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Multivariable Mendelian Randomization Analysis Reveals Potential Causal Effects between Immune Cells and Prostate Cancer Risk

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Abstract: *Background:* Previous studies indicated that immune cells and Metabolites might play an important role in the occurrence and development of Prostate Cancer (PCa). Our study aimed to illustrate the causal effects between immune cells and metabolites and PCa risk, and the mediating role of metabolites between immune cells and PCa. *Methods:* This study utilized immune cells as Exposures, metabolites as Mediators, and PCa as Outcomes. Initially, immune cell and metabolite data were processed. Subsequently, a four-stage approach involved six Mendelian randomization analyses: Stage one focused on batch immune cell to PCa MR (MR 1); Stage two involved immune cell to PCa Reverse MR (MR 2); Stage three performed batch metabolite to PCa MR (MR 3); Stage four included three MR analyses: prostate carcinogenesis related immune cells to PCa-related metabolites MR yielding beta 1 (MR 4), PCa-related metabolites to PCa MR yielding beta 2 (MR 5), and prostate carcinogenesis related immune cells to PCa MR yielding beta All (MR 6). Finally, mediation and direct effects were computed. *Results:* Our research identified twenty-five immune cells and nine metabolites associated with the incidence of PCa. Among the most intriguing associations, genetically predicted "CD25 on IgD^+ $CD38^-$ Unswitched Memory (unsw mem) cells" are linked to an increased risk of PCa, Notably, 14.6% (1.68%, 27.5%) of this risk is mediated through the metabolite "3-hydroxypyridine sulfate levels" with a mediated effect of 0.00235 (0.00027, 0.00442) and a p-value of 0.026751157. This indicates that an increase in "CD25 on IgD^+ $CD38^-$ unsw mem cells" can promote the development of PCa by reducing the levels of "3-hydroxypyridine sulfate". Conclusions: Our findings suggest that 3-hydroxypyridine sulfate mediates the association between CD25 on IgD^+ $CD38^-$ unsw mem and increased PCa risk. This study unexpectedly found that elevated 3-hydroxypyridine sulfate levels may explain how coffee consumption could protect against PCa.

Keywords: Mendelian randomization; Prostate cancer (PCa); Immune Cells; Metabolites; 3-hydroxypyridine sulfate; CD25 on IgD^+ $CD38^-$ unswitched memory (unsw mem) cells

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1. Introduction

PCa is a significant global health concern, with approximately 1.3 million new cases diagnosed each year. Currently, there are around 10 million men living with a PCa diagnosis, with about 700,000 of them having metastatic disease. Metastatic PCa is responsible for over 400,000 deaths yearly, and this number is projected to more than double by 2040 [1]. Therefore, it is imperative to research the molecular mechanisms underlying its development, identify the root causes of the disease, and ultimately work towards preventing its occurrence.

Recent research published in Nature highlights that immune cells can identify cancer cell antigens and present them to T cells and B cells for elimination [2]. Despite PCa typically being considered an immunologically "cold" tumor, it is indeed infiltrated by various types of immune cells [3]. Studies on the immune-related microenvironment indicate that both innate and adaptive immune cells play roles in the development and progression of PCa [4].

Additionally, previous study identified 103 genes associated with immune cell abundance in the tumor microenvironment, illustrating how genetic variations influence the expression of immune genes [5]. Immune cells, especially B cells, undergo various genetic stresses during their maturation and activation processes [6]. Double-strand DNA breaks and mutations are crucial for normal immune function; however, improper management of these DNA damages can lead to serious consequences such as immune deficiencies or cancer [7]. These findings collectively underscore how genetic factors may impact the expression and infiltration of immune cells, thereby potentially influencing the onset and progression of PCa.

Furthermore, relevant investigations have demonstrated a possible relationship between plasma metabolite concentrations and PCa risk [8]. Studied discovered that 49 metabolites were related with PCa survival [9]. As lipid metabolism contributes to the progression of PCa and citrulline metabolites may signal subclinical PCa [10–12]. While lignans and in vivo metabolites, particularly enterolactone (ENL), may be employed as chemo-preventive treatments for PCa [13]. Vitamin D and its metabolites can help cure and prevent PCa [14]. L-methionine (l-Met) and its metabolites play an important role in the development of PCa [15].

Metabolites are highly hereditary, where relevant studies have revealed that the expression of metabolites is affected by genetics, epigenetics, and the environmental factors [16–19]. At the same time, Metabolite concentrations are influenced by both genetic and environmental factors [20]. However, there is still absence of research investigating the potential causal relationship between immunity and metabolism in the pathogenesis of PCa.

Mendelian randomization (MR) serves as a causal inference technique that leverages genetic variants as a surrogate for exposure, resembling a natural randomized controlled trial and mitigating confounding bias and reverse causality often present in observational studies. Multivariable Mendelian randomization (MVMR) is an extended method that enables the investigation of independent effects of correlated exposures on an outcome by include genetic variations of each exposure in the same model. Additionally, since traditional, non-instrumental variable methods for mediation analyses would experience bias due to confounding between an exposure, mediator, and outcome as well as measurement error, a two-step MVMR study can be used to investigate the pathways through which an exposure affects an outcome and improve causal inference in mediating effects [21].

This study examined the independent causal relationships between immune cells and metabolites in the development of PCa, utilizing immune cells as exposure factors, metabolites as mediating factors, and PCa as outcome factors. The focus was on the mediating effect of metabolites on alterations in immune cell numbers during the pathogenesis of PCa, providing a foundation for further research on the disease.

2. Methods

2.1. Study design

This study utilized immune cells as Exposures, metabolites as Mediators, and PCa as Outcomes. Initially, immune cell and metabolite data were processed by correlational analysis of genetic factors and exposures, removal of linkage disequilibrium Single Nucleotide Polymorphisms (SNPs), and exclusion of weak instrumental variables. Subsequently, a four-stage approach involved six Mendelian randomization analyses: First, MR Analysis of batch immune cells to PCa (MR 1). Second, the Reverse MR Analysis: PCa was sent to immune cells for MR Analysis (MR 2). Third, the MR Analysis of batch metabolites to PCa (MR 3). Then, the mediating effect analysis included three MR Analyses including the MR Analysis of prostate carcinogenesis related immune cells (MR 4), the MR Analysis of PCa-related metabolites to PCa (MR 5) and the MR Analysis of prostate carcinogenesis related immune cells of PCa to NAFLD (MR 6). Finally, mediation and direct effects were computed. The research ideas are detailed in **Figure 1**.

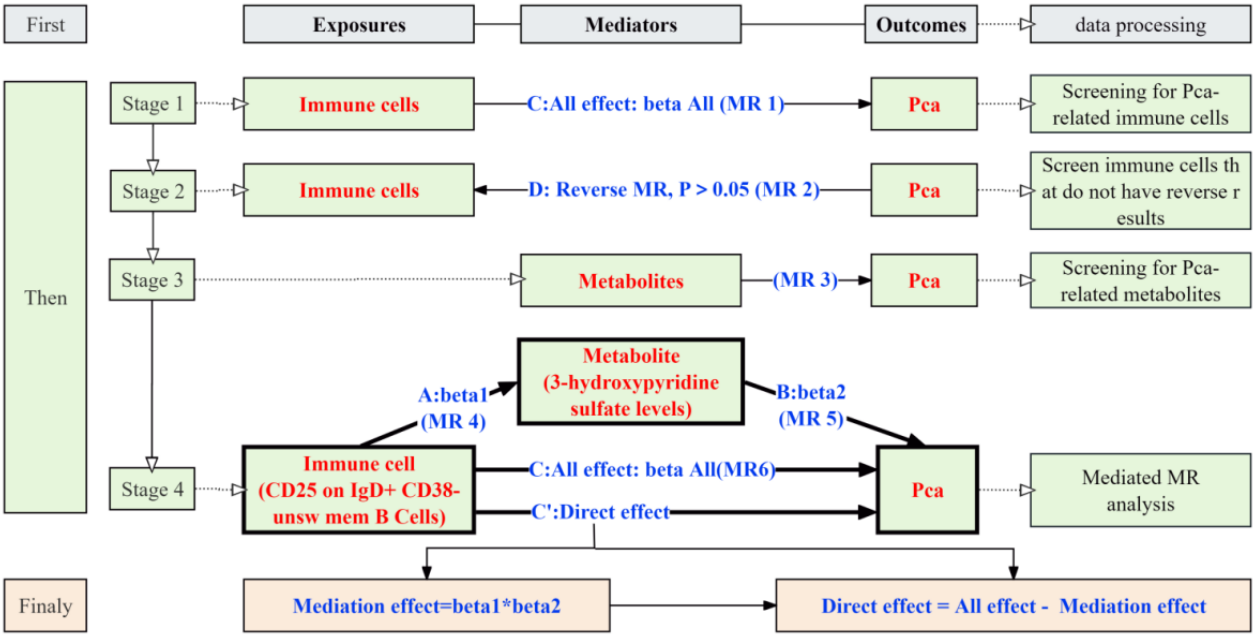

Figure 1. Overview of the study design.

2.2. Data sources of exposures, mediators, and outcomes

The PCa data of the European population published in 2021 from the Genome-Wide Association Study (GWAS) summary date (https://gwas.mrcieu.ac.uk/datasets/ebi-a-GCST90018905/) was obtained, with a total of 211227, and the sample size, 24119306 Number of SNPs [22]. The immune cell data of the European population from the literature was downloaded, including a total of 731 immune cells, numbers from GCST0001391 (https://www.ebi.ac.uk/gwas/studies/GCST0001391) to the GCST0002121 (https://www.ebi.ac.uk/gwas/studies/ GCST0002121) [21]. The metabolite data of the European population from the literature was downloaded, including a total of 1399 metabolites and 34843 SNPs this date which was accessible and available at (http://ftp.ebi.ac.uk/pub/databases/gwas/summary_statistics/) [23]. The above three sets of data were selected from populations of European ancestry to reduce potential bias from population stratification.

This study utilized the R 4.3.2 TwoSampleMR package to filter data with a p-value $< 1 \times 10^{-5}$, extracting

SNPs strongly correlated with "immune cells or metabolites". Parameters were set to kb = 10000 and $r^2 = 0.001$ to filter out SNPs in linkage disequilibrium. Using the 'ieugwasr' R package, and removed weak instrumental variables (defined as those poorly correlated with the phenotype or explaining minimal phenotype variance, with F < 10).

2.3. MR of batch immune cells or metabolites to PCa (Step one, three)

To identify immune cells and metabolites associated with PCa, Mendelian randomization analyses were conducted using the TwoSampleMR, version 0.42, R package. Five methods (MR Egger, Weighted median, Inverse variance weighted (IVW), Simple mode, Weighted mode) were employed for batch MR analysis of immune cell datas or metabolites to PCa, with IVW as the primary method [24]. Results were filtered using p-value < 0.05, and immune cells and metabolites showing consistent directional odds ratios (OR > 1 or OR < 1) across all five MR methods were finally extracted.

2.4. Reverse MR of Immune cells to PCa (Step two)

To screen for immune cells associated with the pathogenesis of PCa, MR Analysis (MR 2) was performed in this study, where the reverse MR Analysis of immune cells to NAFLD, using PCa as the exposure factor and immune cells as the outcome variable. Pleiotropy results were filtered with $p > 0.05$ to obtain negative results, and immune cells with $p < 0.05$ were excluded.

2.5. Mediation MR (Step four)

In Steps one to three, Mendelian Randomization (MR1,2,3) was used to identify prostate carcinogenesis related immune cells and PCa related metabolites. In Step four, three MR analyses was concluded including the MR (MR4) of prostate carcinogenesis related immune cells on PCa related metabolites to identify metabolites mediating the effect of immune cells on PCa development, obtain effect size betal. Next is MR (MR5) of PCa related metabolites on PCa to obtain effect size beta2. Then is MR (MR6) of prostrate carcinogenesis related immune cells on PCa to obtain total effect size betaAll. For these analyses, this study has employed the TwoSample MR package and applied the MR-PRESSO method for pleiotropy testing to identify and remove biased SNPs (significance level < 0.05) [25]. Then performed heterogeneity testing, created scatter plots, forest plots, and funnedl plots and conducted a Leave one out analysis. Finally, calculate the mediating effect beta (1 × 2) = beta 1 × beta 2, direct effect (beta dir) = total effect (beta all) – mediating effect (beta 1 × 2).

3. Result

3.1. Screening of PCa-associated immune cells

In Step one, 731 immune cells were referred from the literature. After removing invalid instrumental variables, this study further filtered it down to 729 immune cells. Using five different Mendelian Randomization (MR1) analysis methods, and 34 immune cells was identified to have a causal relationship with PCa. Further screening revealed 26 immune cells with consistent OR directions and p-value < 0.05 across all five MR methods.

In Step two, reverse Mendelian Randomization analysis was used to identify 25 prostate carcinogenesis related immune cells (revp-value > 0.05) for subsequent mediation Mendelian Randomization (MR2) analysis. These immune cells include $IgD^{-}CD38^{-}$ %lymphocyte, $CD62L^{-}$ monocyte %monocyte, $CD39^{+}$ resting Treg AC, Secreting Treg % CD4 Treg, Activated & resting Treg % CD4 Treg, Monocytic Myeloid-Derived Suppressor

Cell (Mo MDSC AC), $CD14^{-}$ $CD16^{+}$ monocyte %monocyte, Leukocyte AC, CD8br NKT %T cell, CD24 on IgD^{+} $CD24^{+}$, CD24 on IgD^{-} $CD38^{-}$, CD24 on unsw mem, CD25 on IgD^{+} $CD38^{-}$ unsw mem, IgD on IgD^{+} $CD24^{-}$, CD3 on $CD39^{+}$ resting Treg, HVEM on EM (effector memory) CD8br, HVEM on CD8br, CD4 on monocyte, FSC-A (forward scatter area) on plasmacytoid dendritic cell (DC), FSC-A on NK, Side Scatter Area (SSC-A) on granulocyte, CD11c on myeloid DC, CD11c on $CD62L^{+}$ myeloid DC, HLA DR on DC, and HLA DR on CD33dim HLA DR^{+} $CD11b^{-}$.

3.2. Screening of PCa-associated metabolites

In Step three, 1400 metabolites were initially obtained from the literature. After removing instrumental variables, 1400 metabolites were screened. Further analysis using five Mendelian randomization (MR3) methods identified 1399 metabolites with potential causal relationships to PCa. Among these, 9 metabolites consistently showed odds ratios (OR) and p-value ≤ 0.05 across all five MR methods. These metabolites include Cysteinyl glycine disulfide, 3-hydroxypyridine sulfate, Sulfate of piperine metabolite $C_{16}H_{19}NO_3$, Sarcosine, oxidized Cys-gly, Arachidonate (20:4, n-6), X-19438, Phosphate to citrate ratio, and the ratio of Androsterone glucuronide to Etiocholanolone glucuronide.

3.3. The mediation Mendelian randomization analysis

In Steps one to three, prostate carcinogenesis-related immune cells and PCa related metabolites were screened for potential use in intermediate Mendelian randomization analysis. Subsequently, in step four, three Mendelian randomization analyses were conducted.

3.3.1. Result of prostate carcinogenesis related immune cells to PCa-related metabolites MR (MR 4)

A causal relationship between prostate carcinogenesis-related immune cells (CD25 on IgD^{+} $CD38^{-}$ unsw mem) and the PCa-related metabolites (3-hydroxypyridine sulfate levels, CST90199990) was discovered. All five MR methods showed a beta (β) value of < 0, with the IVW method presenting a p-value of < 0.05 and an OR value of < 1. Combining the scatter plot and forest plot, the analysis indicates that CD25 on IgD^{+} $CD38^{-}$ unsw mem is a protective factor for 3-hydroxypyridine sulfate levels. As the number of CD25 on IgD^{+} $CD38^{-}$ unsw mem increases, the levels of 3-hydroxypyridine sulfate decrease; conversely, as the number of CD25 on IgD^{+} $CD38^{-}$ unsw mem decreases, the levels of 3-hydroxypyridine sulfate increase. The leave-one-out analysis suggests that the MR analysis results are reliable and stable. The funnel plot analysis shows no heterogeneity of SNPs. For further details, see **Figure 2**.

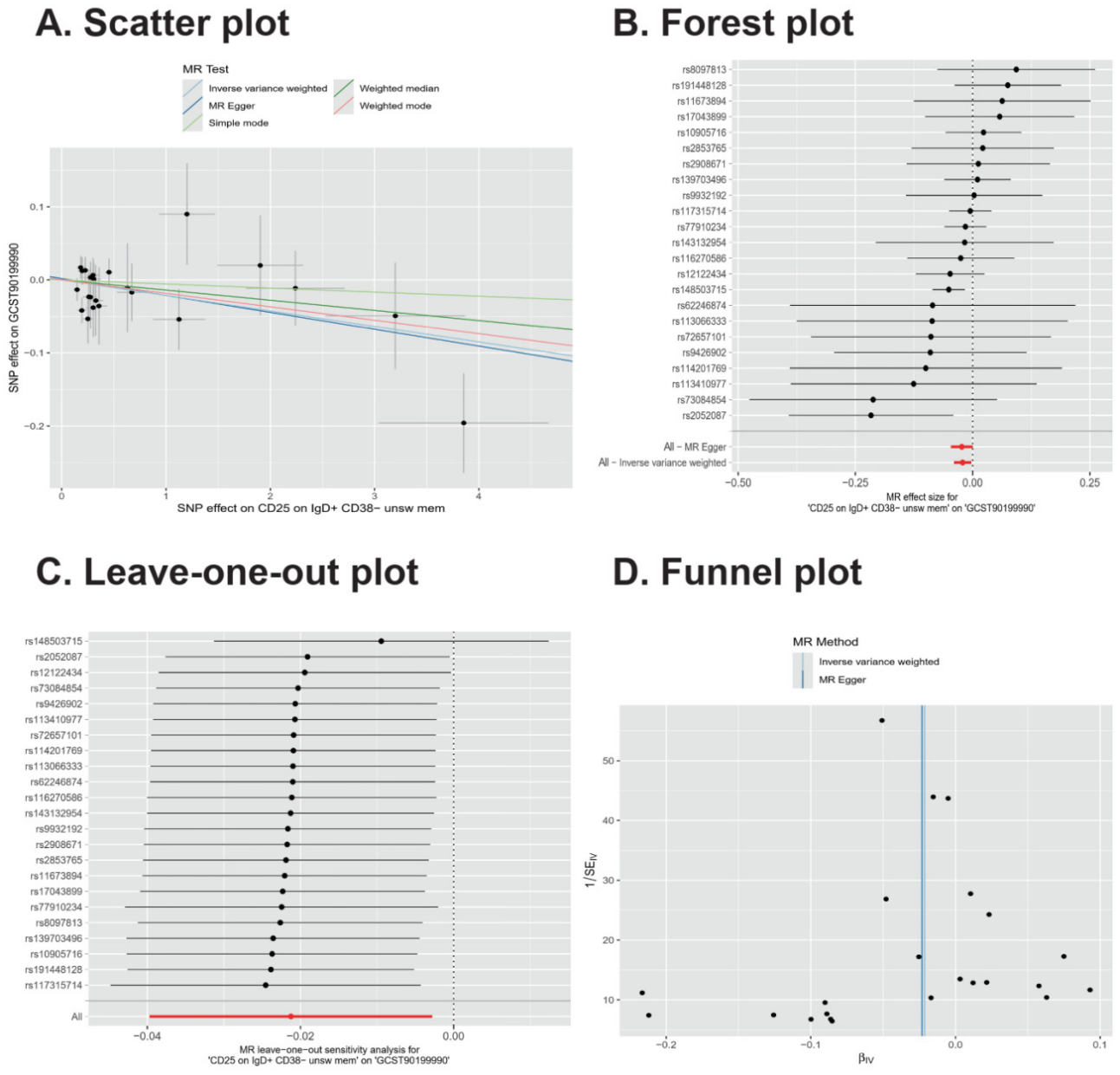

Figure 2. CD25 on IgD+ CD38– unsw mem on 3-hydroxypyridine sulfate levels of MR analysis result. A. Scatter plot; B. Forest plot of SNP; C. Leave one-out-plot plot; D. Funnel plot.

3.3.2. Result of PCa-related metabolites to PCa MR (MR 5)

Through analysis, it was discovered that there is a causal relationship between PCa-related metabolites (3-hydroxypyridine sulfate levels) and PCa. The b (beta 1) values of all five methods were < 0, the p value of the IVW method was < 0.05, and the OR < 1. Combined with the scatter plot and forest plot, the analysis indicates that "3-hydroxypyridine sulfate levels" are a protective factor for PCa. As the levels of "3-hydroxypyridine sulfate levels" increase, the risk of PCa decreases; conversely, as the levels decrease, the risk of PCa increases. The leave-one-out analysis demonstrated the reliability and stability of the MR analysis results. The funnel plot analysis indicated that the SNPs do not exhibit heterogeneity. For further details, refer to **Figure 3**.

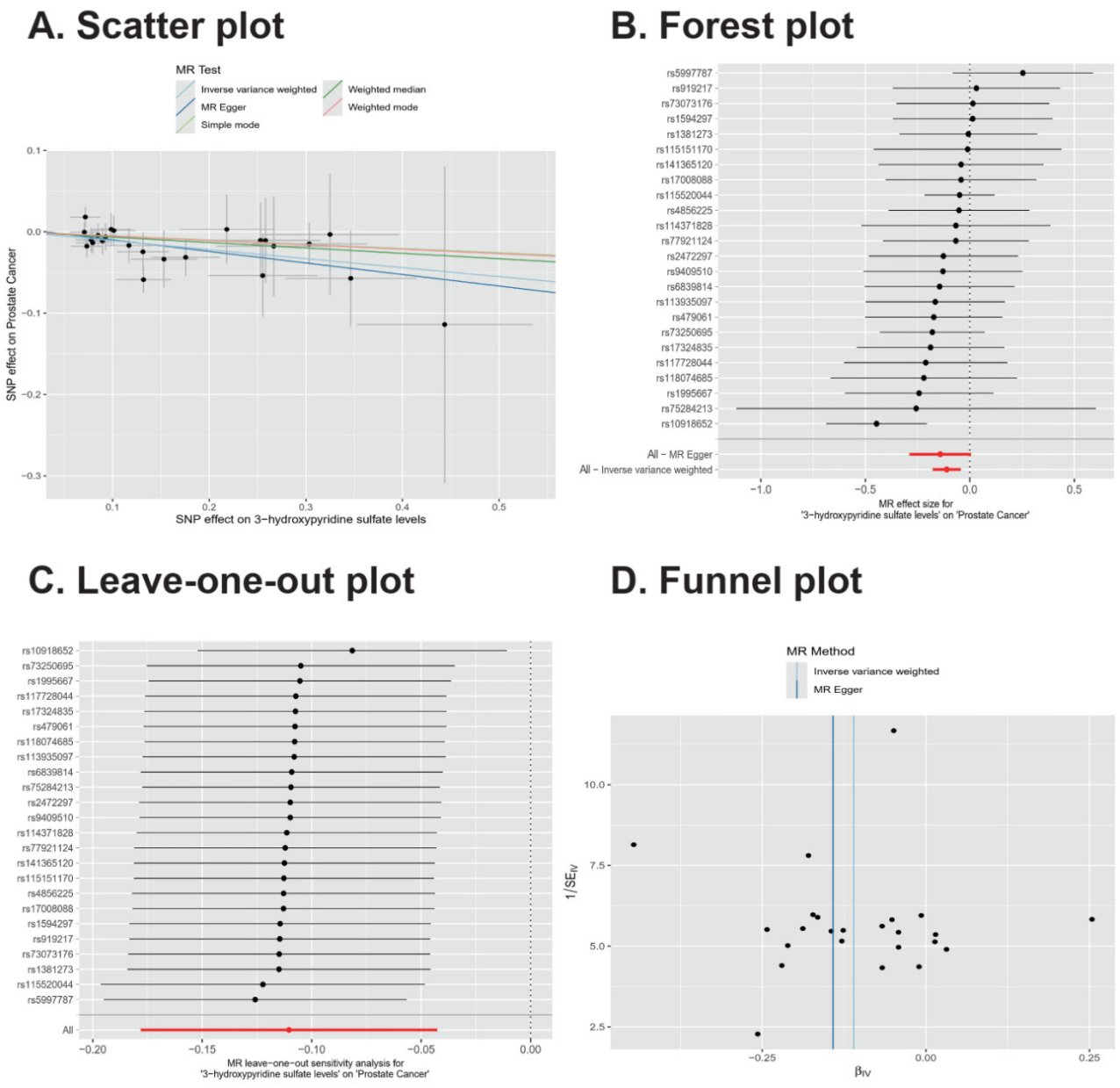

Figure 3. 3-hydroxypyridine sulfate levels on PCa of MR analysis result. A. Scatter plot; B. Forest plot of SNP; C. Leave one-out-plot plot; D. Funnel plot.

3.3.3. Result of prostate carcinogenesis related immune cells to PCa MR (MR 6)

A causal relationship between prostate carcinogenesis and immune cells expressing "CD25 on IgD^+ $CD38^-$ unsw mem" was discovered. The b (beta 1) values of all five methods were > 0, with the IVW method showing a p-value < 0.05 and an OR > 1. Combined scatter plot and forest plot analyses indicate that "CD25 on IgD^+ $CD38^-$ unsw mem" is a risk factor for PCa. As the quantity of "CD25 on IgD^+ $CD38^-$ unsw mem" increases, the risk of PCa also increases; conversely, a decrease in "CD25 on IgD^+ $CD38^-$ unsw mem" quantity lowers the risk. Leave-one-out analysis demonstrates the reliability and stability of MR analysis results. Funnel plot analysis shows no heterogeneity in SNP. See **Figure 4** for details.

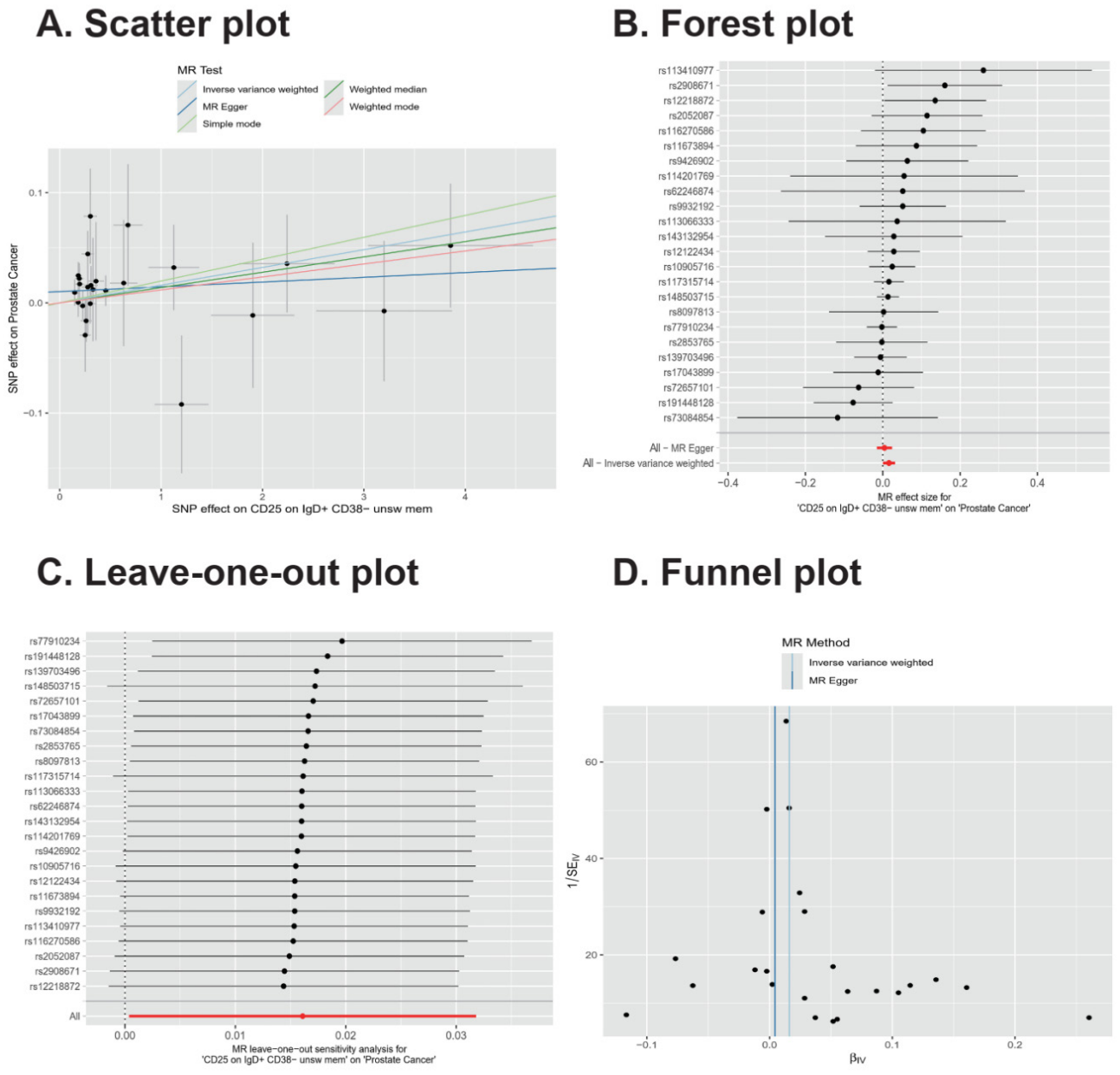

Figure 4. CD25 on IgD+ CD38– unsw mem on PCa of MR analysis result. A. Scatter plot; B. Forest plot of SNP; C. Leave one-out-plot; D. Funnel plot.

3.3.4. Mediation effect, mediation ratio, and direct effect

Based on the formula: Mediation effect (beta12) = beta1 × beta2, Direct effect (beta_dir) = Total effect (beta_all) – Mediation effect (beta12), the mediated effect is 0.00235 (0.00027, 0.00442), the mediated proportion is 14.6% (1.68%, 27.5%), and the p-value is 0.026751157 (**Table 1**). The direct effect is 0.0137478280. In each of the three Mendelian randomization analyses conducted, the IVW method demonstrated a p-value $<$ 0.05. The initial two analyses revealed an odds ratio (OR) $<$ 1, while the third analysis exhibited an OR $>$ 1. This indicates that an increase in the number of "CD25 on IgD^+ $CD38^-$ unswitched memory B cells" can promote the occurrence of PCa by reducing "3-hydroxypyridine sulfate levels", with a mediation effect of 14.6%, which is statistically significant.

See **Figure 5** for details.

Table 1. Multivariable Mendelian randomization and mediation analyses result OF CD25 on IgD^+ $CD38^-$ unsw mem, 3-hydroxypyridine sulfate levels and PCa

Immune cell	Metabolite	Outcome	Mediated effect	Mediated proportion	p-value
CD25 on IgD^+ $CD38^-$ unsw mem	3-hydroxypyridine sulfate levels	PCa	0.00235 (0.00027, 0.00442)	14.6% (1.68%, 27.5%)	0.026751157

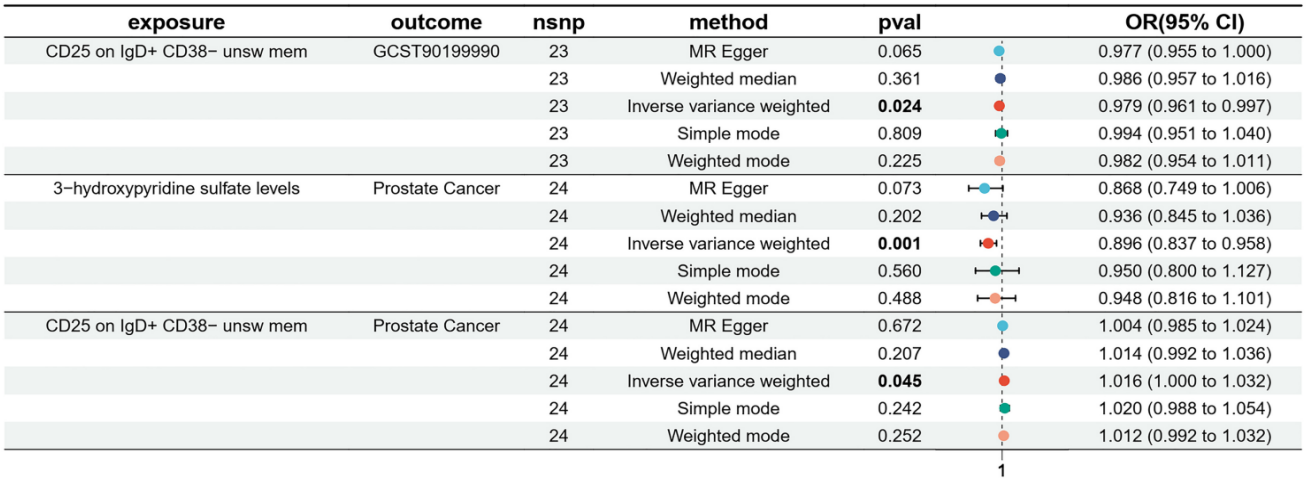

Figure 5. Forest plot of multivariable Mendelian randomization and mediation analyses result for CD25 on IgD+ CD38– unsw mem, 3-hydroxypyridine sulfate levels and PCa.

4. Discussion

Previous studies indicated that immune cells and metabolites might play an important role in the occurrence and development of PCa [12,26–29]. Our study aimed to illustrate the causal effects between immune cells and metabolites and PCa risk, and the mediating role of metabolites between immune cells and PCa. Our results suggested that 25 immune cells and 9 metabolites were associated with PCa. The most intriguing correlation is with the genetically predicted "CD25 on IgD^+ $CD38^-$ Unswitched Memory B cells" was associated with an increased risk of PCa, and 14.6% (1.68%, 27.5%) of this effect was mediated through Metabolite "3-hydroxypyridine sulfate levels".

This research have first identified that the expression level of "CD25 on IgD^+ $CD38^-$ Unswitched Memory B cells" correlates with an elevated risk of PCa incidence. Relevant studies have manifested that compared to the normal prostate tissue, higher B cell infiltration was found in the PCa regions, suggesting that B cells can facilitate the development of PCa and serve as a therapeutic target [30]. According to the expression levels of IgM, IgD, CD10, CD19, CD24, CD27 and CD38, B cells could be divided into eight continuous differentiated subsets, namely Immature B cells, T1 Transitional B cells, T2 Transitional B cells, T3 Transitional B cells, Naive B cells, unswitched-memory B cells (unsw-mem B), switched-memory B cells (unsw-mem B), Plasmablast cells [31,32]. Many studies refer to cells with either the phenotype $CD27^+IgM^+IgD^+$ or $CD27^+IgM^+IgD^-$ as unswitched memory B cells. Among them, IgD serves as a marker for regulatory B cells involved in negative regulation of immune and inflammatory responses; CD38 is a multifunctional receptor and enzyme present on the surface of B lymphocytes [33,34]. It influences B cell functions by cross-linking with relevant cytokines, inducing B lymphocyte proliferation and apoptosis, thereby

affecting immune regulation in the body and promoting tumorigenesis; and CD25 is activated B cell markers, one study shown that CD25 expresses at high level in B-ALL patients with BCR/ABL$^{(+)}$, which may serve as a predictive marker for the presence of BCR/ABL fusion gene, and relates with relapse, CD25$^{(+)}$ may serve as an adjuvant indicator for poor prognosis [35,36]. Therefore, based on the above studies, it is reasonable to hypothesize that elevated levels of CD25 expression on IgD^{+} CD38^{-} Unswitched Memory B cells may indicate a poor prognosis for the disease, which consistent with our research.

3-Hydroxypyridine sulfate serves as a serum marker for coffee consumption, has been reported to be associated with multiple diseases. One study found that the organic compound 3-hydroxypyridine sulfate was significantly associated with an increased risk of Diabetic retinopathy (DR), and some studies have shown that 3-hydroxypyridyl sulfate are inversely associated with the risk of asthma and primary gastric cancer [37–40]. While there are currently no studies investigating the correlation between 3-hydroxypyridine sulfate and prostate cancer, numerous literature reports exist examining the relationship between coffee intake and prostate cancer. Some studies suggest an increased intake of coffee may be linked to a reduced risk of PCa, however, some studies indicate no clear evidence of an association with PCa incidence. Nevertheless, they collectively suggest that coffee consumption is associated with a reduced risk of fatal PCa, and possibly due to non-caffeine components of coffee [41–44]. So, what are the main components of coffee consumption that play a protective role against prostate cancer? Our research may answer this question. Relevant studies have shown that 3-hydroxypyridine sulfate may affect the proliferation and apoptosis process of prostate cells by affecting the availability and activity of androgens [45]. Future investigations, including prospective cohort studies and in vitro/in vivo experiments, are necessary to explore the association between levels of 3-hydroxypyridine sulfate in coffee metabolites in the blood and PCa. Such efforts aim to contribute to our understanding and prevention strategies against PCa.

For the first time, 3-hydroxypyridine sulfate was identified to mediate the effect of "CD25 on IgD^{+} CD38^{-} Unswitched Memory B cells" on the onset of prostate cancer. The mechanism may be that 3-hydroxypyridine sulfate affects CD25 expression level in the context of B cell activation and memory formation [46].

A multivariate Mendelian randomization (MVMR) approach was used to analyze the results, which is similar to a natural randomized controlled trial, which avoids the confounding bias and reverse causality of some observational studies, and also incorporates the genetic variants of each exposure into the same model to investigate the independent effects of related exposures on the results. The results are consistent with those of previous studies. To date, this study are the first to investigate the causal relationship between immune cells and metabolites and the risk of PCa, and our study is also the first to demonstrating that the number of CD25 on IgD^{+} CD38^{-} Unswitched Memory B cells increase, which may increase the risk of PCa by reducing 3-hydroxypyridine sulfate levels.

However, the study has three limitations. Firstly, our analysis is based solely on European populations, which may limit the generalizability of our findings to other regions [21–23]. In future studies, including populations from other regions for analysis should be considered. Secondly, our prostate cancer data only discussed overall prostate cancer cases and did not perform clinical staging of prostate cancer. Thirdly, our study shows that 3-hydroxypyridine sulfate levels mediated genetic prediction in PCa rate of 14.6%, still needs more research to quantify other medium.

5. Conclusion

In summary, this study revealed the causal relationship between immune cells and metabolites and PCa through

mediated Mendelian randomization analysis, and findings indicate that 3-hydroxypyridine sulfate levels mediate the observed association between CD25 on IgD^+ $CD38^-$ unsw mem and heightened risk of PCa. This study speculate that increased levels of the metabolite 3-hydroxypyridine sulfate may serve as a potential mechanism by which coffee consumption exerts a protective effect against the occurrence or progression of PCa. This hypothesis presented novel avenues for future research in this field.

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Disclosure statement

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Reference

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