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Original Article

Causal effects and metabolite mediation of immune cells in preterm birth: a Mendelian randomization study

Tong Zhou[1,2*], Yanqiu Zhang[3*], Sheng Zhang[1], Jun Cao[1], Bin Feng[1], Jieyu Jin[1], Qingqin Tang[1], Jun Qiu[1], Longwei Qiao[4], Yuting Liang[1,5]

[1]Center for Clinical Laboratory, The First Affiliated Hospital of Soochow University, Suzhou 215002, Jiangsu, China; [2]Medical College of Soochow University, Suzhou 215123, Jiangsu, China; [3]School of Pharmacy, Anhui Medical University; Key Laboratory of Anti-inflammatory and Immune Medicine, Ministry of Education; Institute of Clinical Pharmacology, Anhui Medical University, Hefei 230032, Anhui, China; [4]Center for Reproduction and Genetics, School of Gusu, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Nanjing Medical University, Suzhou 215002, Jiangsu, China; [5]Molecular Oncology Laboratory, Department of Orthopedic Surgery and Rehabilitation Medicine, The University of Chicago Medical Center, Chicago, IL 60637, USA. [*]Equal contributors.

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Abstract: Background: Preterm birth poses significant risks to neonatal health. Although immune dysregulation has been implicated in its etiology, the causal roles of specific immune cell phenotypes and the potential mediating effects of metabolites remain unclear. This study applied Mendelian randomization (MR) to investigate causal relationships between immune cell phenotypes and preterm birth, and to assess whether plasma metabolites mediate these associations. Methods: Two-sample and mediation MR analyses were performed using genetic variants from genome-wide association studies (GWAS) of immune cells and plasma metabolites. Causal estimates were primarily derived using inverse variance weighting (IVW), with sensitivity analyses conducted via MR-Egger, MR-PRESSO, and leave-one-out validation. Results: A total of 28 immune cell phenotypes and 47 metabolites were robustly associated with preterm birth ($P < 0.05$). Reverse MR analysis revealed no evidence of reverse causality for the identified immune phenotypes. Among these, $CD28^-$ $CD8^{br}$ AC exhibited the strongest association with increased preterm birth risk. Mediation analysis demonstrated that the effect of $CD28^-$ $CD8^{br}$ AC on preterm birth (total effect: 0.148; IVW OR [95% CI]: 1.160 [1.056-1.274], $P = 0.002$) was partially mediated by isoleucine levels (mediation proportion: 6.79%; $P = 0.027$) and the acetylcarnitine-to-propionylcarnitine (C2/C3) ratio (mediation proportion: 7.35%; $P = 0.029$). Sensitivity analyses confirmed the robustness of these findings. Conclusion: This study establishes causal links between immune cell phenotypes, metabolites, and genetic susceptibility to preterm birth. Specifically, $CD28^-$ $CD8^{br}$ AC may elevate preterm birth risk through modulation of isoleucine and the C2/C3 ratio, providing novel insights into disease mechanisms and potential therapeutic targets.

Keywords: Mendelian randomization, immune cells, metabolites mediator, preterm labour and delivery

Introduction

Preterm birth, defined as delivery before 37 weeks of gestation, is a common pregnancy complication. Epidemiological studies report that preterm birth occurs in approximately 12% of pregnancies worldwide [1]. In recent years, the global incidence of preterm birth has risen, making it the second leading direct cause of mortality among children under five years of age [2]. This condition not only significantly affects neonatal health but also imposes a growing socioeconomic burden. The pathogenic mechanisms of preterm birth are not fully understood and are currently considered a complex syndrome arising from multiple factors, including infection or inflammation, uteroplacental ischemia or hemorrhage, excessive uterine distension, stress, and immune-mediated processes [3]. Given the involvement of both innate and adaptive immunity in maintaining pregnancy tolerance, recent research has increasingly highlighted the critical role of immune dysfunction in the pathogenesis of pre-

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term birth [4, 5]. Therefore, a thorough investigation into the contributions of immune cells to the onset and progression of preterm birth is essential for advancing diagnostic and therapeutic approaches.

Pregnancy entails a finely regulated maternal immune response, in which maternal effector T cells, regulatory T cells (Tregs), and macrophages play crucial roles [6]. Clinical studies have reported reductions in both the number and function of Tregs in the maternal circulation of women undergoing preterm birth [7]. Macrophage depletion during pregnancy has been shown to impair fetal development and lead to early pregnancy loss [8]. Additionally, elevated levels of invariant natural killer T (iNKT) cells have been observed in pregnant women with pregnancy complications compared to those with normal pregnancies [9]. Although previous studies have explored associations between immune cells and preterm birth, the molecular mechanisms through which these cells contribute to preterm birth remain incompletely characterized, and the regulatory functions of other immune cell types in the initiation and progression of preterm birth have yet to be elucidated.

Beyond the direct involvement of immune cells, metabolic factors are also recognized as significant contributors to the risk of preterm birth. Growing evidence suggests that metabolites may act as key mediators through which immune cells influence disease pathogenesis. Studies have identified significant differences in metabolites related to amino acid metabolism, liver function, and fatty acid metabolism between preterm and term cases [10]. Maternal lipid profiles have been linked to preterm birth risk, with most studies indicating that dyslipidemia increases this risk [11, 12]. However, most existing studies are retrospective or observational in nature. Thus, the causal relationship between metabolites and preterm birth remains unclear, and the specific mechanisms by which immune cells may modulate metabolite levels or activities to influence preterm birth have not been established.

Mendelian randomization (MR) is an innovative statistical approach that uses genetic variants as instrumental variables (IVs) to minimize biases from confounding and reverse causality commonly present in conventional observational studies [13, 14]. MR has emerged as a robust methodological framework for assessing causal relationships between exposures and outcomes. It typically employs genetic variants, particularly single nucleotide polymorphisms (SNPs), as IVs [15]. These instruments serve as proxies for modifiable exposures, allowing for more precise and reliable estimation of causal effects compared to traditional observational analyses. This study aims to systematically evaluate the causal associations between immune cell phenotypes and preterm birth and to investigate the potential mediating role of plasma metabolites.

Materials and methods

Study design

Based on a bidirectional MR analysis using two independent samples and mediation analysis framework, this study systematically assessed the causal relationship between immune cell phenotypes and genetic predisposition to preterm birth, and further investigated the potential mediating role of plasma metabolites [16, 17]. We first conducted two-sample MR analyses to estimate the causal effects of immune cell phenotypes on preterm birth while evaluating and excluding potential reverse causation. Subsequently, we assessed the causal relationships between plasma metabolites and preterm birth, as well as the causal effects of preterm birth-related immune cell phenotypes on preterm birth-associated plasma metabolites. Finally, we quantified the indirect effects of immune cell phenotypes on preterm birth mediated through specific metabolite. The overall study design is illustrated in **Figure 1**.

Data sources

The details of all GWAS summary statistics data used in this study are summarized in **Table 1**. All datasets were based on European populations. Genetic data for 731 immune cell phenotypes were obtained from a cohort study including 3,757 individuals of European ancestry (accession numbers: GCST90001391 to GCST90002121), which comprised approximately 22 million genetic variants [18]. These immune cell phenotypes were categorized into six panels: B cells, conventional dendritic cells (cDCs), T cell maturation stages, monocytes, myeloid cells, TBNK (B cells, natural killer cells, T cells), and regulatory T cells (Tregs). They were

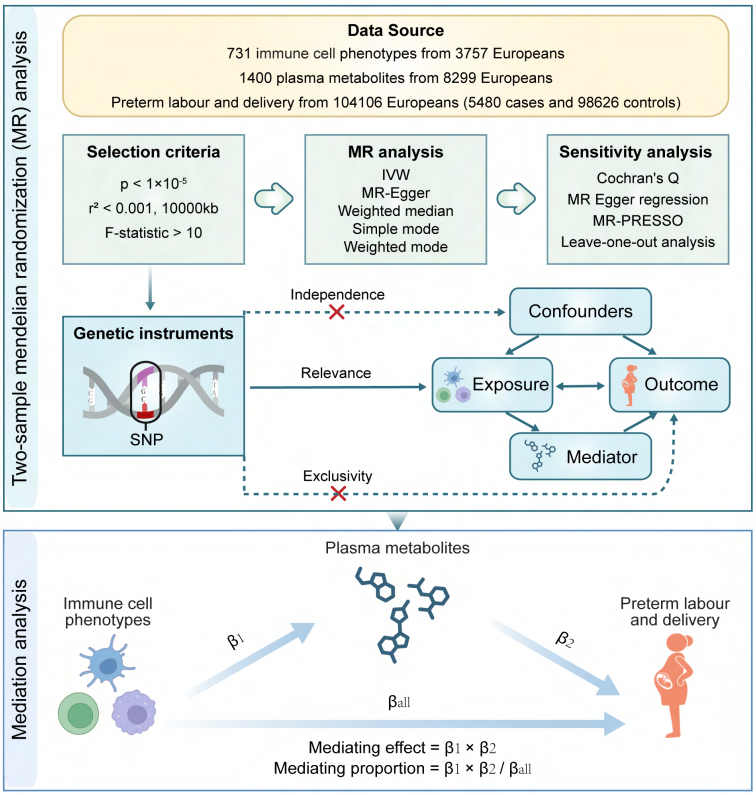


Figure 1. Flowchart of Mendelian Randomization (MR) analysis conducted in this study.

further classified into four measurement types: absolute cell count (ACC, n = 118), median fluorescence intensity (MFI, n = 389), morphological parameters (MP, n = 32), and relative cell count (RCC, n = 192).

Data on 1,400 metabolites were sourced from a GWAS comprising 8,299 European participants (accession numbers: GCST90199621 to GCST90201020), including 1,091 plasma metabolites and 309 metabolite ratios [19].

Summary statistics for preterm birth were derived from the FinnGen consortium (finn-b-015_PRETERM; https://www.finngen.fi/en). This dataset defines preterm birth as overall preterm delivery without distinguishing between spontaneous and medically indicated subtypes. The analysis included 104,106 European-ancestry participants (5,480 cases and 98,626 controls) and 16,379,340 single nucleotide polymorphisms (SNPs). Cases were defined as females with a documented history of preterm birth, while controls consisted of females with no history of preterm delivery.

Identification of instrumental variables

The identification process of IVs in MR analysis adheres to three fundamental assumptions (relevance, independence, and exclusivity assumptions). First, SNPs strongly associated with exposure factors were selected as IVs ($P < 1 \times 10^{-5}$). This threshold was chosen to secure a sufficient number of instruments while maintaining a strong genetic association with the exposure. Linkage disequilibrium (LD) pruning was conducted with a distance threshold of 10,000 kb and an R^2 threshold of 0.001 to identify independent SNPs [20]. Additionally, the F-statistic, an indicator of the instrument's strength, was calculated using the following formula: $F = (N - K - 1)/K \times R^2/(1 - R^2)$, where N denotes the total sample size, K represents the number of SNPs, and R^2 suggests the variance explained by all SNPs, and SNPs with an F-statistic below 10 were excluded to minimize bias arising from weak instruments [21]. Furthermore, to enhance the accuracy of the analysis, SNPs that could potentially influence the outcome through pleiotropic effects were excluded. This systematic identification process establishes a robust IV foundation for MR analysis, thereby improving its reliability in evaluating the causal relationship between exposure factors and outcome variables.

Mendelian randomization analysis

In this study, five MR methods - inverse variance weighting (IVW), MR-Egger, weighted median, simple mode, and weighted mode - were employed to assess potential causal relationships between immune cell phenotypes, plasma metabolites, and preterm birth risk [22-25]. Among these, the IVW method was selected as the primary analytical approach due to its robustness and widespread acceptance in causal inference. This method was applied to estimate the effect of each SNP on both expo-

Table 1. Summary of GWAS datasets used in this study

Traits	Datasets source	Sample size Total (cases/controls)	Ancestry	Accession codes
731 immune cell phenotypes	Cucca et al. [18]	3,757	European	GCST90001391 - GCST90002121
1091 plasma metabolites and 309 plasma metabolite ratios	Richards et al. [19]	8,299	European	GCST90199621 - GCST90201020
Preterm labour and delivery	FinnGen	104,106 (5,480/98,626)	European	finn-b-O15_PRETERM

sure and outcome, with weights assigned based on their variances to derive pooled causal estimates [22]. To address potential reverse causality, reverse MR analyses were also conducted to examine the effect of preterm birth on immune cell phenotypes, thereby validating the main findings.

Sensitivity analysis

Sensitivity analyses were performed to evaluate the potential influence of heterogeneity and pleiotropy on the accuracy of the causal estimates [26]. Heterogeneity among the selected IVs was assessed using Cochran's Q statistic based on both the IVW and MR-Egger methods, with a *p*-value greater than 0.05 indicating the absence of significant heterogeneity. Horizontal pleiotropy was examined via the MR-Egger intercept test and the MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) method; if the *p*-value greater than 0.05, horizontal pleiotropy is considered to be absent [27]. In addition, the MR-PRESSO framework was applied to identify and remove outlying variants, after which causal estimates were reassessed. Leave-one-out sensitivity analysis was conducted to determine whether the overall causal estimate was driven by any single SNP [28]. Funnel plots were also generated to visually inspect the symmetry of genetic associations and further evaluate potential heterogeneity across IVs.

Analysis of mediating effect

Within the framework of MR analysis, a series of systematic and rigorous steps were implemented to investigate the potential mediating role of plasma metabolites in the relationship between immune cell phenotypes and preterm birth. First, we used two-sample MR analysis to estimate the total effect (βall) of immune cell phenotypes (exposure) on preterm birth (outcome). Second, we applied two-sample MR to assess the causal effect (β_1) of selected immune cell phenotypes (exposure) on plasma metabolites (mediator), as well as the causal effect (β_2) of these metabolites on preterm birth (outcome). After confirming the direction and consistency of these effects along the hypothesized pathway, the indirect (mediated) effect was quantified using the coefficient product method ($\beta_1 \times \beta_2$). The proportion of the total effect mediated by metabolites was then calculated as ($\beta_1 \times \beta_2$)/βall [29].

Statistical analysis

All statistical analyses and data visualization were performed using R software (version 4.3.1) with the following packages: TwoSampleMR, MR-PRESSO, forestploter, and ggplot2. "TwoSampleMR" package was utilized for conducting two-sample MR analysis, facilitating data integration and result visualization. "MR-PRESSO" package was employed to detect and correct for horizontal pleiotropy, ensuring the robustness of causal inference. "forestploter" package was used for generating forest plots. "ggplot2" package was used for general data visualization, generating statistical plots. Associations are presented as odds ratios (OR) with 95% confidence intervals (CI). A significance threshold of $P < 0.05$ was applied. These analytical approaches were implemented to systematically evaluate the potential causal relationships between immune cell phenotypes and preterm birth.

Results

Causality between immune cell phenotypes and preterm labour and delivery

Using two-sample MR analysis, this study systematically investigated the potential causal relationships between multiple immune cell phenotypes and the risk of preterm labour and delivery. To ensure the robustness and reliability of the analysis results, MR-Egger regression

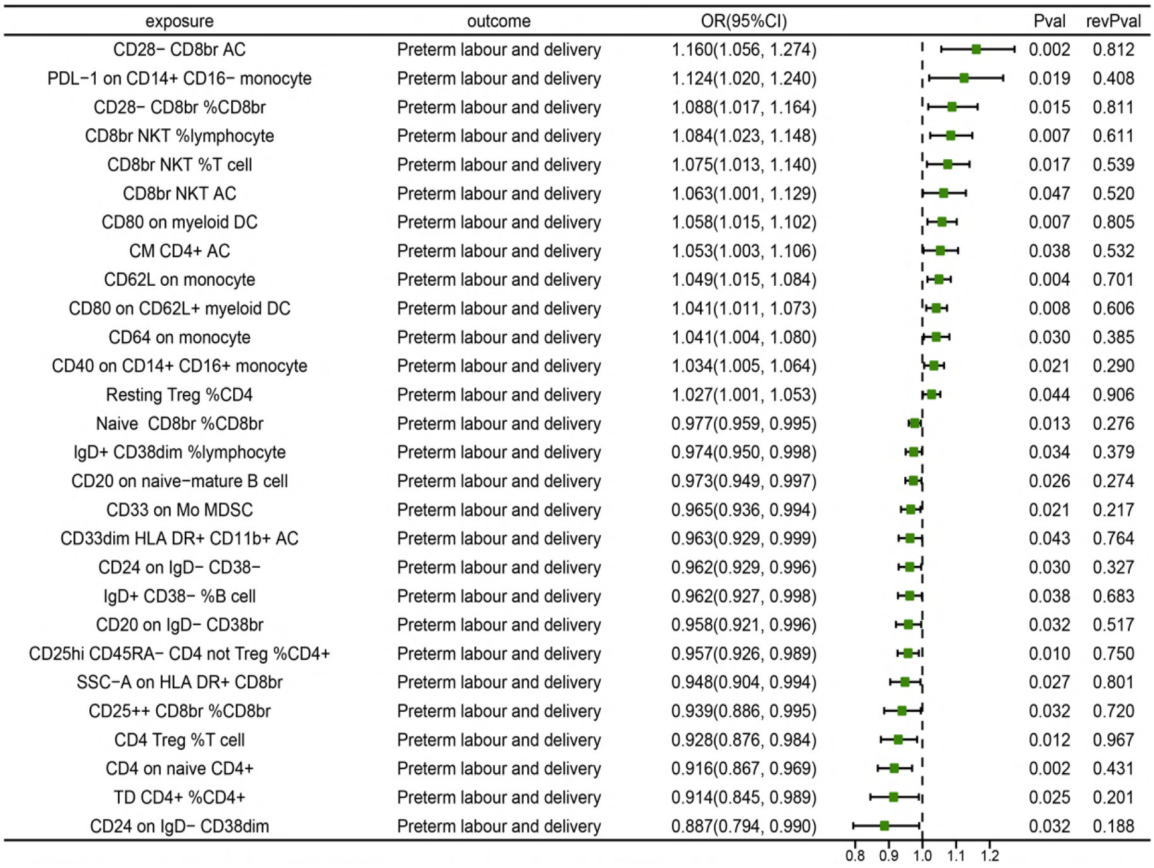


Figure 2. Forest plot of causal associations between immune cell phenotypes and preterm labour and delivery.

was employed to detect and mitigate biases arising from horizontal pleiotropy. Following an initial screening process, 28 immune cell phenotypes were identified as being significantly associated with the risk of preterm labour and delivery ($P < 0.05$) (**Figure 2**). Whereas 13 were potential risk factors, involving $CD28^{-}$ $CD8^{br}$ AC (OR [95% CI]: 1.160 [1.056-1.274], P = 0.002), PDL-1 on $CD14^{+}$ $CD16^{-}$ monocyte (OR [95% CI]: 1.124 [1.020-1.240], P = 0.019), $CD28^{-}$ $CD8^{br}$ $\%CD8^{br}$ (OR [95% CI]: 1.088 [1.017-1.164], P = 0.015), $CD8^{br}$ NKT %lymphocyte (OR [95% CI]: 1.084 [1.023-1.148], P = 0.007), $CD8^{br}$ NKT %T cell (OR [95% CI]: 1.075 [1.013-1.140], P = 0.017), $CD8^{br}$ NKT AC (OR [95% CI]: 1.063 [1.001-1.129], P = 0.047), CD80 on myeloid DC (OR [95% CI]: 1.058 [1.015-1.102], P = 0.007), CM $CD4^{+}$ AC (OR [95% CI]: 1.053 [1.003-1.106], P = 0.038), CD62L on monocyte (OR [95% CI]: 1.049 [1.015-1.084], P = 0.004), CD80 on $CD62L^{+}$ myeloid DC (OR [95% CI]: 1.041 [1.011-1.073], P = 0.008), CD64 on monocyte (OR [95% CI]: 1.041 [1.004-1.080], P = 0.030), CD40 on $CD14^{+}$ $CD16^{+}$ monocyte (OR [95% CI]: 1.034 [1.005-1.064], P = 0.021), Resting Treg %CD4 (OR [95% CI]: 1.027 [1.001-1.053], P = 0.044). And 15 phenotypes were potential protective factors for preterm labour and delivery, including Naive $CD8^{br}$ $\%CD8^{br}$ (OR [95% CI]: 0.977 [0.959-0.995], P = 0.013), IgD^{+} $CD38^{dim}$ %lymphocyte (OR [95% CI]: 0.974 [0.950-0.998], P = 0.034), CD20 on naive-mature B cell (OR [95% CI]: 0.973 [0.949-0.997], P = 0.026), CD33 on Mo MDSC (OR [95% CI]: 0.965 [0.936-0.994], P = 0.021), $CD33^{dim}$ HLA DR^{+} $CD11b^{+}$ AC (OR [95% CI]: 0.963 [0.929-0.999], P = 0.043), CD24 on IgD^{-} $CD38^{-}$ (OR [95% CI]: 0.962 [0.929-0.996], P = 0.030), IgD^{+} $CD38^{-}$ %B cell (OR [95% CI]: 0.962 [0.927-0.998], P = 0.038), CD20 on IgD^{-} $CD38^{br}$ (OR [95% CI]: 0.958 [0.921-0.996], P = 0.032), $CD25^{hi}$ $CD45RA^{-}$ CD4 not Treg $\%CD4^{+}$ (OR [95% CI]: 0.957 [0.926-0.989], P = 0.010), SSC-A on HLA DR^{+} $CD8^{br}$ (OR [95% CI]: 0.948 [0.904-0.994], P = 0.027), $CD25^{++}$ $CD8^{br}$ $\%CD8^{br}$ (OR [95% CI]: 0.939 [0.886-0.995], P = 0.032), CD4 Treg %T cell (OR [95% CI]: 0.928 [0.876-0.984], P = 0.012), CD4 on naive $CD4^{+}$ (OR [95% CI]: 0.916

[0.867-0.969], P = 0.002), TD $CD4^+$ %$CD4^+$ (OR [95% CI]: 0.914 [0.845-0.989], P = 0.025), CD24 on IgD^- $CD38^{dim}$ (OR [95% CI]: 0.887 [0.794-0.990], P = 0.032).

Subsequently, a reverse MR analysis was performed, treating preterm labour and delivery as an exposure factor and 28 immune cells as the outcomes of the MR analysis. The IVW results showed that there was no reverse causal relationship between preterm labour and delivery and these 28 immune cells (P > 0.05), indicating that further analysis could be performed (**Figure 2**).

Causality between plasma metabolites and preterm labour and delivery

47 plasma metabolites were found to have causal association with preterm labour and delivery using IVW (P < 0.05) (**Table 2**). Among them, 5-dodecenoylcarnitine (C12:1) levels, Glycodeoxycholate levels, Acetylcarnitine (C2) to propionylcarnitine (C3) ratio, Arachidonate (20:4n6) to paraxanthine ratio, Adenosine 5'-monophosphate (AMP) to tyrosine ratio, Spermidine to ergothioneine ratio, X-12740 levels, Xylose levels, Adenosine 5'-monophosphate (AMP) to palmitate (16:0) ratio, Margaroylcarnitine (C17) levels, Sphingomyelin (d18:1/20:1, d18:2/20:0) levels, 8-methoxykynurenate levels, X-11372 levels, Hydroxy-N6,N6,N6-trimethyllysine levels, Arachidonate (20:4n6) to caffeine ratio, 12,13-DiHOME levels, Vanillic acid glycine levels, Adenosine 5'-diphosphate (ADP) to glucose ratio, Adenosine 5'-diphosphate (ADP) to ornithine ratio, 2-ketocaprylate levels, X-17690 levels, Pregnenetriol sulfate levels, N-acetylglucosaminylasparagine levels, (N(1) + N(8))-acetylspermidine levels, Androstenediol (3beta,17-beta) disulfate (1) levels, 3-methylcytidine levels, 5alpha-androstan-3alpha,17beta-diol monosulfate (1) levels, Androsterone sulfate levels were identified as risk factors for preterm labour and delivery (OR > 1).

On the other hand, Ferulic acid 4-sulfate levels, Pyridoxal levels, 1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1) levels, Isoleucine levels, Gamma-glutamylglutamine levels, 3-methyl catechol sulfate (1) levels, Phosphate to linoleoyl-arachidonoyl-glycerol (18:2 to 20:4) [2] ratio, Cholate to adenosine 3',5'-cyclic monophosphate (cAMP) ratio, Cysteinylglycine levels, 2-palmitoleoyl-GPC (16:1) levels, 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (p-16:0/20:4) levels, 5,6-dihydrothymine levels, Mannose to mannitol to sorbitol ratio, Mannose to fructose ratio, Adenosine 5'-monophosphate (AMP) to histidine ratio, Threonine to alpha-ketobutyrate ratio, Alpha-ketobutyrate levels, Dodecanedioate levels, Taurine to glutamate ratio were protective factors against preterm labour and delivery (OR < 1).

Mediating analysis reveals metabolite-mediated causal pathways linking immune cell phenotypes to preterm labour and delivery

Based on the OR values, $CD28^-$ $CD8^{br}$ AC was the most significant risk factor for preterm labour and delivery (OR [95% CI]: 1.160 [1.056-1.274], β_{all} = 0.148, P = 0.002) (**Table 3**; **Figure 3A**, **3B**). Furthermore, sensitivity analysis confirmed the robustness of the results. No significant horizontal pleiotropy was found (MR-Egger intercept (P = 0.872) and MR-PRESSO global pleiotropy test (P = 0.346)) (**Table 4**). No significant heterogeneity was observed, with p values of 0.316 and 0.253 for the Q-statistic in the IVW and MR Egger analysis, respectively (**Table 4**). Additionally, the funnel plot exhibited a predominantly symmetric distribution of causal effects, indicating minimal bias (**Figure 3C**). Leave-one-out sensitivity analyses confirmed that no single SNP significantly influenced the overall causal estimate (**Figure 3D**).

Therefore, $CD28^-$ $CD8^{br}$ AC was selected for in-depth subsequent analysis to further explore its mechanism in preterm labour and delivery. With $CD28^-$ $CD8^{br}$ AC as the exposure variable and 47 preterm labour and delivery-related plasma metabolites as the outcome variables, MR analysis was performed to estimate β_1. Based on the IVW estimates, a significant negative causal relationship was identified between $CD28^-$ $CD8^{br}$ AC and isoleucine levels (OR [95% CI]: 0.893 [0.839-0.950], β_1 = -0.113, P < 0.001) (**Table 3**; **Figure 4A**, **4C**), while a significant positive causal relationship was observed with C2/C3 ratio (OR [95% CI]: 1.080 [1.016-1.147], β_1 = 0.007, P = 0.013) (**Table 3**; **Figure 4B**, **4D**). In addition, we assessed the heterogeneity and pleiotropy of $CD28^-$ $CD8^{br}$ AC to isoleucine levels and C2/C3 ratio (**Table 4**). IVW and MR-Egger Cochran's Q test showed that there was no significant heterogeneity in the effect of $CD28^-$ $CD8^{br}$ AC on isoleucine level (P = 0.718 and 0.673, respectively) and on C2/

Table 2. Causal link between plasma metabolites and preterm labour and delivery

Exposure	Outcome	nsnp	OR (95% CI)	pval
5-dodecenoylcarnitine (C12:1) levels	Preterm labour and delivery	25	1.192 (1.049, 1.353)	0.007
Glycodeoxycholate levels	Preterm labour and delivery	20	1.163 (1.037, 1.304)	0.010
Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	Preterm labour and delivery	19	1.153 (1.001, 1.328)	0.049
Arachidonate (20:4n6) to paraxanthine ratio	Preterm labour and delivery	22	1.146 (1.039, 1.264)	0.006
Adenosine 5'-monophosphate (AMP) to tyrosine ratio	Preterm labour and delivery	18	1.140 (1.014, 1.281)	0.029
Spermidine to ergothioneine ratio	Preterm labour and delivery	19	1.139 (1.032, 1.256)	0.010
X-12740 levels	Preterm labour and delivery	13	1.139 (1.003, 1.293)	0.045
Xylose levels	Preterm labour and delivery	25	1.138 (1.019, 1.271)	0.022
Adenosine 5'-monophosphate (AMP) to palmitate (16:0) ratio	Preterm labour and delivery	19	1.136 (1.004, 1.286)	0.044
Margaroylcarnitine (C17) levels	Preterm labour and delivery	26	1.131 (1.023, 1.250)	0.016
Sphingomyelin (d18:1/20:1, d18:2/20:0) levels	Preterm labour and delivery	28	1.131 (1.031, 1.241)	0.009
8-methoxykynurenate levels	Preterm labour and delivery	24	1.118 (1.012, 1.236)	0.029
X-11372 levels	Preterm labour and delivery	27	1.117 (1.012, 1.232)	0.028
Hydroxy-N6,N6,N6-trimethyllysine levels	Preterm labour and delivery	26	1.116 (1.013, 1.229)	0.027
Arachidonate (20:4n6) to caffeine ratio	Preterm labour and delivery	23	1.115 (1.012, 1.227)	0.028
12,13-DiHOME levels	Preterm labour and delivery	28	1.112 (1.005, 1.232)	0.041
Vanillic acid glycine levels	Preterm labour and delivery	23	1.112 (1.033, 1.197)	0.005
Adenosine 5'-diphosphate (ADP) to glucose ratio	Preterm labour and delivery	16	1.107 (1.003, 1.222)	0.043
Adenosine 5'-diphosphate (ADP) to ornithine ratio	Preterm labour and delivery	23	1.103 (1.021, 1.191)	0.012
2-ketocaprylate levels	Preterm labour and delivery	23	1.097 (1.008, 1.194)	0.031
X-17690 levels	Preterm labour and delivery	27	1.095 (1.001, 1.198)	0.048
Pregnenetriol sulfate levels	Preterm labour and delivery	35	1.092 (1.018, 1.171)	0.014
N-acetylglucosaminylasparagine levels	Preterm labour and delivery	24	1.088 (1.014, 1.167)	0.019
(N(1) + N(8))-acetylspermidine levels	Preterm labour and delivery	35	1.081 (1.003, 1.165)	0.042
Androstenediol (3beta,17beta) disulfate (1) levels	Preterm labour and delivery	30	1.078 (1.000, 1.161)	0.049
3-methylcytidine levels	Preterm labour and delivery	19	1.073 (1.014, 1.136)	0.015
5alpha-androstan-3alpha,17beta-diol monosulfate (1) levels	Preterm labour and delivery	21	1.051 (1.005, 1.099)	0.031
Androsterone sulfate levels	Preterm labour and delivery	35	1.049 (1.006, 1.093)	0.024
Ferulic acid 4-sulfate levels	Preterm labour and delivery	33	0.963 (0.930, 0.998)	0.038
Pyridoxal levels	Preterm labour and delivery	41	0.925 (0.861, 0.994)	0.034
1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1) levels	Preterm labour and delivery	34	0.920 (0.854, 0.991)	0.027
Isoleucine levels	Preterm labour and delivery	22	0.915 (0.845, 0.991)	0.029
Gamma-glutamylglutamine levels	Preterm labour and delivery	30	0.914 (0.838, 0.997)	0.042
3-methyl catechol sulfate (1) levels	Preterm labour and delivery	27	0.907 (0.827, 0.996)	0.041
Phosphate to linoleoyl-arachidonoyl-glycerol (18:2 to 20:4) [2] ratio	Preterm labour and delivery	27	0.906 (0.839, 0.978)	0.012
Cholate to adenosine 3',5'-cyclic monophosphate (cAMP) ratio	Preterm labour and delivery	22	0.899 (0.808, 1.000)	0.050
Cysteinylglycine levels	Preterm labour and delivery	20	0.899 (0.816, 0.990)	0.030
2-palmitoleoyl-GPC (16:1) levels	Preterm labour and delivery	25	0.890 (0.801, 0.988)	0.029
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (p-16:0/20:4) levels	Preterm labour and delivery	22	0.883 (0.789, 0.988)	0.030
5,6-dihydrothymine levels	Preterm labour and delivery	19	0.879 (0.777, 0.995)	0.042
Mannose to mannitol to sorbitol ratio	Preterm labour and delivery	21	0.879 (0.778, 0.993)	0.038
Mannose to fructose ratio	Preterm labour and delivery	18	0.876 (0.771, 0.996)	0.043
Adenosine 5'-monophosphate (AMP) to histidine ratio	Preterm labour and delivery	23	0.874 (0.772, 0.990)	0.034
Threonine to alpha-ketobutyrate ratio	Preterm labour and delivery	19	0.833 (0.742, 0.936)	0.002
Alpha-ketobutyrate levels	Preterm labour and delivery	17	0.827 (0.717, 0.954)	0.009
Dodecanedioate levels	Preterm labour and delivery	22	0.822 (0.729, 0.926)	0.001
Taurine to glutamate ratio	Preterm labour and delivery	17	0.813 (0.692, 0.956)	0.012

C3 ratio (P = 0.948 and 0.923, respectively). Additionally, there was no horizontal pleiotropy in the effect of $CD28^{-}$ $CD8^{br}$ AC on isoleucine level (MR-Egger intercept (P = 0.579) and the MR-PRESSO global pleiotropy test (P = 0.744)) and on C2/C3 ratio (MR-Egger intercept (P = 0.820) and the MR-PRESSO global pleiotropy test (P = 0.960)). The funnel plots and leave-one-out analysis also indicated reliable data (**Figure 4E-H**).

Table 3. Mediation analyses for the association between metabolic mediators, immune cell phenotypes and preterm labour and delivery

Exposure	Outcome	Method	nsnp	β	OR (95% CI)	pval
CD28$^-$ CD8^{br} AC	Preterm labour and delivery	Inverse variance weighted	15	0.148	1.160 (1.056, 1.274)	0.002
CD28$^-$ CD8^{br} AC	Isoleucine levels	Inverse variance weighted	15	-0.113	0.893 (0.839, 0.950)	< 0.001
CD28$^-$ CD8^{br} AC	Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	Inverse variance weighted	15	0.077	1.080 (1.016, 1.147)	0.013
Isoleucine levels	Preterm labour and delivery	Inverse variance weighted	22	-0.089	0.915 (0.845, 0.991)	0.029
Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	Preterm labour and delivery	Inverse variance weighted	19	0.142	1.153 (1.001, 1.328)	0.049

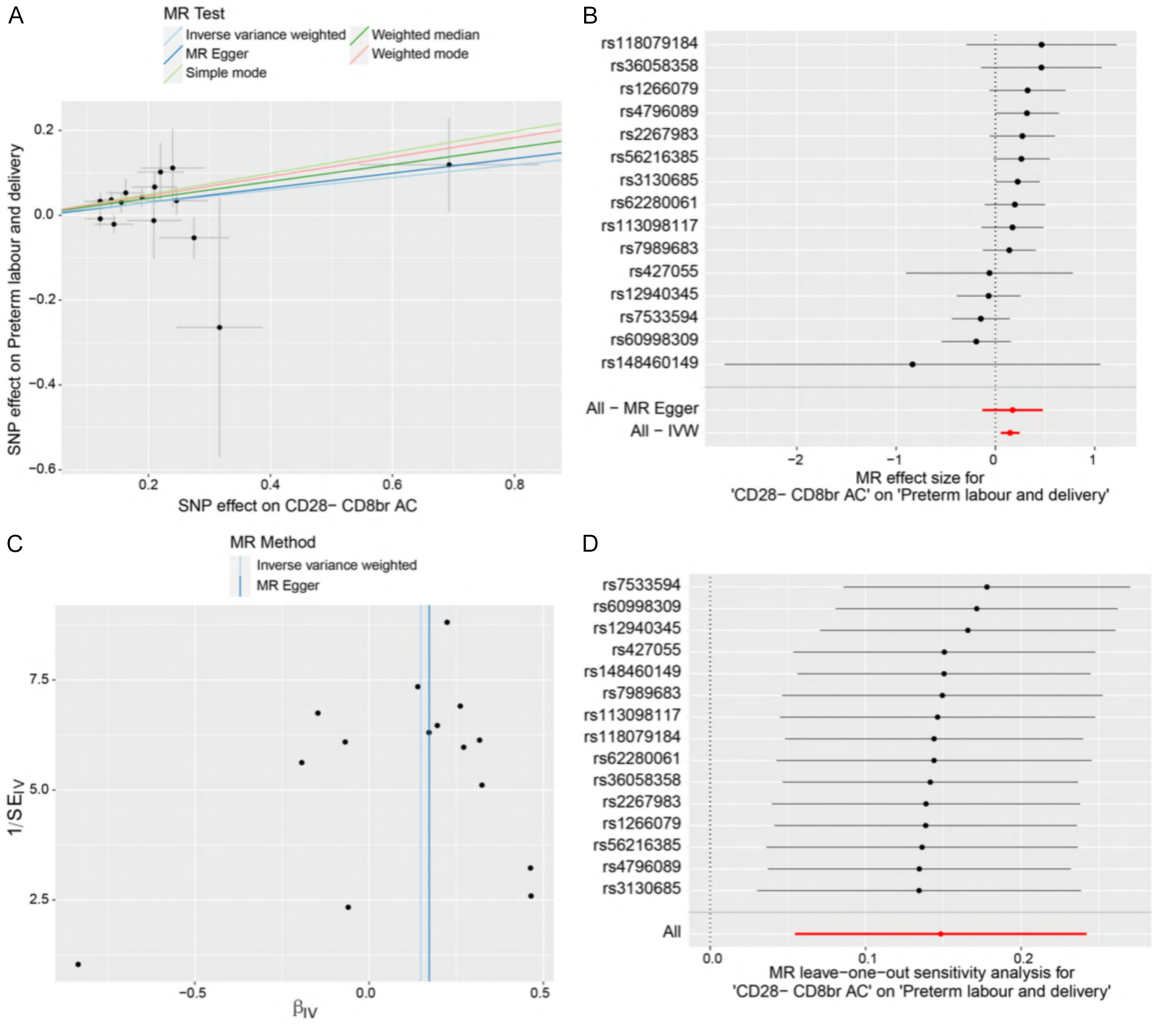
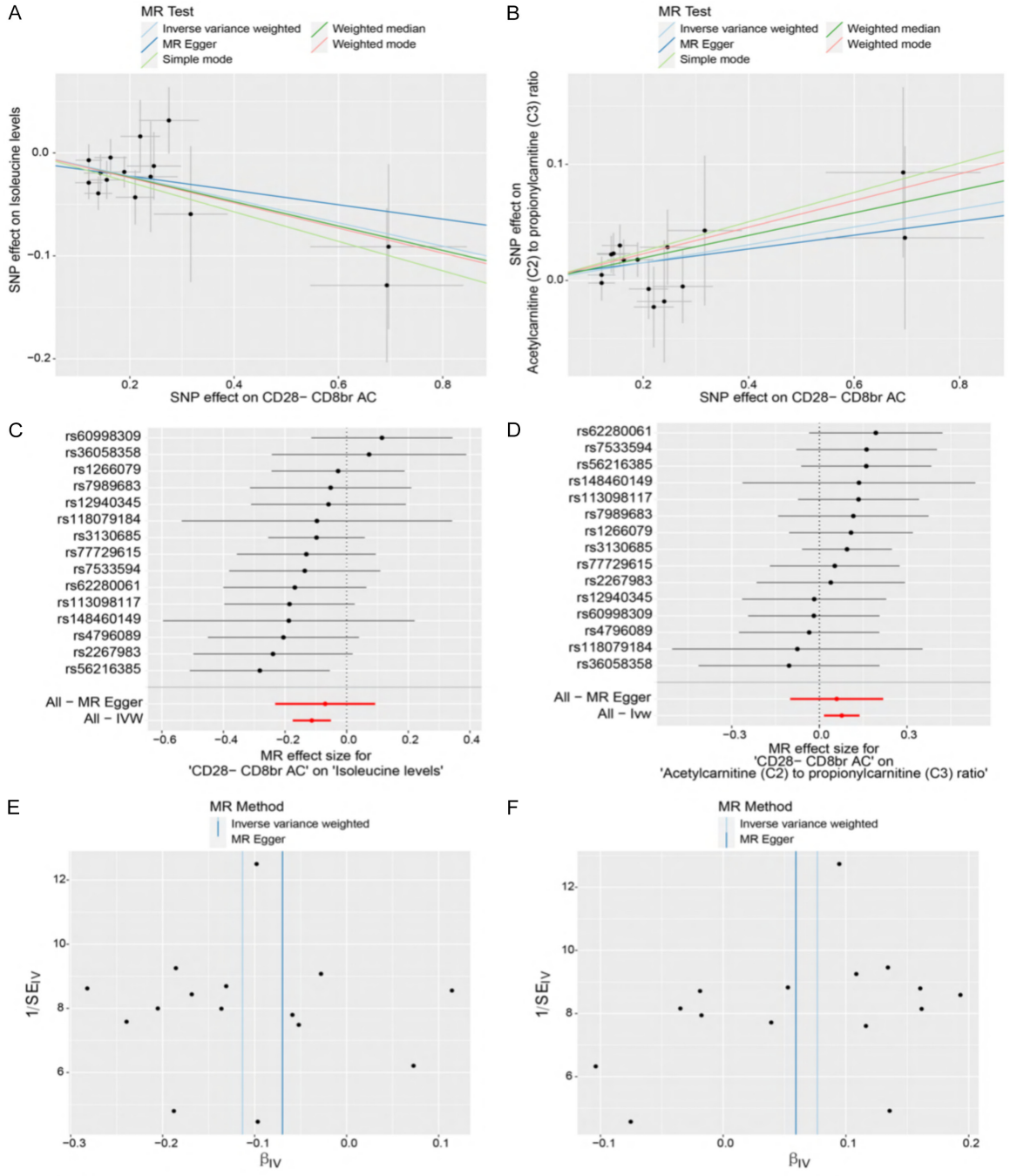


Figure 3. MR results of immune cell phenotypes and preterm labour and delivery. A. Scatter plot of MR for CD28$^-$ CD8^{br} AC on preterm labour and delivery. B. Forest plot of MR for CD28$^-$ CD8^{br} AC on preterm labour and delivery. C. Funnel plot of MR for CD28$^-$ CD8^{br} AC on preterm labour and delivery. D. Leave-one-out analysis of MR for CD28$^-$ CD8^{br} AC on preterm labour and delivery.

In addition, MR analysis indicate that isoleucine level was associated with a reduced risk of preterm labour and delivery (OR [95% CI]: 0.915 [0.845-0.991], β_2 = -0.089, P = 0.029) (**Table 3**; **Figure 5A**, **5C**). While C2/C3 ratio was associated with an increased risk of preterm labour and delivery (OR [95% CI]: 1.153 [1.001-1.328], β_2 = 0.142, P = 0.049) (**Table 3**; **Figure 5B**, **5D**). The results of the sensitivity analyses for isoleucine levels and C2/C3 ratio to preterm

Table 4. The heterogeneity and pleiotropy in MR analysis

exposure	outcome	Heterogeneity test				Pleiotropy test				
		IVW		MR Egger		MR Egger regression			MR PRESSO	
		Cochran Q	pval	Cochran Q	pval	Intercept	se	pval	RSSobs	pval
CD28$^-$ CD8^{br} AC	Preterm labour and delivery	15.965	0.316	15.932	0.253	-0.004	0.026	0.872	18.382	0.346
CD28$^-$ CD8^{br} AC	Isoleucine levels	10.583	0.718	10.260	0.673	-0.009	0.015	0.579	12.171	0.744
CD28$^-$ CD8^{br} AC	Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	6.632	0.948	6.578	0.923	0.003	0.015	0.820	7.509	0.960
Isoleucine levels	Preterm labour and delivery	18.904	0.591	18.230	0.572	0.009	0.011	0.421	20.286	0.679
Acetylcarnitine (C2) to propionylcarnitine (C3)	Preterm labour and delivery	26.308	0.093	26.298	0.069	0.001	0.019	0.937	28.400	0.118



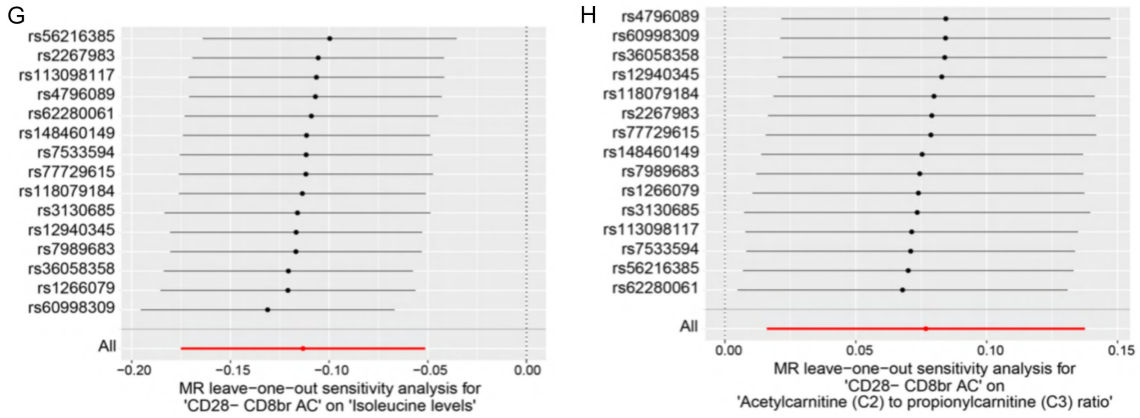


Figure 4. MR results of metabolic mediators and immune cell phenotypes. Scatter plot of MR for (A) CD28$^-$ CD8^{br} AC on Isoleucine levels and (B) CD28$^-$ CD8^{br} AC on Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio. Forest plot of MR for (C) CD28$^-$ CD8br AC on Isoleucine levels and (D) CD28$^-$ CD8^{br} AC on Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio. Funnel plot of MR for (E) CD28$^-$ CD8^{br} AC on Isoleucine levels and (F) CD28$^-$ CD8^{br} AC on Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio. Leave-one-out analysis of MR for (G) CD28$^-$ CD8^{br} AC on Isoleucine levels and (H) CD28$^-$ CD8^{br} AC on Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio.

labour and delivery are presented in **Table 4**. The Cochran's Q statistic of IVW (P = 0.591 for isoleucine levels and P = 0.093 for C2/C3 ratio) and MR-Egger (P = 0.572 for isoleucine levels and P = 0.069 for C2/C3 ratio) suggesting that heterogeneity was not statistically significant. Additionally, no evidence of horizontal pleiotropy was detected using the MR-Egger regression (P = 0.421 for isoleucine levels and P = 0.937 for C2/C3 ratio), and the MR-PRESSO method (P = 0.679 for isoleucine levels and P = 0.118 for C2/C3 ratio). Finally, funnel plots and leave-one-out analysis were created, all of which showed no outliers (**Figure 5E-H**).

These results suggest that increased levels of CD28$^-$ CD8^{br} AC may reduce isoleucine levels or elevate C2/C3 ratio, thereby contributing to increase the risk of preterm labour and delivery.

Mediating effects analysis of plasma metabolites

To quantify these mediating analysis, the mediating effect was calculated as $\beta_1 \times \beta_2$. Accordingly, the effect of CD28$^-$ CD8^{br} AC on preterm labour and delivery via modulation of isoleucine levels was estimated to have a mediating effect of 0.010 (95% CI, 0.001 to 0.019) and a mediation effect ratio of 6.79% (95% CI, 0.777% to 12.8%; P = 0.027) (**Table 5**; **Figure 6A**). In parallel, a mediating effect of 0.011 (95% CI, 0.001 to 0.021) and a mediation effect ratio of 7.35% (95% CI, 0.736% to 14%; P = 0.029) was observed for CD28$^-$ CD8^{br} AC via C2/C3 ratio (**Table 5**; **Figure 6B**).

Discussion

This study systematically investigated the causal relationships between immune cell phenotypes, plasma metabolites, and preterm labour and delivery using MR methods. A total of 28 immune cell phenotypes were identified as causally associated with preterm birth, among which the CD28$^-$ CD8^{br} AC phenotype was established as a prominent risk factor. Additionally, 47 plasma metabolites were found to be causally linked to preterm birth. Mediation analysis revealed that isoleucine and the C2/C3 ratio partially mediated the effect of CD28$^-$ CD8^{br} AC on preterm birth, with mediation proportions of 6.79% and 7.35%, respectively. It was thus suggested that CD28$^-$ CD8^{br} AC increases the risk of preterm birth through modulating levels of isoleucine and the C2/C3 ratio.

The immune system plays a critical role in the pathogenesis of preterm birth, although the underlying mechanisms remain incompletely understood. In this MR study, the CD28$^-$ CD8^{br} AC T-cell phenotype was identified as being positively associated with an increased risk of preterm birth. CD28$^-$ CD8^{br} AC represents a subset of CD8$^+$ T cells characterized by the absence of CD28 surface expression and high levels of CD8. CD8$^+$ T cells are primarily involved in cellular immunity through cytotoxic activity,

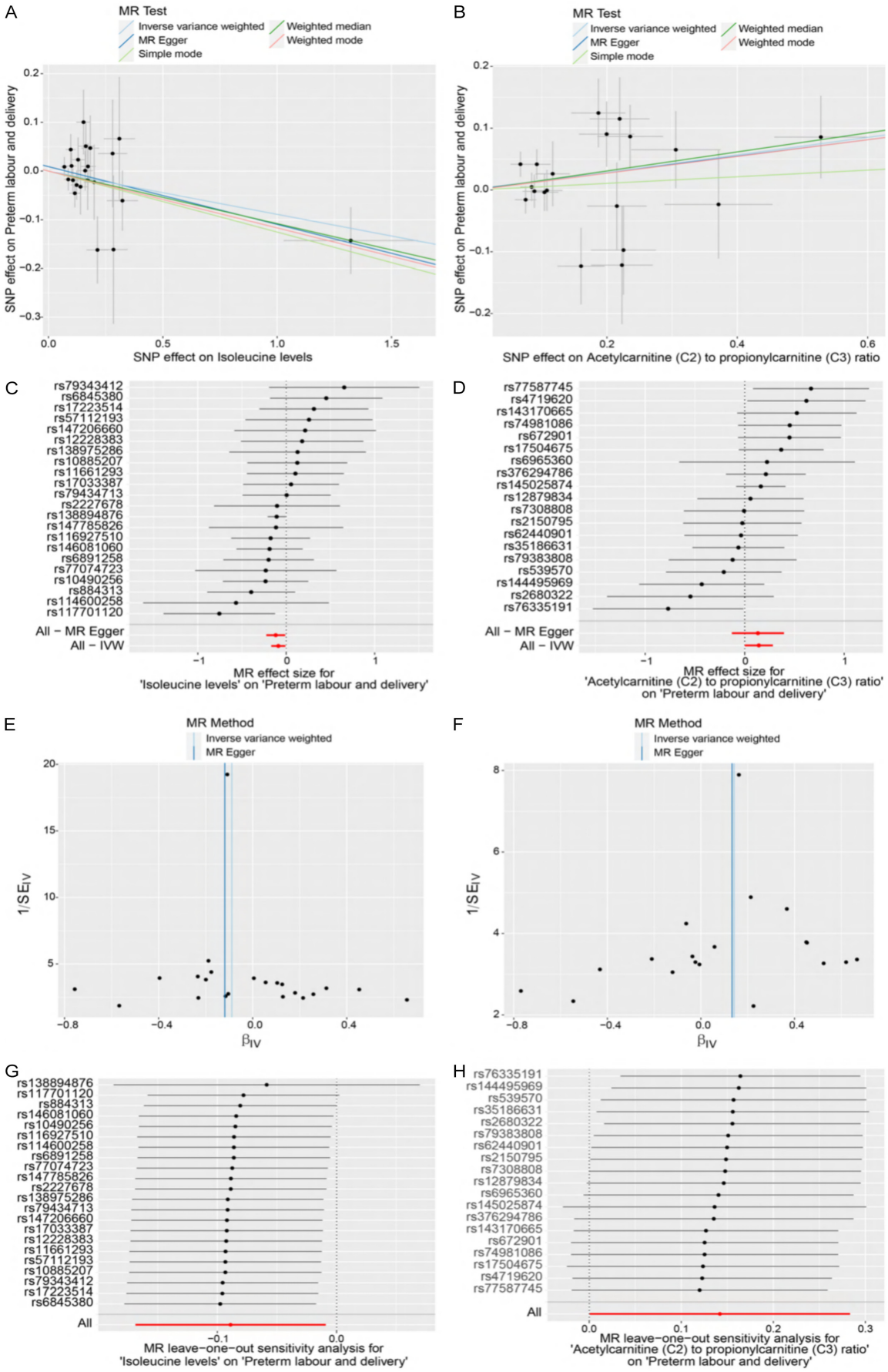

A
MR Test
Inverse variance weighted
MR Egger
Simple mode
Weighted median
Weighted mode
SNP effect on Preterm labour and delivery
SNP effect on Isoleucine levels
B
MR Test
Inverse variance weighted
MR Egger
Simple mode
Weighted median
Weighted mode
SNP effect on Preterm labour and delivery
SNP effect on Acetylcarnitine (C2) to propionylcarnitine (C3) ratio
C
rs79343412
rs6845380
rs17223514
rs57112193
rs147206660
rs12228383
rs138975286
rs10885207
rs11661293
rs17033387
rs79434713
rs2227678
rs138894876
rs147785826
rs116927510
rs146081060
rs6891258
rs77074723
rs10490256
rs884313
rs114600258
rs117701120
All – MR Egger
All – IVW
MR effect size for
'Isoleucine levels' on 'Preterm labour and delivery'
D
rs77587745
rs4719620
rs143170665
rs74981086
rs672901
rs17504675
rs6965360
rs376294786
rs145025874
rs12879834
rs7308808
rs2150795
rs62440901
rs35186631
rs79383808
rs539570
rs144495969
rs2680322
rs76335191
All – MR Egger
All – IVW
MR effect size for
'Acetylcarnitine (C2) to propionylcarnitine (C3) ratio'
on 'Preterm labour and delivery'
E
MR Method
Inverse variance weighted
MR Egger
1/SEIV
βIV
F
MR Method
Inverse variance weighted
MR Egger
1/SEIV
βIV
G
rs138894876
rs117701120
rs884313
rs146081060
rs10490256
rs116927510
rs114600258
rs6891258
rs77074723
rs147785826
rs2227678
rs138975286
rs79434713
rs147206660
rs17033387
rs12228383
rs11661293
rs57112193
rs10885207
rs79343412
rs17223514
rs6845380
All
MR leave–one–out sensitivity analysis for
'Isoleucine levels' on 'Preterm labour and delivery'
H
rs76335191
rs144495969
rs539570
rs35186631
rs2680322
rs79383808
rs62440901
rs2150795
rs7308808
rs12879834
rs6965360
rs145025874
rs376294786
rs143170665
rs672901
rs74981086
rs17504675
rs4719620
rs77587745
All
MR leave–one–out sensitivity analysis for
'Acetylcarnitine (C2) to propionylcarnitine (C3) ratio'
on 'Preterm labour and delivery'

Figure 5. MR results of metabolic mediators and preterm labour and delivery. Scatter plot of MR for (A) Isoleucine levels on preterm labour and delivery and (B) Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio on preterm labour and delivery. Forest plot of MR for (C) Isoleucine levels on preterm labour and delivery and (D) Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio on preterm labour and delivery. Funnel plot of MR for (E) Isoleucine levels on preterm labour and delivery and (F) Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio on preterm labour and delivery. Leave-one-out analysis of MR for (G) Isoleucine levels on preterm labour and delivery and (H) Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio on preterm labour and delivery.

Table 5. The mediation effect of plasma mtabolites on immune cell phenotypes to preterm labour and delivery

Immune cell phenotypes	Plasma mtabolites	Outcome	Mediated effect	Mediated proportion	pval
CD28$^-$ CD8^{br} AC	Isoleucine levels	Preterm labour and delivery	0.010 (0.001, 0.019)	6.79% (0.777%, 12.8%)	0.027
CD28$^-$ CD8^{br} AC	Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	Preterm labour and delivery	0.011 (0.001, 0.021)	7.35% (0.736%, 14%)	0.029

A

exposure	outcome	nsnp	method	pval	OR(95% CI)
		15	MR Egger	0.415	0.933 (0.793 to 1.097)
		15	Weighted median	**0.005**	0.888 (0.817 to 0.966)
CD28− CD8br AC	Isoleucine levels	15	Inverse variance weighted	**<0.001**	0.893 (0.839 to 0.950)
		15	Simple mode	0.067	0.867 (0.753 to 0.998)
		15	Weighted mode	0.090	0.885 (0.777 to 1.009)
		22	MR Egger	**0.042**	0.888 (0.798 to 0.988)
		22	Weighted median	0.083	0.898 (0.795 to 1.014)
Isoleucine levels	Preterm labour and delivery	22	Inverse variance weighted	**0.029**	0.915 (0.845 to 0.991)
		22	Simple mode	0.305	0.882 (0.699 to 1.114)
		22	Weighted mode	**0.038**	0.890 (0.803 to 0.987)
		15	MR Egger	0.287	1.188 (0.876 to 1.611)
		15	Weighted median	**0.001**	1.220 (1.080 to 1.379)
CD28− CD8br AC	Preterm labour and delivery	15	Inverse variance weighted	**0.002**	1.160 (1.056 to 1.274)
		15	Simple mode	**0.029**	1.281 (1.050 to 1.562)
		15	Weighted mode	**0.019**	1.257 (1.062 to 1.489)

B

exposure	outcome	nsnp	method	pval	OR(95% CI)
		15	MR Egger	0.478	1.061 (0.905 to 1.244)
		15	Weighted median	**0.026**	1.102 (1.012 to 1.200)
CD28− CD8br AC	Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	15	Inverse variance weighted	**0.013**	1.080 (1.016 to 1.147)
		15	Simple mode	0.111	1.135 (0.981 to 1.312)
		15	Weighted mode	0.080	1.122 (0.996 to 1.263)
		19	MR Egger	0.333	1.142 (0.879 to 1.484)
		19	Weighted median	0.093	1.167 (0.975 to 1.396)
Acetylcarnitine (C2) to propionylcarnitine (C3) ratio	Preterm labour and delivery	19	Inverse variance weighted	**0.049**	1.153 (1.001 to 1.328)
		19	Simple mode	0.738	1.055 (0.774 to 1.438)
		19	Weighted mode	0.235	1.145 (0.923 to 1.421)
		15	MR Egger	0.287	1.188 (0.876 to 1.611)
		15	Weighted median	**0.001**	1.220 (1.080 to 1.379)
CD28− CD8br AC	Preterm labour and delivery	15	Inverse variance weighted	**0.002**	1.160 (1.056 to 1.274)
		15	Simple mode	**0.029**	1.281 (1.050 to 1.562)
		15	Weighted mode	**0.019**	1.257 (1.062 to 1.489)

Figure 6. A. Forest plot of the causal relationship between CD28$^-$ CD8^{br} AC, Isoleucine levels, and preterm labour and delivery. B. Forest plot of the causal relationship between CD28$^-$ CD8^{br} AC, Acetylcarnitine (C2) to Propionylcarnitine (C3) ratio, and preterm labour and delivery.

recognizing and eliminating virus-infected cells and certain tumor cells [30]. CD28 functions as a key co-stimulatory molecule on T cells, providing essential signals for T-cell activation. Tregs help maintain immune homeostasis through the secretion of inhibitory cytokines such as IL-10 and TGF-β. Disruption of CD28 signaling may impair interactions between Tregs and

antigen-presenting cells, thereby compromising immune regulation [31]. Multiple mechanisms have been reported to contribute to the establishment of fetal tolerance, including the induction of anti-inflammatory Tregs that suppress anti-fetal immune responses [32]. Multiple earlier studies have confirmed that Treg cells play a critical role in maintaining maternal-fetal immune tolerance. Specifically, studies have demonstrated that in the peripheral blood of women with preterm birth, Treg cells not only exhibit abnormal proportions but also show significantly reduced capacity to suppress immune responses [7, 33, 34]. Our findings, which suggest that $CD28^-$ $CD8^{br}$ AC T cells may promote preterm birth by compromising the protective functions of Treg cells, are closely aligned with these previous observations. The decidua at the maternal-fetal interface is enriched with a specialized subset of natural killer cells (dNK). These cells typically exhibit low cytotoxicity and contribute actively to establishing an immunotolerant microenvironment through cytokine secretion, while also supporting placental growth and development. Multiple studies have shown that, compared to dNK cells from age-matched healthy controls, dNK cells from women with recurrent pregnancy loss (RPL) display reduced CD49a expression and elevated levels of perforin, granzyme B, and IFN-γ. Such alterations impair early dNK adhesion and migration into trophoblasts and enhance their cytotoxic potential, thereby compromising endometrial receptivity [35, 36]. Although our study focuses on circulating $CD8^+$ T cells rather than uterine dNK cells, both lines of evidence collectively reinforce the fundamental principle that precise immunoregulation at the maternal-fetal interface is essential for successful pregnancy. Therefore, it is plausible that $CD28^-$ $CD8^{br}$ AC cells may promote preterm birth by attenuating the protective effects of Tregs on fetal tolerance, potentially due to the absence of CD28-mediated co-stimulation.

To further elucidate the mechanisms through which $CD28^-$ $CD8^{br}$ AC influences preterm birth, the causal relationship between metabolite levels and preterm birth risk was investigated. Isoleucine, a branched-chain amino acid, plays essential roles in systemic physiological processes including growth, immune function, protein and fatty acid metabolism, and glucose transport [37]. Previous studies have reported significantly lower plasma isoleucine levels in women with preterm birth compared to those with term deliveries, suggesting a potential link between maternal isoleucine status and preterm birth risk [38]. This observation is consistent with the present MR analysis, which identified a causal relationship between decreased isoleucine levels and preterm birth. Given its role in energy metabolism, isoleucine can be oxidatively catabolized to produce energy [39]. Thus, preterm birth may be associated with disruptions in maternal energy metabolism. Reduced isoleucine availability could lead to inadequate energy supply for the fetus, potentially compromising fetal development and increasing preterm birth risk. Moreover, as an essential substrate for protein synthesis [40], isoleucine deficiency may impair fetal tissue and organ development, further contributing to fetal growth restriction and elevated preterm birth risk. In this study, isoleucine was identified as a metabolite associated with preterm birth, and MR mediation analysis indicated that $CD28^-$ $CD8^{br}$ AC contributes to preterm birth partly through the reduction of circulating isoleucine levels.

Furthermore, a potential causal relationship was identified between $CD28^-$ $CD8^{br}$ AC and preterm birth, which appeared to be mediated by an increased C2/C3 acylcarnitine ratio. Although direct evidence linking the C2/C3 ratio to preterm birth remains limited, it may indirectly influence preterm birth risk through several biological mechanisms. C2 plays a crucial role in fatty acid oxidation by facilitating the transport of acyl groups into mitochondria for energy production [41], while C3 is involved in the metabolism of branched-chain fatty acids [42]. An elevated C2/C3 ratio may reflect disrupted fatty acid metabolism, potentially leading to mitochondrial dysfunction and inadequate energy supply. Such metabolic disturbances could impair normal fetal development and placental function, thereby increasing preterm birth risk. Given that proper fatty acid metabolism is essential for placental development and function [43], an altered C2/C3 ratio might indicate compromised placental fatty acid oxidation, affecting blood flow and nutrient delivery to the fetus and ultimately contributing to fetal growth restriction and preterm birth. Although the C2/C3 ratio has not been established as an independent diagnostic biomarker for preterm birth, its integration with other clini-

cal indicators and metabolic profiles may improve comprehensive risk assessment, particularly in pregnant women with suspected metabolic abnormalities.

The findings of this study suggest that energy metabolism and branched-chain amino acid metabolism may function as critical mediators through which immune cells influence preterm birth. A deeper understanding of this immunometabolic pathway could provide valuable insights into novel mechanisms underlying preterm birth. Targeted regulation of specific immune cell subsets - such as inhibition of pathogenic $CD8^+$ T cell populations - or correction of metabolic disturbances, such as modulation of carnitine metabolism, may offer potential strategies for preventing preterm birth. These results establish a new framework for investigating the immunometabolic mechanisms involved in preterm birth. Future studies integrating multi-omics data could further elucidate these pathways and facilitate the identification of clinically relevant biomarkers or therapeutic targets. We plan to further validate these findings in longitudinal pregnancy cohorts by employing high-dimensional immune phenotyping and metabolomic profiling approaches. Such efforts may help establish temporally resolved immunometabolic networks and provide mechanistic insights into trimester-specific dysregulations associated with preterm birth.

Several limitations of this study should be acknowledged. The analysis was conducted exclusively in European populations, which may limit the generalizability of the findings to other ethnic groups. Furthermore, the absence of stratified data regarding sex-specific differences or gestational age variations represents an important constraint, potentially influencing the interpretability and broader applicability of the results. In addition, the MR approach does not provide direct mechanistic evidence; thus, the proposed pathways involving $CD28^-$ $CD8^{br}$ AC and associated metabolites require validation through functional studies in experimental models, such as animal or cellular systems. Moreover, the lack of in vitro or in vivo experimental verification limits the causal inference that can be drawn from the current analyses. Finally, the identified metabolite biomarkers remain preliminary, and their predictive and therapeutic utility must be evaluated in large, prospective clinical cohorts.

Conclusion

In this study, 28 immune cell phenotypes and 47 metabolites were identified as being associated with genetic susceptibility to preterm birth. Notably, $CD28^-$ $CD8^{br}$ AC (from the Treg panel) was found to potentially increase preterm birth risk by reducing isoleucine levels or elevating the C2/C3 acylcarnitine ratio. The application of MR methods revealed a causal immunometabolic network in preterm birth, providing a critical foundation for elucidating its etiology and developing targeted interventions. These findings may contribute to improved diagnostic and therapeutic strategies, ultimately offering more effective clinical solutions for patients at risk of preterm birth.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuting Liang, Center for Clinical Laboratory, The First Affiliated Hospital of Soochow University, No. 899, Pinghai Street, Suzhou 215002, Jiangsu, China. E-mail: liangyuting666@126.com; Dr. Longwei Qiao, School of Gusu, The Affiliated Suzhou Hospital of Nanjing Medical University, Nanjing Medical University, No. 26, Daoqian Street, Suzhou 215002, Jiangsu, China. E-mail: qiaolongwei1@126.com

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