ImmunoTargets and Therapy

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ORIGINAL RESEARCH

Dissecting Causal Relationship Among Immune Cells, Plasma Metabolites and Coronary Atherosclerosis: A Mendelian Randomization Study

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Background: Circulating immune cells and metabolites are linked to coronary atherosclerosis, but the specific causal relationships and the role of metabolites as mediators remain unclear.

Methods: Summary statistics from GWAS datasets on immune cells (n=3,757), circulating metabolites (n=8,299), and coronary atherosclerosis (cases n=51,589; controls n=343,079) were analyzed using bidirectional Mendelian randomization. Two-step and multivariate Mendelian randomization were employed to identify mediating metabolites, with inverse variance weighting (IVW) as the primary method.

Results: We identified nine immune cell phenotypes, including specific T-cell and monocyte populations, with significant causal links to coronary atherosclerosis. Additionally, 41 plasma metabolites across four metabolic pathways were identified, including 3-hydroxy-2-ethylpropionate and trans-2-hexenoylglycine. Mediation analysis revealed that 3-hydroxy-2-ethylpropionate mediated the effect of IgD+ CD24+ B-cells on coronary atherosclerosis (mediating effect: 0.961; 95% CI: 0.955–0.967), while trans-2-hexenoylglycine regulated IgD+ CD24+ B-cells, showing a mediation ratio of 16.7% (mediating effect: 0.983; 95% CI: 0.981–0.986).

Conclusion: Key immune cell phenotypes and plasma metabolites were linked to coronary atherosclerosis. The roles of 3-hydroxy-2-ethylpropionate and trans-2-hexenoylglycine in regulating B-cell function suggest potential therapeutic targets for prevention and treatment.

Keywords: immune cells, coronary atherosclerosis, plasma metabolites, Mendelian randomization, B-cell function

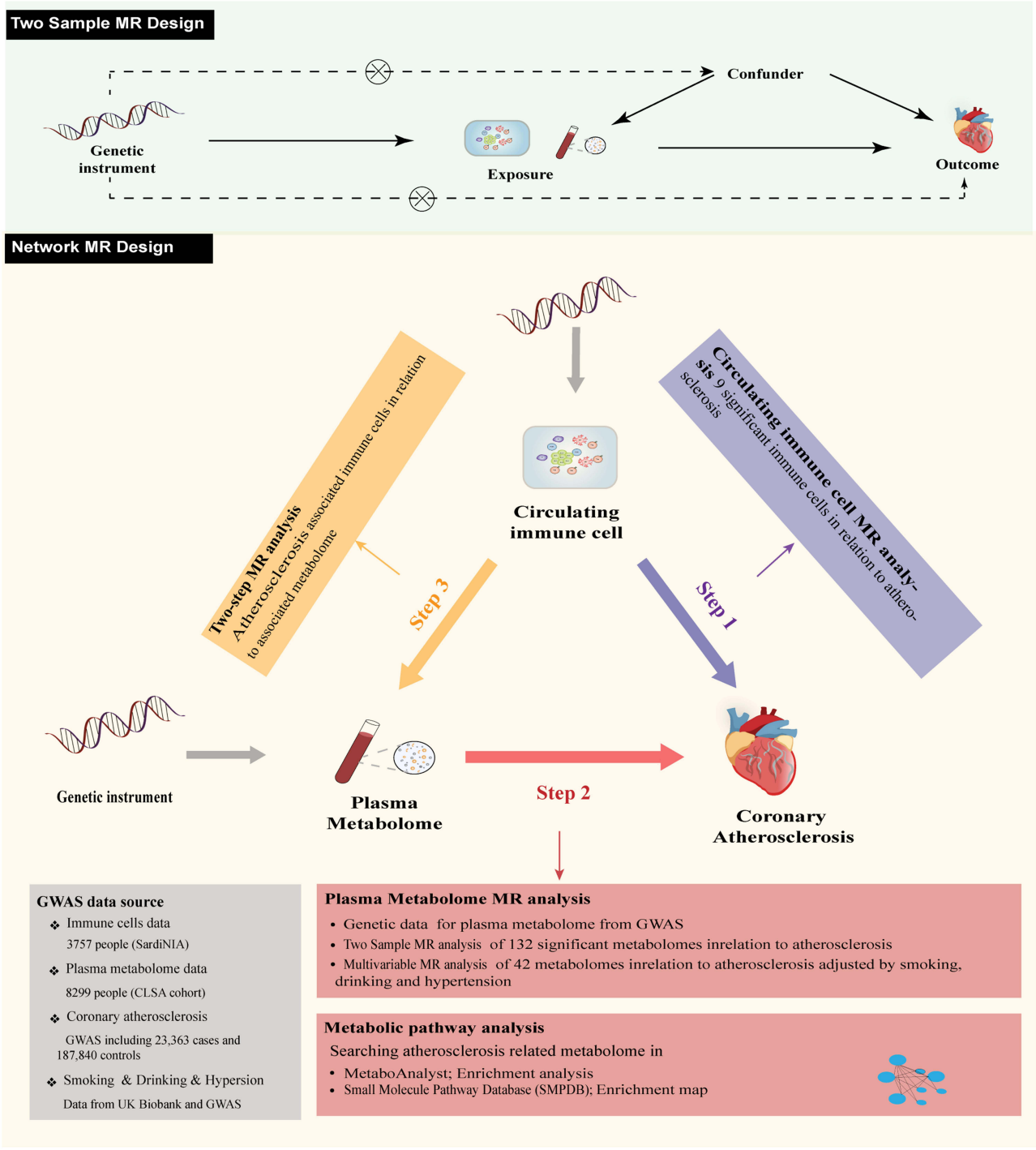
Introduction

Coronary atherosclerotic heart disease (CAD), responsible for substantial mortality and disability globally, involves a complex pathogenesis that involves the interaction of genetic, environmental, and metabolic factors.[1] Although extensive epidemiologic research has identified a plethora of risk factors associated with CAD, the inherent limitations of observational studies, including their susceptibility to confounding and reverse causality, hinder the ability to establish definitive causal relationships and thoroughly elucidate the etiological mechanisms underlying CVD.

Immune cells and related metabolites perform critical roles in CVD health maintenance and disease modification, particularly in the formation and development of CVD.[2] Dysregulated immune responses and chronic inflammation are central to the pathogenesis of CAD, directly contributing to plaque formation, progression, and rupture.[3–5] For instance, the ingestion of oxidized low-density lipoprotein (LDL) by macrophages leads to the formation of foam cells, a hallmark of early atherosclerotic development, and their accumulation within the arterial wall further exacerbates local

Received: 22 November 2024
Accepted: 4 February 2025
Published: 6 March 2025

Graphical Abstract



inflammation.[6,7] Moreover, this inflammatory environment is amplified by the activation and polarization of T cells, which modulate macrophage function and contribute to plaque instability.[8,9] Pro-inflammatory T-cell subsets, such as Th1 and Th17 cells, release cytokines like interferon-gamma (IFN-γ) and interleukin-17 (IL-17), which promote macrophage activation, enhance foam cell formation, and drive atherogenesis. In contrast, regulatory T cells (Tregs)

exert anti-inflammatory effects by suppressing excessive immune responses and stabilizing plaques, thus protecting against disease progression.[10] The interplay between these immune subsets reflects a delicate balance: pro-inflammatory responses, if unchecked, drive plaque growth and destabilization, whereas anti-inflammatory mechanisms counteract these effects to maintain vascular integrity. Dysregulation of this balance shifts the immune environment toward chronic inflammation, fostering plaque formation and rendering plaques more prone to rupture. This immune-mediated disruption underscores the critical role of immune cells in CAD pathogenesis and highlights their potential as therapeutic targets.[11] In addition, circulating metabolites, such as short-chain fatty acids and cholesterol crystals, contribute to plaque instability and inflammation by activating the NLRP3 inflammasome and promoting the release of inflammatory cytokines.[12,13] However, extensive research investigating the precise mechanisms of circulating metabolites in CVD remains insufficient.

Mendelian Randomization (MR) is an advanced analytical method that uses genetic variants as instrumental variables to assess causal relationships between exposures and outcomes. This approach is particularly valuable in overcoming limitations inherent in observational studies, such as confounding and reverse causality. By leveraging the random allocation of genetic variants at conception, MR provides a robust framework to infer causality in complex biological systems, offering insights that are less biased than those derived from traditional observational methods.[14] In this research, a 2-sample bidirectional Mendelian randomization analysis was performed to explore the causal relationship among specific immune cells and cardiovascular disease according to the aggregated statistics from the largest and current genome-wide association study (GWAS). Then, we examined whether plasma metabolites mediate the pathway from immune cells to cardiovascular disease (CVD).

Methods

Study Design

Figure 1 depicts the study design. First, we performed bidirectional Mendelian randomization (BMR) to determine the causal effect of 731 immune cell types on coronary atherosclerosis.

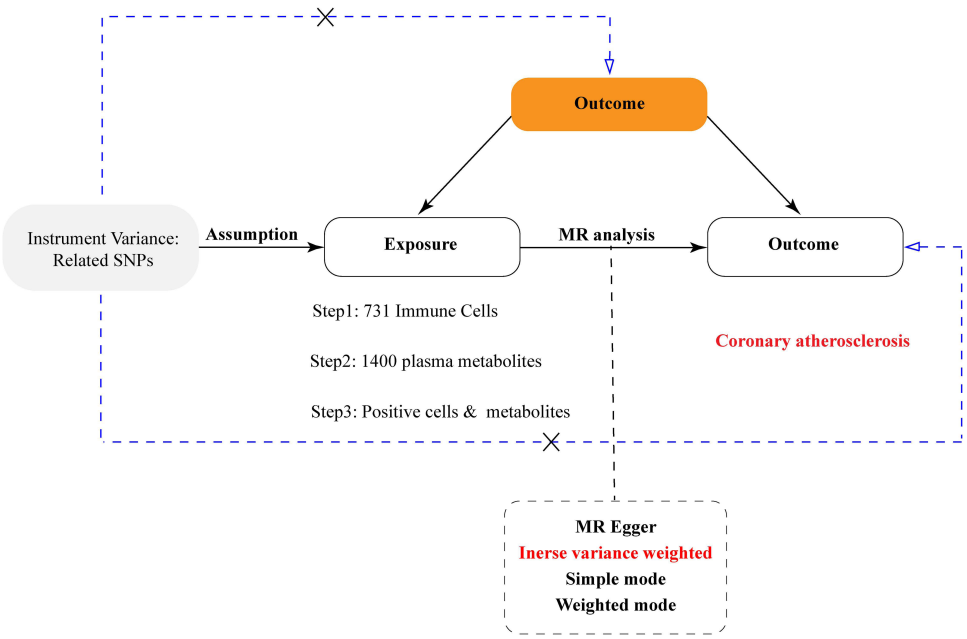


Figure 1 Flowchart of this study. The SNPs from GWAS applied as instrument variables (IVs) were related to exposure. Dashed lines indicate that IV is not associated with confounders and that IV affects the risk of the outcome directly through exposure rather than other alternative pathways. Dashed lines depict irrelevancy, and solid lines depict correlation.

https://doi.org/10.2147/ITT.S508042

Subsequently, we performed a two-sample Mendelian randomization to pinpoint and prioritize the lipids contributing to CVD from 1400 plasma metabolites. Finally, with two-step Mendelian randomization and multivariate Mendelian randomization methods, we develop a causal relationship from immune cells to CVD through mediator lipids.

Data Sources

The latest GWAS summary data provided circulating immune cell genetic information, collected and analyzed by the SardiNIA consortium from 3757 individuals. The findings comprise 118 absolute cell counts (ACs), 389 median fluorescence intensities (MFIs) for surface antigen levels, 32 morphologic features (MPs), and 192 relative cell counts (RCs).[15]

Summary statistics for the plasma metabolome analysis were sourced from the GWAS Catalog at the European Bioinformatics Institute (EBI), encompassing 1,091 metabolites and 309 ratios from 8,299 European individuals (accession numbers GCST90199621 to GCST90201020).[16] The dataset included 850 identified metabolites, classified into eight categories: lipids (395), amino acids (210), xenobiotics (130), nucleotides (33), cofactors and vitamins (31), carbohydrates (22), peptides (21), and energy metabolites (8), with the remaining being partially characterized (21) and unidentified molecules (220).

The GWAS summary data for atherosclerosis was obtained from the tenth release of the FinnGen consortium (https://r10.risteys.finngen.fi/). This dataset originates from a prospective cohort study, which employed International Classification of Diseases (ICD) diagnostic codes to identify cases of atherosclerosis. Specifically, we accessed genetic data for coronary artery atherosclerosis, encompassing 23,363 cases and 187,840 controls from the FinnGen database.

The GWAS datasets used in this study underwent rigorous quality control procedures implemented by the original consortia. These procedures included imputation to a reference panel, filtering for single nucleotide polymorphisms (SNPs) with high imputation quality scores ($R^2 > 0.8$), and excluding variants with low minor allele frequency (MAF < 0.01) or high missingness rates. Additionally, sample overlap was minimized across datasets to prevent confounding effects, and results were derived from large, well-characterized cohorts with robust phenotypic and genotypic data.

The GWAS datasets, all publicly available with limited sample overlap, received ethical approval from the original studies. Table S1 provides details of the data used in this study.

Statistical Analysis

We performed two-sample MR analyses to determine the causal relationships between circulating immune cells, plasma metabolites, and coronary atherosclerosis. The principal method employed was the inverse variance weighted (IVW) method, a meta-analysis approach that combines Wald ratio estimates for each IV and restricts the intercept to zero.[17] Causal association effect estimates were presented as risk ratios with 95% confidence intervals for binary outcomes. Additional methods such as MR-Egger, weighted median (WM), simple mode, and weighted mode were employed as supplementary approaches to assess causal effects and correct for horizontal pleiotropy. Other sensitivity analysis methods, including MR-Egger, weighted median methods, and simple effects modeling, were employed to ensure the reliability of the results. The MR method allows testing for violations of the assumption of unbiasedness (ie, the absence of unobserved interactions of confounders between the instrumental variables and the outcome) and permits bias adjustment of the results.[18] The weighted median approach utilizes the median statistic to provide robust estimates even if up to 50% of the instrumental variables are subject to invalid effects.[19] Simple effects models estimate causality by directly using the predictive effects of individual instrumental variables on exposure and outcome, which is particularly effective whenever the underlying data satisfy strong instrumental variable assumptions.

Heterogeneity of the outcomes was assessed using the Cochran Q statistic, which evaluates the consistency of causal estimates across all instrumental variables (IVs).[20] Significant heterogeneity, indicated by a high Cochran Q value ($p < 0.05$), suggests potential violations of the Mendelian Randomization (MR) assumptions, such as horizontal pleiotropy or variability in the strength of genetic instruments. Such heterogeneity implies that the genetic variants may influence the outcome through pathways independent of the exposure of interest. To further explore the robustness of the results and the influence of individual single nucleotide polymorphisms (SNPs), leave-one-out sensitivity analysis was conducted. This method systematically removes each SNP and re-evaluates the causal estimates, identifying whether any single

variant disproportionately drives the overall results. In addition, the potential horizontal pleiotropy between instrumental variables (IVs) and outcomes was further tested through the MR-Egger intercept test and the Mendelian randomized pleiotropic residuals (MR-PRESSO) global test.

Finally, a two-step Mendelian Randomization (MR) analysis was conducted to investigate the potential mediating role of plasma metabolites in the causal pathway between circulating immune cells and coronary atherosclerosis. This approach involved two steps: first, estimating the causal effect of circulating immune cells on plasma metabolites (β1), and second, estimating the causal effect of plasma metabolites on coronary atherosclerosis (β2). The direct effect of circulating immune cells on coronary atherosclerosis was denoted as β3. The percentage of the total effect mediated by plasma metabolites was calculated as the ratio of the indirect effect (β1 × β2) to the total effect (β3), providing a quantitative measure of the mediation effect. To ensure robust estimation of standard errors and confidence intervals, the bootstrap resampling method was employed. This method involved 10,000 iterations to account for variability in the estimated parameters, providing accurate and reliable effect estimates from the two-sample MR analysis.

Enrichment analysis of metabolites was conducted using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/). Additionally, two-sample Mendelian randomization analysis utilized R (version 4.2.1) with the "TwoSampleMR (0.5.6)" and "MR-PRESSO (1.0)" packages.

An OR greater than 1 indicated a positive association, where increased exposure corresponded to a higher likelihood of the outcome. Conversely, an OR less than 1 suggested a protective effect, while an OR of 1 indicated no association. Statistical significance was defined as $P < 0.05$, providing strong evidence against the null hypothesis. Associations with $0.05 \leq P < 0.10$ were considered suggestive and warranting further investigation.

Results

Selection of IVs

We screened 731 immune cell phenotypes and 1400 plasma metabolite GWAS data for IVs in this study, ensuring all had F values greater than 10, eliminating weak instrumental variable bias. The number of SNPs screened for all positive results is detailed in Tables S2 and S3.

Identifying the Causal Effects of Circulating Immune Cells on Coronary Atherosclerosis

Thirty-five immune cell types were found to have a causal role in coronary atherosclerosis with nominal significance. Higher levels of 19 immune cells and lower levels of 16 were linked to an increased risk of the disease. These included B cells (10 types), T cells (8 types), monocytes (6 types), dendritic cells (5 types), natural killer (NK) cells (3 types), granulocytes (2 types), and myeloid cells (1 type), as shown in Figure 2A. MR-Egger, weighted median, simple mode, and weighted mode analyses validated the IVW findings for all immune cells except CD127 on CD4+ T cells (Figure 2B). Additionally, the MR-Egger intercepts (Table S4) and the global test from MR-PRESSO (Table S5) confirmed the absence of horizontal pleiotropy, while Cochran's Q and Rucker's Q tests indicated significant heterogeneity for traits like myeloid dendritic cells, NK cells, and CX3CR1 on monocytes (Tables S6 and S7). To address this heterogeneity, we employed robust Mendelian Randomization (MR) methods, including MR-Egger regression and weighted median analysis, to ensure the reliability of the causal estimates. These methods provided consistent results with the primary analysis. Furthermore, leave-one-out sensitivity analysis demonstrated that removing individual single nucleotide polymorphisms (SNPs) did not substantially alter the causal estimates.

In the context of well-established risk factors for coronary atherosclerosis, including smoking, alcohol consumption, and hypertension, multivariate Mendelian randomization was performed to assess the strength of causal associations across 30 promising immune cell markers, while accounting for potential confounding variables. With inverse variance weighting (IVW) methods and complementary sensitivity analyses, a noteworthy inverse association was observed between 11 immune cell phenotypes and coronary atherosclerosis risk including B-cell %lymphocyte (OR = 0.919, 95% CI: 0.863–0.978, p =0.007), B-cell Absolute Count (OR = 0.920, 95% CI:0.865–0.979, p = 0.008), HLA DR on B cells (OR = 0.916, 95% CI: 0.860–0.975, p = 0.006), IgD+CD24+B-cell% lymphocyte (OR = 0.917, 95% CI:

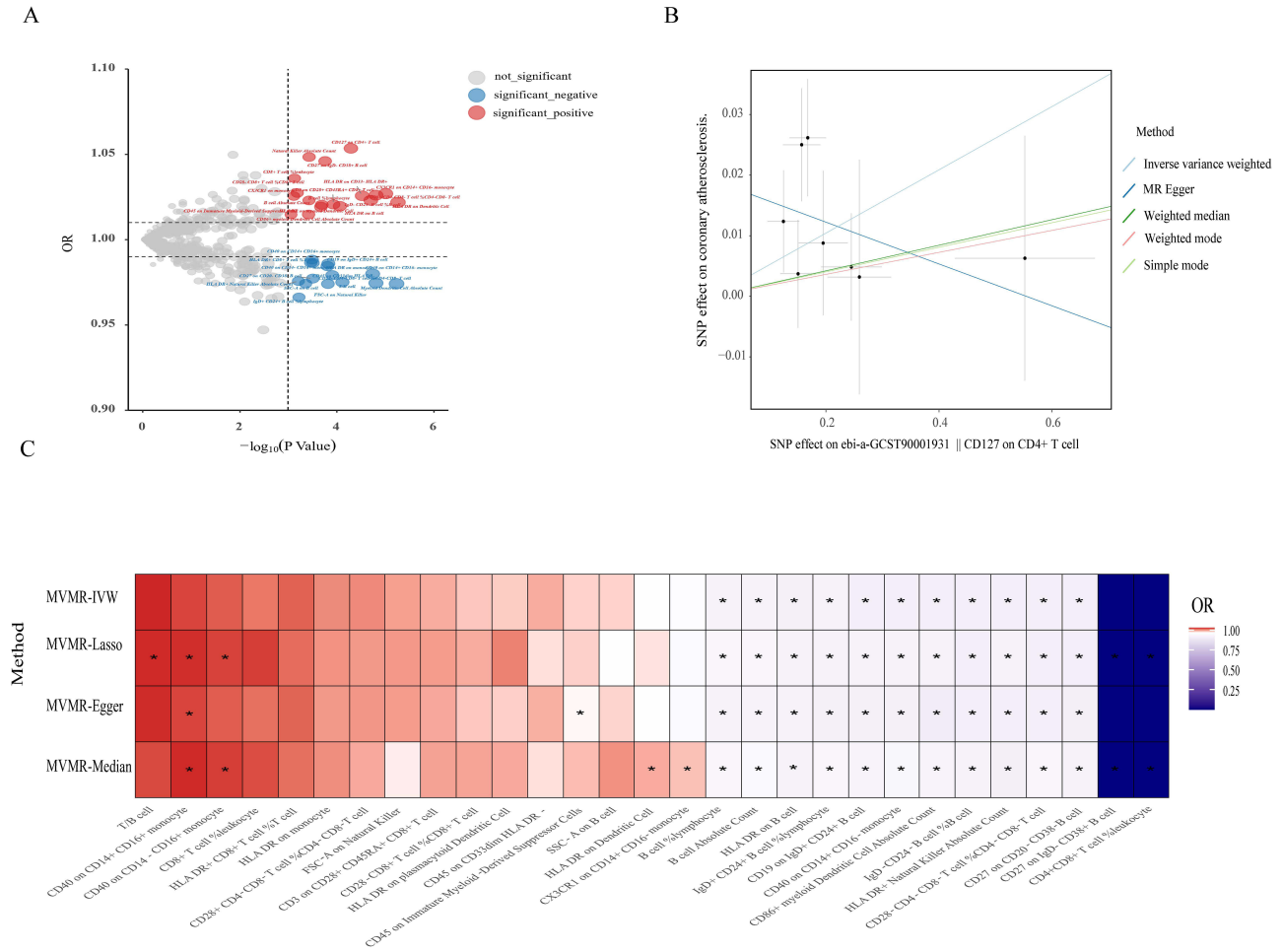


Figure 2 Causal associations between circulating immune cells and coronary atherosclerosis. (**A**) Volcano plot of two-sample MR analysis between immune cells and coronary atherosclerosis. (**B**) Scatter plot for causal associations between the effect of CD127 on CD4+ T cells effect of the outcome. (**C**) Heatmap of multivariate MR analysis between 29 promising immune cells and coronary atherosclerosis. Associations with *P* values ≤ 0.05 are depicted with *.

0.863–0.975, $p = 0.005$), CD19 on IgD+CD24+B cells (OR = 0.911, 95% CI: 0.857–0.969, $p = 0.003$), CD40 on CD14+ CD16-monocytes (OR = 0.910, 95% CI: 0.856–0.968, $p = 0.003$), CD86+ myeloid Dendritic Cell Absolute Count (OR = 0.910, 95% CI: 0.855–0.967, $p = 0.003$), IgD-CD24-B cell %B cell (OR = 0.911, 95% CI: 0.856–0.970, $p = 0.003$), HLA DR+ Natural Killer Absolute Count (OR = 0.909, 95% CI: 0.855–0.966, $p = 0.002$), CD28-CD4-CD8-T cell%CD4-CD8 -T cell (OR = 0.911, 95% CI: 0.858–0.967, $p = 0.002$), CD27 on CD20-CD38-B cell (OR = 0.908, 95% CI: 0.854–0.965, $p = 0.002$) (Figure 2C and Table S8).

Reverse Mendelian randomization analysis was performed to assess the effects of coronary atherosclerosis on the immunophenotype. Significant reverse causal effects were detected on T/B cells (OR = 1.20, 95% CI: 1.03–1.41, p = 0.023), B-cell absolute counts (OR = 0.79, 95% CI: 0.68–0.93, p = 0.004), B-cell %lymphocyte (OR = 0.84, 95% CI: 0.71–0.98, p = 0.028), and HLA DR on dendritic cells (OR = 0.83, 95% CI: 0.69–0.99, p = 0.036) (Figure 3 and Table S9).

Identifying Causal Effects of Plasma Metabolites on Coronary Atherosclerosis

With extensive metabolomics data, we examined the causal link between genetically predicted metabolic profiles and coronary atherosclerosis. Table S10 outlines the serum metabolic factors identified as either deleterious or protective. IVW analysis identified 68 metabolites with protective effects and another 68 with adverse effects on coronary atherosclerosis. Multivariate MR analysis further confirmed significant roles of 42 plasma metabolites (Table S14). Specifically, elevated levels of acetylcarnitine (C2) (OR = 1.119, 95% CI: 1.035–1.209, $p = 0.005$), 3-hydroxy-2-ethylpropionate (OR = 1.124, 95% CI: 1.048–1.206, $p = 0.001$), 1-ribosyl-imidazoleacetate (OR = 1.084, 95% CI: 1.016–1.157, p = 0.014), and 1-stearoyl-

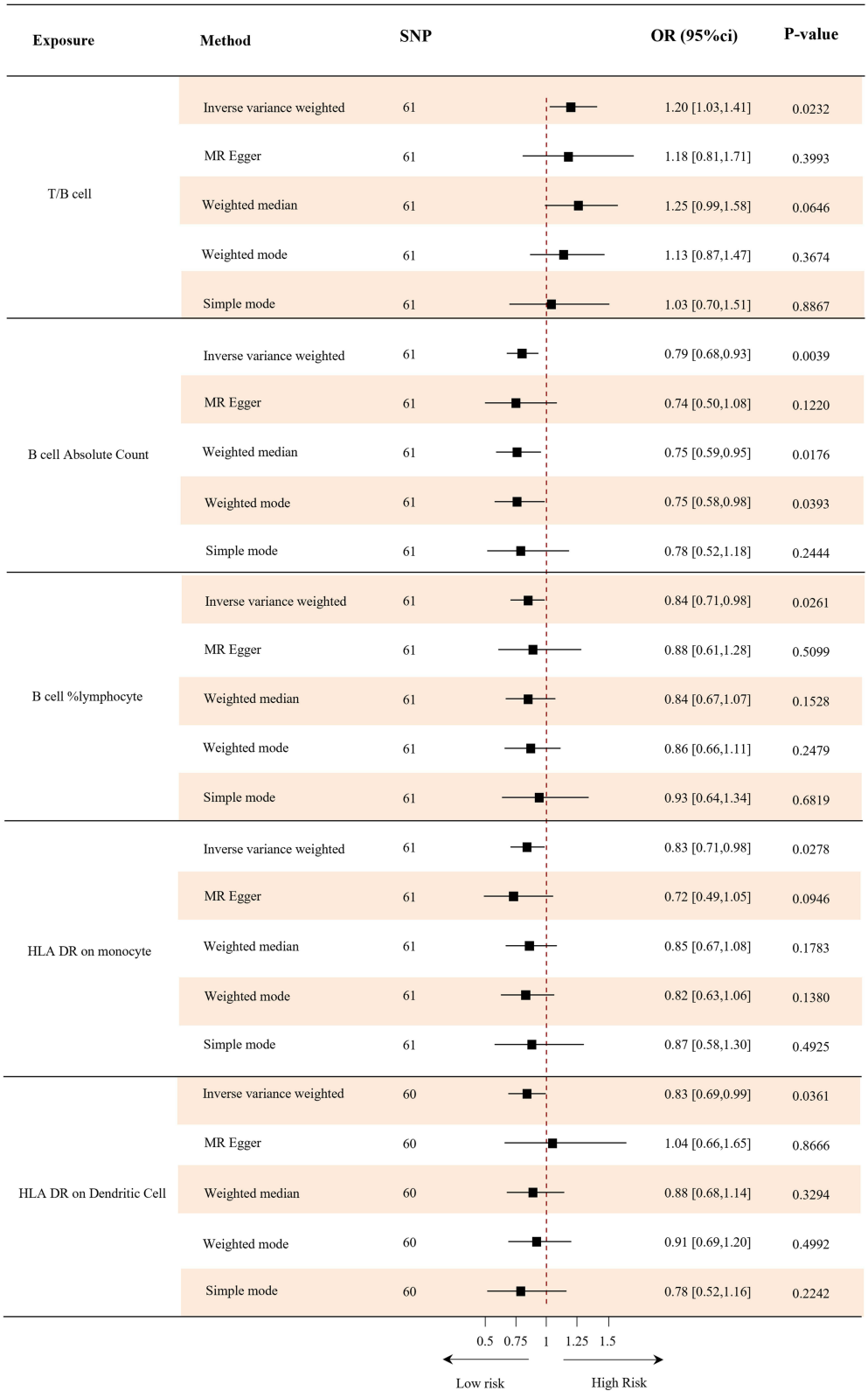


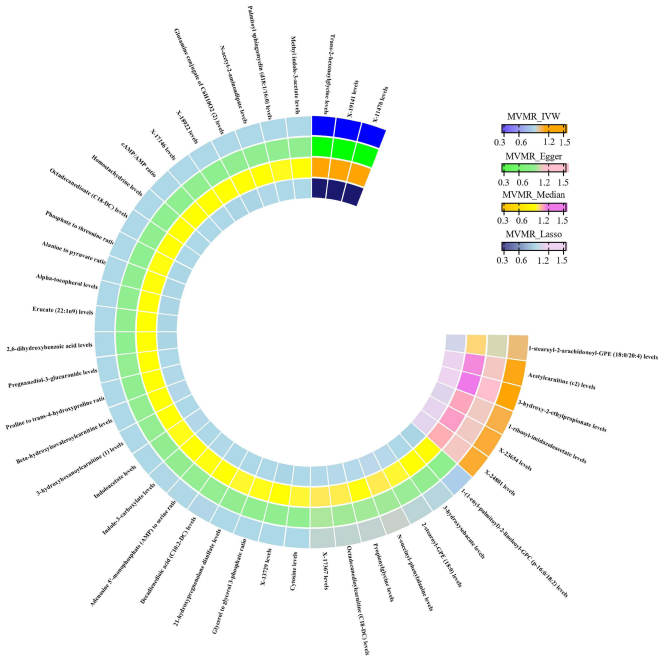
Figure 3 Causal associations between coronary atherosclerosis and circulating immune cells according various methods.

https://doi.org/10.2147/ITT.S508042

2-arachidonoyl-GPE (18:0/20:4) (OR = 1.037, 95% CI: 1.000–1.074, $p = 0.047$) were linked to increased risk. Additionally, metabolites X-23654 (OR = 1.097, 95% CI: 1.016–1.185, $p = 0.018$) and X-24801 (OR = 1.102, 95% CI: 1.031–1.178, $p = 0.004$) showed significant promotive effects, while 36 other metabolites were found to have significant adverse effects on coronary atherosclerosis (Figure 4A and Tables S11–S13).

Metabolic pathway analysis, as shown in Figure 4B and Table S15, identified four significant pathways involved in the pathogenesis of coronary atherosclerosis: "glycine and serine metabolism" (FDR < 0.001), "methionine metabolism" (FDR = 0.0021), "phenylalanine and tyrosine metabolism" (FDR = 0.0021), and "homocysteine degradation" (FDR = 0.0093). Finally, reverse MR showed a significant reverse causal effect of the proline to trans-4-hydroxyproline ratio (OR = 1.11, 95% CI: 1.00–1.22, $p = 0.044$) (Figure 4C).

A



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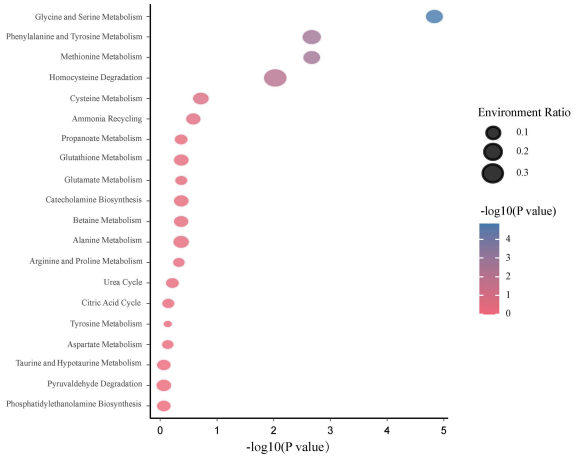

C

Outcome	Method	SNP	OR	P-value
Proline to trans-4-hydroxyproline ratio	Inverse variance weighted	65	1.11 (1.00-1.22)	0.044
	MR Egger	65	1.04 (0.79-1.37)	0.772
	Weighted median	65	1.05 (0.91-1.22)	0.508
	Weighted mode	65	1.02 (0.79-1.31)	0.903
	Simple mode	65	0.96 (0.70-1.32)	0.825

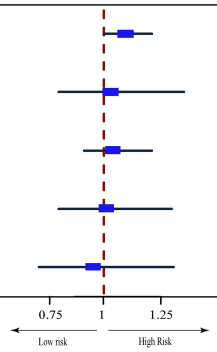

Figure 4 Causal associations between plasma metabolites and coronary atherosclerosis. (**A**) Heatmap of multivariate MR analysis between 42 promising plasma metabolites and coronary atherosclerosis. (**B**) Dot plot of metabolite pathway enrichment. (**C**) Forest plot for causal associations between coronary atherosclerosis and circulating immune cells by using different methods.

Mediation Analysis of Coronary Atherosclerosis by Circulating Immune Cells and Plasma Metabolites

Given the individual causal impacts of circulating immune cells and metabolites on coronary atherosclerosis, we conducted a two-step Mendelian Randomization (MR) analysis to investigate the potential mediating effects of plasma metabolites on the relationship between circulating immune cells and coronary atherosclerosis. Initially, we evaluated the causal effects of nine immune cell phenotypes on 41 plasma metabolites. Seven immune cells were found to have significant causal effects on plasma metabolites, and 12 plasma metabolites were identified as potential mediators (Figure 5). These included notable associations such as HLA DR+ Natural Killer Absolute Count and 3-hydroxysebacate levels (OR = 0.946, 95% CI: 0.901–0.994, p = 0.027), and CD19 expression on IgD+ CD24+ B cells with trans-2-hexenoylglycine levels (OR = 0.959, 95% CI: 0.927–0.991, p = 0.014). Subsequently, we calculated the indirect effects of circulating immune cells on coronary atherosclerosis mediated by these metabolites. While no mediators reached the conventional significance threshold of $P < 0.05$, two metabolites showed suggestive mediation effects at $P < 0.10$. This threshold was deemed reasonable given the exploratory nature of the analysis and the likelihood of subtle mediating effects in complex biological pathways. Specifically, 3-hydroxy-2-ethylpropionate was involved in the effect of IgD+ CD24+ B-cell % lymphocyte on coronary atherosclerosis, with a mediating effect of 0.961 (95% CI:0.955 −0.967), and the mediation ratio was 15.34% (Figure 6A). Additionally, CD19 on IgD+ CD24+ B cells appeared to exert a protective effect against atherosclerosis by mediating Trans-2-hexenoylglycine levels, with a mediating effect of 0.983 (95% CI:0.981–0.986) and a mediating ratio of 16.7% (Figure 6B).

Discussion

Extensive published genetic data were employed in this study to investigate the causal relationship between 731 immune cell characteristics and 1400 plasma metabolites in coronary atherosclerosis. To date, this is the pioneering MR study to

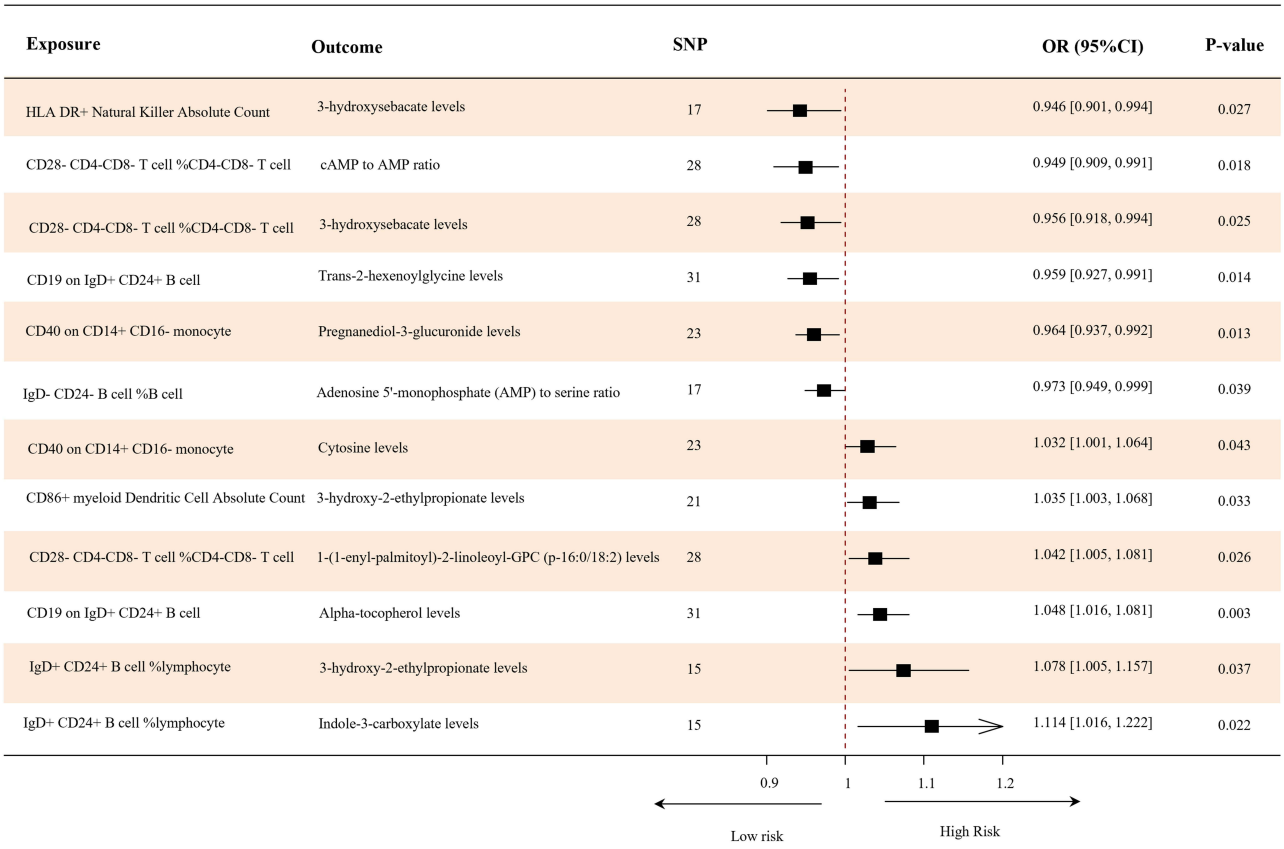

Figure 5 Inverse variance weighted Mendelian randomization estimation of the association between positive circulating immune cells and plasma metabolites.

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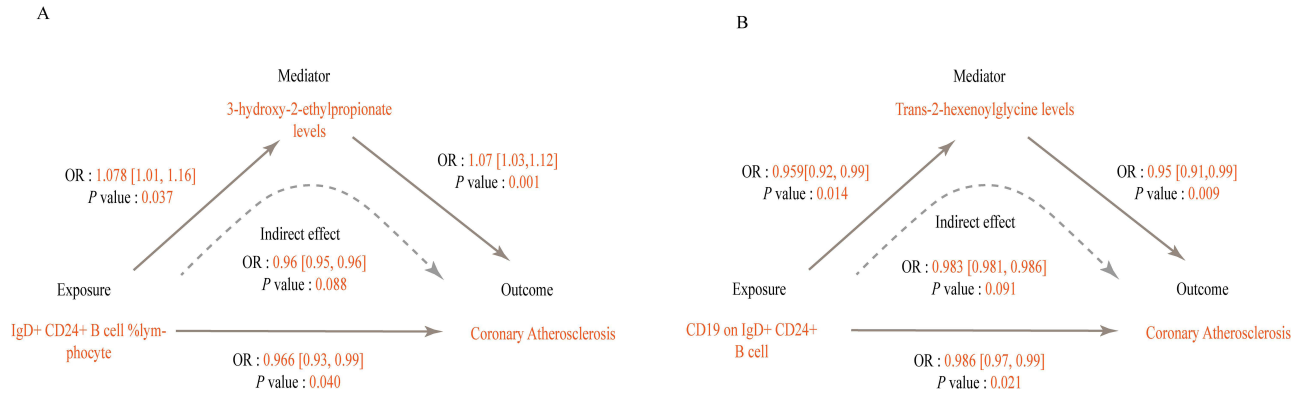

Figure 6 Mediation analysis of the impact of circulating immune cells on coronary atherosclerosis using a two-step Mendelian randomization framework. The "Direct effect" refers to the impact of IgD+ CD24+ B-cell % lymphocytes and CD19 on IgD+ CD24+ B cells on coronary atherosclerosis risk, adjusted for the mediator (3-hydroxy-2-ethylpropionate levels in (**A**) or trans-2-hexenoylglycine levels in (**B**)). The "Indirect effect" denotes the impact of IgD+ CD24+ B-cell % lymphocytes and CD19 on IgD+ CD24+ B cells on coronary atherosclerosis risk mediated through 3-hydroxy-2-ethylpropionate levels in (**A**) or trans-2-hexenoylglycine levels in (**B**).

detect the causal association between multiple immune phenotypes and metabolites in coronary atherosclerosis. MR analyses definitively pinpointed 9 immune cell phenotypes potentially impacting coronary atherosclerosis, along with 41 pertinent plasma metabolites. Moreover, our findings suggest potential roles for seven immune cell types in modulating twelve specific metabolites.

Our study revealed that elevated levels of CD40 on CD14+ CD16- monocytes, HLA DR+ natural killer absolute count and CD86+ myeloid dendritic cell absolute count in the peripheral blood reduced the risk of atherogenesis. In particular, monocytes and macrophages the immune sentinels of the innate immune system, are categorized into three subpopulations based primarily on CD14 and CD16 expression: classical (CD14+ CD16-), intermediate (CD14+ CD16+), and atypical (CD14- CD16+).[21] Classical monocytes have been extensively studied in autoimmune diseases, and CD40 can enhance phagocytosis and anti-inflammatory cytokine production in these monocytes, thereby modulating the local inflammatory milieu and inhibiting the progression of atherosclerosis.[22,23] Moreover, HLA DR+ phenotypic natural killer cells were able to effectively remove apoptotic cells and other cellular debris, which prevented the accumulation of fatty streaks in the arteries, an indispensable function in the prevention of atherosclerosis, and our results further support a protective response to atherosclerosis by HLA DR+ natural killer cells.[24] Dendritic cells with CD86+ phenotype are critical in antigen presentation and T-cell activation, especially in the atherosclerotic milieu. It has been shown that these dendritic cells have a significant effect on disease management in atherosclerosis by enhancing T-cell responsiveness.[25]

Furthermore, our study revealed for the first time the protective role of specific B-cell subtypes in atherosclerosis. In particular, IgD+ CD24+ B cells and IgD- CD24- B cells have not been sufficiently explored in previous studies. Recent studies demonstrate that B-lymphocytes contribute critically to the pathogenesis of atherosclerosis by producing specific antibodies against oxidized LDL, thereby reducing foam cell formation and safeguarding vascular integrity from further damage.[26,27] CD24, as a special antigen on the surface of B lymphocytes, serves an important role in the proliferation, differentiation and signaling of B lymphocytes.[27] With an adoptive transfer assay in an ApoE$^{-/-}$ mouse model, Strom et al found that CD24-expressing B cells could inhibit atherogenesis in an IL-10-dependent manner[28] underscoring the potential protective role of CD24+ B cells in the regulation of atherosclerosis. In addition, the costimulatory receptor CD27 and the ligand CD70 are members of the TNF family of receptors and ligands that are predominantly found in T cells, B cells, and NK cells.[29,30] Both of which are vitally important in establishing T-cell responses and memory. In an experimental setting, mice lacking CD27 exhibited exacerbated atherosclerotic lesions and increased inflammation, underscoring the protective role of CD27 in cardiovascular health. Moreover, systemic and bone marrow-derived CD27 deficiency has been linked to a decrease in Treg abundance, further implicating CD27 in the modulation of atherosclerotic disease dynamics.[31] Our study not only confirmed the role of classical monocytes, HLA DR+ NK cells, and CD86+ myeloid dendritic cells in reducing atherosclerosis, but also firstly discloses the critical protective role of IgD+ CD24+ and IgD- CD24- B cells in atherosclerosis. These results not only extend our understanding of B cell function,

but also provide new directions for future therapeutic strategies, emphasizing the importance of these specific B-cell subtypes in protecting arterial health.

Next, we employed multivariate Mendelian randomization to investigate the dependent effects of 1400 plasma metabolites on coronary atherosclerosis. After adjusting for latent confounders of smoking, alcohol consumption, and hypertension, we identified 41 metabolites that were positively associated with coronary atherosclerosis. Our findings confirm the existence of a unique metabolic signature of atherosclerosis and further characterize a few pivotal metabolites and metabolic pathways involved in the pathogenesis of atherosclerosis. Notably, four metabolites in the amino acid metabolic pathway were found to have a significant causal relationship with atherosclerosis, emphasizing their possible contribution to the development of this cardiovascular disease. Many small molecules (eg, nitric oxide, dopamine, 5-hydroxytryptamine, polyamines, and glutathione) have irreplaceable physiological functions, since amino acids are not only the basic components of peptides and proteins, but are also precursors for many small molecules. During the development of atherosclerosis, amino acids serve as nutrients, immunomodulators, and antioxidants, affecting the structure and function of the arterial wall.[32] Glycine is a nonessential amino acid involved in glutathione synthesis and glycine/serine metabolism. In the human body, glycine is not only involved in the synthesis of peptide chains and proteins, but is also associated with the metabolism of one-carbon units through the glycine cleavage enzyme pathway, which in turn is involved in the synthesis of nucleic acids and other biomolecules.[33] Several studies have demonstrated that glycine and related metabolites can protect against atherosclerosis by suppressing diverse inflammatory pathways and reducing immune cell infiltration.[34,35] Oren Rom and colleagues observed a significant downregulation of glycine biosynthesis genes in the liver, particularly the expression of alanine-glyoxylate aminotransferase (AGXT), in models of atherosclerosis in both humans and nonhuman primates. Furthermore, dietary and genetic methods that limit the availability of glycine exacerbated the development of nonalcoholic fatty liver disease (NASH) and atherosclerosis. Conversely, supplementation with glycine or overexpression of AGXT was found to reverse these pathological changes,[36] suggesting a novel therapeutic target for coronary atherosclerosis.

Our research provides additional insight into the critical effects of methionine metabolism and homocysteine in the potential treatment of atherosclerosis. Methionine is a necessary amino acid that participates in the urea cycle and methionine metabolism. In humans, methionine is metabolized primarily through the methionine cycle, where it is broken down into homocysteine and other essential metabolites. Extensive research has established that elevated levels of homocysteine are closely related to a heightened risk of cardiovascular diseases like atherosclerosis. An increase in homocysteine is considered as affecting the development of atherosclerosis through several mechanisms, including promoting inflammatory responses, increasing oxidative stress, and impairing endothelial cell function.[37,38] Furthermore, metabolomic studies have demonstrated that the increase in homocysteine correlates with enhanced consumption of methionine in patients with atherosclerosis, suggesting hyperactivity in the methionine metabolic pathway in these individuals.[39] Recent studies indicate that increased intake of B vitamins correlates with a reduction in plasma homocysteine levels, thereby reducing the risk of cardiovascular disease.[40] These findings underscore the role of methionine and homocysteine metabolism in atherosclerosis and suggest that modulating this pathway may enhance cardiovascular health. In addition, we also detected a potential role for phenylalanine and tyrosine metabolism in atherosclerosis. Recent research highlights the intricate connection between phenylalanine and tyrosine metabolism and the development of atherosclerosis. Disorders such as phenylketonuria (PKU) and tyrosinemia, which disrupt these metabolic pathways, can significantly influence cardiovascular health. An imbalance in phenylalanine hydroxylase activity leads to an accumulation of phenylalanine and a consequential deficiency in tyrosine, the precursor for dopamine synthesis. This disruption in neurotransmitter balance extends beyond neurological impacts, influencing systemic functions such as vascular tone and blood pressure, key components in the pathogenesis of atherosclerosis.[41] Elevated phenylalanine levels and altered tyrosine metabolism may enhance oxidative stress and inflammatory responses, exacerbating the development of atherosclerotic lesions.[42] Moreover, inherent metabolic disorders affecting these amino acids often correlate with an increased risk of cardiovascular diseases, highlighting the necessity for balanced amino acid levels.

To investigate whether circulating immune cells contribute to atherogenesis through plasma metabolites, we employed a two-step Mendelian randomization approach. While no mediators were identified at the conventional

significance threshold of $P < 0.05$, relaxing the threshold to $P < 0.10$ revealed two suggestive mediators. This adjustment is commonly applied in exploratory studies to detect trends that may not achieve strict significance due to limited statistical power or the complexity of biological interactions.[43] The absence of significant mediators at $P < 0.05$ suggests that the mediating role of plasma metabolites may be subtle or constrained by sample size. However, using a relaxed threshold of $P < 0.10$ allows for the identification of trends that warrant further investigation, particularly in studies examining complex pathways or small effect sizes. Although this increases the risk of Type I errors, it reduces the likelihood of overlooking potential mediators that could offer valuable insights into causal mechanisms. These findings should therefore be interpreted cautiously and validated in larger studies. Notably, the levels of 3-hydroxy-2-ethylpropionate mediated the influence of the IgD+ CD24+ B-cell ratio on coronary atherosclerosis, demonstrating a mediating effect of 0.961. This finding suggested that this metabolite might modulate disease progression through its impact on the functionality of this specific B-cell subset. Additionally, CD19 expression on IgD+ CD24+ B cells could play a protective role against atherosclerosis by regulating trans-2-hexenoylglycine levels, evidenced by a mediating effect of 0.983 and a mediation ratio of 16.7%. This finding underscores the potential role of CD19 in regulating specific metabolic pathways, which could be pivotal in combatting atherosclerosis.

Despite the strengths of our study, several limitations must be acknowledged. First, the exploratory nature of the analysis and the adoption of a relaxed significance threshold ($P < 0.10$) increase the risk of Type I errors, and the findings should be interpreted with caution until validated in larger, independent cohorts. Second, while the two-sample Mendelian randomization approach assumes no horizontal pleiotropy, residual confounding from pleiotropic effects cannot be completely excluded despite conducting sensitivity analyses. Third, the study utilized metabolite and immune cell data from specific populations, which may limit the applicability of the findings to other ethnic or demographic groups. Lastly, although potential mediators were identified, this study does not elucidate the precise biological mechanisms underlying these associations, which will require further experimental investigation.

Conclusion

In conclusion, this study identified multiple immune cell phenotypes and plasma metabolites as key contributors to the pathogenesis of atherosclerosis through extensive Mendelian randomization analyses. In addition to uncovering the mediating roles of 3-hydroxy-2-ethylpropionate and trans-2-hexenoylglycine via specific B-cell phenotypes, we also highlighted protective roles of specific immune cell subtypes, including CD40+ monocytes and HLA DR+ NK cells. Furthermore, the identification of amino acid metabolic pathways, such as glycine and methionine metabolism, underscores their potential as novel therapeutic targets. These findings provide comprehensive insights into immune-metabolite interactions in atherosclerosis and open new directions for future research and therapeutic strategies.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

Ethics Statement

According to items 1 and 2 of Article 32 of "The Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects" (effective February 18, 2023), this study is exempt from ethical review and approval. The exemption applies as the research exclusively utilized summary statistics obtained from publicly available GWAS studies, which do not involve the collection or use of individual-level human data.

Acknowledgments

We thank all studies for providing genotype and phenotype data supporting the original work of all GWASs used in the study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work is supported by the grants National Natural Science Foundation of China (No. 82271624 and No. 82073681), Key R&D projects in Hebei Province (No. 19277762D), Key Program of Hebei Natural Science Foundation for Precision Medicine (No. H2022206300), and Key Science and Technology Research Projects of Colleges and Universities in Hebei Province (No. ZD2019100). Hebei Natural Science Foundation (No. H2022206327).

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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