e32073. doi: 10.1016/j.heliyon.2024.e32073

26. Wang Y, Tong X, Zhang J, Ye X. The complement C1qA enhances retinoic acidinducible gene-I-mediated immune signalling. *Immunology*. (2012) 136:78–85. doi: 10.1111/j.1365-2567.2012.03561.x

27. Sasaki S, Nishihira K, Yamashita A, Fujii T, Onoue K, Saito Y, et al. Involvement of enhanced expression of classical complement C1q in atherosclerosis progression and plaque instability: C1q as an indicator of clinical outcome. *PloS One*. (2022) 17: e0262413. doi: 10.1371/journal.pone.0262413

28. Kim SJ, Chae S, Kim H, Mun DG, Back S, Choi HY, et al. A protein profile of visceral adipose tissues linked to early pathogenesis of type 2 diabetes mellitus. *Mol Cell Proteomics*. (2014) 13:811–22. doi: 10.1074/mcp.M113.035501

29. Bhatia VK, Yun S, Leung V, Grimsditch DC, Benson GM, Botto MB, et al. Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am J Pathol*. (2007) 170:416–26. doi: 10.2353/ajpath.2007.060406

30. Durst R, Neumark Y, Meiner V, Friedlander Y, Sharon N, Polak A, et al. Increased risk for atherosclerosis of various macrophage scavenger receptor 1 alleles. *Genet Test Mol Biomarkers*. (2009) 13:583–7. doi: 10.1089/gtmb.2009.0048

31. Gudgeon J, Marín-Rubio JL, Trost M. The role of macrophage scavenger receptor 1 (MSR1) in inflammatory disorders and cancer. *Front Immunol*. (2022) 13:1012002. doi: 10.3389/fimmu.2022.1012002

32. Kong FQ, Zhao SJ, Sun P, Liu H, Jie J, Xu T, et al. Macrophage MSR1 promotes the formation of foamy macrophage and neuronal apoptosis after spinal cord injury. *J Neuroinflamm*. (2020) 17:62. doi: 10.1186/s12974-020-01735-2

33. Naito M, Suzuki H, Mori T, Matsumoto A, Kodama T, Takahashi K. Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. *Am J Pathol*. (1992) 141:591–9.

34. Li H, Freeman MW, Libby P. Regulation of smooth muscle cell scavenger receptor expression *in vivo* by atherogenic diets and *in vitro* by cytokines. *J Clin Invest*. (1995) 95:122–33. doi: 10.1172/JCI117628

35. Kelley JL, Ozment TR, Li C, Schweitzer JB, Williams DL. Scavenger receptor-A (CD204): a two-edged sword in health and disease. *Crit Rev Immunol*. (2014) 34:241–61. doi: 10.1615/CritRevImmunol.v34.i3

36. Zhang Z, Jiang YJ, Zhou ZQ, Huang JN, Chen SC, Zhou WY, et al. Scavenger receptor A1 attenuates aortic dissection via promoting efferocytosis in macrophages. *Biochem Pharmacol*. (2019) 168:392–403. doi: 10.1016/j.bcp.2019.07.027

37. Hiltunen TP, Luoma JS, Nikkari T, Yä-Herttuala S. Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit aortic lesions. *Circulation*. (1998) 97:1079–86. doi: 10.1161/01.CIR.97.11.1079

38. Chatzigeorgiou A, Kandaraki E, Piperi C, Livadas S, Papavassiliou AG, Koutsilieris M, et al. Dietary glycotoxins affect scavenger receptor expression and the hormonal profile of female rats. *J Endocrinol*. (2013) 218:331–7. doi: 10.1530/JOE-13-0175

39. Mussbacher M, Salzmann M, Brostjan C, Hoesel B, Schoergenhofer C, Datler H, et al. Cell type-specific roles of NF-κB linking inflammation and thrombosis. *Front Immunol*. (2019) 10:85. doi: 10.3389/fimmu.2019.00085

40. Osipo C, Golde TE, Osborne BA, Miele LA. Off the beaten pathway: the complex cross talk between Notch and NF-kappa B. *Lab Invest*. (2008) 88:11–7. doi: 10.1038/labinvest.3700700

41. Stephens LA, Thomas HE, Ming L, Grell M, Darwiche R, Volodin L, et al. Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. *Endocrinology*. (1999) 140:3219–27. doi: 10.1210/endo.140.7.6873

42. Cheng W, Cui C, Liu G, Ye C, Shao F, Bagchi AK, et al. NF-κB, A potential therapeutic target in cardiovascular diseases. *Cardiovasc Drugs Ther*. (2023) 37:571–84. doi: 10.1007/s10557-022-07362-8

43. Trusca VG, Fuior EV, Kardassis D, Simionescu M, Gafencu AV. The opposite effect of c-Jun transcription factor on apolipoprotein E gene regulation in hepatocytes and macrophages. *Int J Mol Sci*. (2019) 20:1471. doi: 10.3390/ijms20061471

44. Riera-Sans L, Behrens A. Regulation of alphabeta/gammadelta T cell development by the activator protein 1 transcription factor c-Jun. *J Immunol*. (2007) 178:5690–700. doi: 10.4049/jimmunol.178.9.5690

45. Obsilova V, Honzejkova K, Obsil T. Structural insights support targeting ASK1 kinase for therapeutic interventions. *Int J Mol Sci*. (2021) 22:13395. doi: 10.3390/ijms222413395

46. Deng Y, Adam V, Nepovimova E, Heger Z, Valko M, Wu Q, et al. c-Jun N-terminal kinase signaling in cellular senescence. *Arch Toxicol*. (2023) 97:2089–109. doi: 10.1007/s00204-023-03540-1

47. Burda P, Laslo P, Stopka T. The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. *Leukemia*. (2010) 24:1249–57. doi: 10.1038/leu.2010.104

48. Liu Z, Huang S. Upregulation of SPI1 during myocardial infarction aggravates cardiac tissue injury and disease progression through activation of the TLR4/NFkappaB axis. *Am J Transl Res*. (2022) 14:2709–27. doi: 10.1093/eurheartj/ehad655.3068

49. Huo S, Wang Q, Shi W, Peng L, Jiang Y, Zhu M, et al. ATF3/SPI1/SLC31A1 signaling promotes cuproptosis induced by advanced glycosylation end products in diabetic myocardial injury. *Int J Mol Sci*. (2023) 24:1667. doi: 10.3390/ijms24021667

50. Zhang ZY, Miao LF, Qian LL, Wang N, Qi MM, Zhang YM, et al. Molecular mechanisms of glucose fluctuations on diabetic complications. *Front Endocrinol (lausanne)*. (2019) 10:640. doi: 10.3389/fendo.2019.00640

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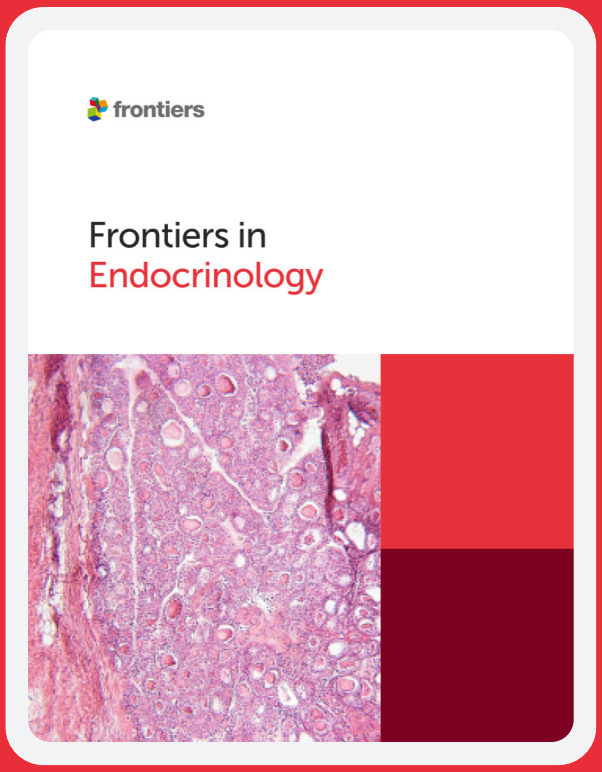

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