Peer Review File

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Reviewer A

The authors aimed to better understanding the causal relationship between inflammation and ischemic heart disease, by exploring through a MR the relationship between T regulation and IHD.
The method they used is well established and the authors made significant efforts to apply it as best as possible. They probably have been too liberal with respect to variants selections (see below), but this can be adjusted.
My main concerns relate to
1) The lack of description of the GWAS used and of analyses of differences and similarities. In particular, the T regulation is influenced by aging, and it is difficult to ensure that results obtained on the exposure GWAS are applicable to the outcome one, i.e. as the immune systems, in particular those based on T-cell population wanes with age, results obtained on a younger or older population may not be applicable to another one with different age.
2) The authors are testing various variants influencing T cells, but without grouping them in clusters/phenotypes and providing guidance. This multiplies the tests that may lead to false discovery, even with adjusted statistics as proposed and prevent the reader to really get robust insights from the work.
3) The CD4 on CD4 and CD25 on CD4 are also important findings, they are not stated in the conclusion, nor in the abstract. This prevent any comprehensive assessment of the results and may lead to partial conclusions.
Comments on the various parts of the text can be found below:

Comment 1: Introduction: While the role of inflammation in IHD has been well established by various studies, the relationship between T cells subtypes and IHD is less established (as underlined by the authors), but this part of the text is not supported by any reference and needs to be better explicated. The last part of the introduction may be re-written to make it clear that the objective is to explore the causal association between T reg subtypes and IHD using two different GWAS (for exposure and outcomes).
Reply 1: Thank you for your insightful feedback. We have revised the introduction section, adding relevant references to support the relationship between T cell subtypes and IHD. Furthermore, the last part of the introduction has been rewritten to clarify the objective of exploring the causal association between Treg subtypes and IHD using two different GWAS.
Changes in the text: In Page 4/lines 99-102, we have added the citations (PMID: 30586716; PMID: 32777294; PMID: 28450656). In page4/line110, we have changed the the last part of the introduction to the sentence: “Therefore, this study aims to conduct a two-sample MR study using two different genome-wide association studies (GWAS) to further explore the causal association between molecular characteristics in peripheral Treg and IHD risk. Through this study, we hope to gain a deeper understanding of the role of Treg-related molecules in the pathogenesis of IHD and provide new ideas and methods for the prevention and treatment of IHD.”.

Comment 2: Methods: In general, both GWAS are not sufficiently described, especially with respect to population characteristics beyond just being from European origin. The traits used for the GWAS are not explained, in particular the definition of IHD in the outcome GWAS is not provided, for the exposure GWAS, the way traits are assessed is not provided. The correspondence between both populations cannot be assessed.

Reply 2: Regarding your concerns about the insufficient description of the two GWAS studies, we have now provided additional information on the population characteristics. Furthermore, we have elaborated on the traits used in the GWAS, including a clear definition of IHD. For the exposure GWAS, we have explained the method used to assess the molecular traits in Page 5/line 148"the phenotype ascertainment method involves antibody staining of peripheral blood from normal individuals and flow cytometry processing".

Changes in the text: In Page 5/line 141,"The comprehensive Treg subtypes (refer to Supplementary File 1) were obtained from the SardiNIA study, which consists of GWAS data collected from 3,757 individuals (57% female) belonging to the general population residing on the central east coast of Sardinia, Italy" to the sentence "The comprehensive Treg subtypes (refer to Supplementary File 1) were obtained from the SardiNIA study, which consists of GWAS data collected from 3,757 individuals (57% female) representing the general population residing on the central east coast of Sardinia, Italy (10). This cohort included 118 absolute cell counts, 389 MFIs of surface antigens, 32 morphological parameters, and 192 relative counts (ratios between cell levels), amounting to a total of 731 cell traits evaluated in this population." In Page 5/line 159, we added the sentence "IHD is described as coronary thrombosis, which is "the coagulation of blood in any of the coronary vessels". The presence of a blood thrombus usually leads to myocardial infarction.".

Comment 3: Several IHD GWAS exist and the rationale for the selection of the outcome GWAS is not provided. As no reference is provided, it is difficult to assess its representativeness and larger IHD GWAS exist and have been previously used for publication (see Tcheandjeu et al. Nature medicine 2022).

Reply 3: Thank you for pointing out this important aspect of our study. We acknowledge that the sample size of the IHD GWAS we used in our study is not the largest available. However, we chose this particular GWAS dataset for several reasons. Firstly, the sample size of this GWAS was sufficient to achieve the desired statistical power for our analysis. While larger GWAS datasets are available, we believe that the size of our chosen dataset was adequate to address the research questions we posed. Secondly, the GWAS data we used were obtained from the IEU open GWAS project, which provides a widely accessible and standardized resource for genetic association studies. The use of this dataset allowed us to ensure a stable and reproducible analysis process, as the data can be directly analyzed using commonly available R packages. We greatly appreciate your suggestions. Incorporating updated and more comprehensive datasets can further improve the robustness and generalizability of research results. In future studies, we will consider using the latest, largest sample size, and more representative GWAS studies.

Changes in the text: None

Comment 4: Exposure GWAS: the authors used a classic 5X10-8 threshold which is applicable to large GWAS testing a single trait. There several traits are tested, and the author of the SardiNIA study used a more stringent threshold (6.9X10-9) after determination of the genome-wide significance for Sardinians. Furthermore, the authors of the referenced paper, took in account various additional parameters including cell counts to calculate a final threshold of 1.3X10-11. Therefore, considering these calculations and the multi traits analyses, the threshold of 5X10-8 may be too lenient and may lead conclusion not sufficiently robust. The table 1 suggest that a significant proportion of SNPs could still be used the most stringent p value.
Instrument selection: the description of clumping procedure is unclear, the sentence should be re-written to specify that non independent SNPs (SNPs in LD) have been selected, and not the reverse.
Reply 4: Thank you for your thoughtful comments and suggestions. We appreciate your feedback on our choice of threshold value and the clarity of our instrument selection process.
Regarding the threshold value, we agree that the selection of an appropriate threshold is crucial in GWAS and MR studies. In our study, we used a threshold of 5E-8, which is a common and standard screening threshold in GWAS and MR studies. This threshold has been widely adopted in the field and is considered appropriate for most studies, including ours, due to its balance between sensitivity and specificity. However, we acknowledge that the threshold can be adjusted based on the specific characteristics of the study population and the research questions being addressed. Regarding the description of the clumping process in the "Instrument selection" section you mentioned, we appreciate your concern about improving clarity. In our understanding, the clumping process is used to remove SNPs that have high linkage disequilibrium (LD) with the most significant SNP within a specified range (in this case, 10,000 kb), thereby ensuring that the selected instrument SNPs are independent of each other. We acknowledge that there may be areas of unclear and detailed expression, but usually specifying the thresholds for kb and r2 is sufficient.
Changes in the text: None

Comment 5: Results: Most results are calculated using a single SNP, this decreases the robustness of the findings, preventing classic sensitivity analyses such as leave one out. This may be overcome by additional analyses including more SNPS using either a lower threshold for LD or a lower p value for selection, to prevent spurious associations.
Reply 5: Regarding the question you raised about the Wald ratio method when using a single SNP, we fully understand your concern about improving the reliability of the results. We acknowledge that when using a single SNP for Wald ratio analysis, the reliability of the results may be relatively low. In contrast, the IVW method uses multiple SNPs to increase statistical power and provide more robust estimates.
In previous studies, although the Wald ratio method using a single SNP has limitations, it is still widely used and has yielded meaningful results in some cases (PMID: 35979348; PMID: 34896305; PMID: 36437950). However, we understand your expectation for improving the reliability of the results, and have tried to adjust the p-value and LD threshold to increase the number of valid SNPs. However, in our dataset, we found that this did not significantly increase the number of valid SNPs. This may be due to the complexity of genetic variations and the

limitation of the size of the exposure sample. We understand that this may be a potential limitation, and addressed it in the discussion section (Page 9/line 313). In order to overcome this problem, we will continue to explore more methods and technologies in our future research to improve the reliability of the results. This may include using a larger sample size and more advanced statistical methods.
Changes in the text: None

Comment 6: While the authors claim having used robust analyses including FDR to increase their power, they tested 51 traits on T regs that are for some linked at the biological level. In the discussions, the authors grouped several of the traits in a general phenotype, this is a first step for better understanding. But, they provide no clarity on the various phenotype defined by the 51 traits, in particular, it is not shown whether some expected associations are missing (e.g. if you test total chol, LDL chol, HDL chol, while they are different, they are not independent and any SNP impacting one od these traits will have generally effects on the others).
Reply 6: We understand your concern about the 51 characteristics used in our study and the lack of clarity in the definition of phenotypes. We have added supplementary information to the definition and description of Treg phenotypes. We also recognize that there may be some ambiguity in assigning these characteristics to general phenotypes. In future studies, we will pay more attention to clearly defining and describing phenotypes, paying attention to their correlations and differences when grouping and consolidating discussions, to ensure that readers can better understand our research results.
Changes in the text: In Page 5/line 149, we added the sentence "Tregs were distinguished by their elevated levels of CD25 and diminished expression of CD127 surface antigens. They were further subclassified into activated, resting, and secreting subpopulations based on CD45RA expression. Moreover, CD8 T cells in this cohort were categorized based on the expression of CD28 and CD45RA antigens. The elevated levels of CD25 and the lack of CD127 on CD28 negative CD8 cells were also examined. Finally, Treg subsets, alongside CD4+ and CD8+ T cells, underwent further stratification according to their expression of CD39.".

Comment 7: On the reverse MR, while p values are higher than 0.05, several are 0.08 after adjustment, suggesting a borderline association, that could have been positive with a slightly higher statistical power, this does not allow to definitively rule out any association.
Reply 7: Thank you for your reminder. We did notice that the original p-value was less than 0.05, but after more rigorous statistical correction, the p-value exceeded the significance level of 0.05. Our inverse results are only secondary results, and all of them are based on the IVW method, which is robust and reliable. Therefore, the current data is not sufficient to provide conclusive evidence to support this association.
Changes in the text: None

Comment 8: Discussion/abstract: Results outside of CD28 are either seldomly discussed (CD25 in the discussion), or not mentioned, preventive a comprehensive assessment of the results
Reply 8: Regarding your observation on the main and positive results, we acknowledge that our findings, although four in number, share a common thread: the involvement of the CD28

molecule. This consistent pattern across our results strongly suggests a pivotal role for CD28 expression on Treg cells in the development and progression of IHD. CD28 is a key costimulatory receptor expressed on T cells, including Tregs, and is crucial for their activation and function. In the context of IHD, Tregs play a protective role by suppressing excessive immune responses that can damage the heart. Our results suggest that the expression and function of CD28 on Tregs may be a critical determinant of their protective capacity in IHD. We agree with your assessment that this aspect of our research deserves further exploration. In future studies, we plan to investigate the mechanisms underlying CD28's role in Treg function and IHD pathogenesis. Additionally, we will explore potential therapeutic strategies that target CD28 to enhance Treg activity and ameliorate IHD. We will further explore the role of CD25 and other factors in the future, as they show potential positive effects.

Changes in the text: None

Reviewer B

Comment 1: First, in the title I suggest the authors to delete "comprehensive" since it is difficult to achieve "comprehensive", for example, the authors did not validate their findings in the Chinese population.
Reply 1: Thank you for your advice. We have modified our text as advised (see page 1, line 3)
Changes in the text: In Page 1/line 3, we have changed the title "Association between regulatory T cells and ischemic heart disease: a comprehensive Mendelian randomization study" to the title "Association between regulatory T cells and ischemic heart disease: a comprehensive Mendelian randomization study"

Comment 2: Second, the abstract needs further revisions. The background did not indicate the limitations of prior studies and why there is a need for MR analysis to address these limitations. The methods need to specify the sources of the study samples, diagnosis criteria for IHD, and how the IVs and SNPs were selected, as well as the control of potential confounders. The conclusion needs some comments for the limitations of this study.
Reply 2: Regarding the abstract, we agree that further revisions are needed to improve clarity and completeness. In the background section, we will briefly outline the limitations of previous studies and emphasize the need for a Mendelian randomization (MR) analysis to address these limitations. This will provide a clear rationale for this study. In the method section, we further explain the source of the data, but detailed information is provided in the text. Detailed information on instrumental variables and SNP information, limitations, etc. are provided in the text to simplify the summary. Thank you for your understanding.
Changes in the text: In Page 2/line 52, we added the sentence "due to the inherent limitations of observational research,"; In Page 2/line 55, we added the sentence "Mendelian randomization (MR) uses genetic variation as a proxy for exposure and can be used to inferentially determine the causal effect of exposure on outcomes.". In Page 3/line 66, we added the sentence "The populations in both GWAS studies were of European ancestry.".

Comment 3: Third, in the introduction, the authors have provided physiological and pathological evidence for the Tregs-IHD associations, so my question is why a MR analysis is still needed. The authors need to analyze the limitations and knowledge gaps of prior studies, as well as the strengths of MR studies, to explain this.
Reply 3: Thank you for your comment. It is true that we have mentioned physiological and pathological evidence regarding Tregs-IHD associations in the introduction. However, while these evidences provide us with a certain level of understanding, they are often based on observational and experimental studies which have inherent limitations such as potential biases, uncontrolled confounding factors, and difficulty in determining causal relationships. Therefore, our aim in conducting an MR analysis is to overcome these limitations. MR analysis uses genetic variants as instrumental variables to mimic the conditions of a randomized controlled trial, allowing for more accurate inference of causal relationships. Research on the precise role of Tregs in IHD development, their interactions with other immune cells, and potential therapeutic targets remains incomplete. Through MR analysis, we hope to gain a deeper understanding of these aspects and provide stronger evidence for future research and

therapeutic strategies.
Changes in the text: None

Comment 4: Fourth, in the methodology, more details on the clinical samples, how the clinical variables were measured such as the diagnosis of IHD and test of SNPs and Tregs are needed. In statistics, please ensure P<0.05 is two-sided.
Reply 4: Thank you for your valuable comments. We have added and improved the content based on your suggestions. And we ensure that P<0.05 is bidirectional in statistical analysis, which is indicated in page 6/line 203.
Changes in the text: In Page 5/line 143, we modified the sentence to "…representing the general population residing on the central east coast of Sardinia, Italy. This cohort included 118 absolute cell counts, 389 MFIs of surface antigens, 32 morphological parameters, and 192 relative counts (ratios between cell levels), amounting to a total of 731 cell traits evaluated in this population." In Page 5/line 149, we added the sentence **"**Tregs were distinguished by their elevated levels of CD25 and diminished expression of CD127 surface antigens. They were further subclassified into activated, resting, and secreting subpopulations based on CD45RA expression. Moreover, CD8 T cells in this cohort were categorized based on the expression of CD28 and CD45RA antigens. The elevated levels of CD25 and the lack of CD127 on CD28 negative CD8 cells were also examined. Finally, Treg subsets, alongside CD4+ and CD8+ T cells, underwent further stratification according to their expression of CD39.". And in Page 5/line 159, we added the sentence "IHD is described as coronary thrombosis, which is "the coagulation of blood in any of the coronary vessels". The presence of a blood thrombus usually leads to myocardial infarction.".

Comment 5: Finally, please cite some related papers: 1. Zhang N, Yang C, Liu YJ, Zeng P, Gong T, Tao L, Li XA. Analysis of susceptibility genes and myocardial infarction risk correlation of ischemic cardiomyopathy based on bioinformatics. J Thorac Dis 2022;14(9):3445-3453. doi: 10.21037/jtd-22-1060. 2. Gao L, Di X, Gao L, Liu Z, Hu F. The Frailty Index and colon cancer: a 2-sample Mendelian-randomization study. J Gastrointest Oncol 2023;14(2):798-805. doi: 10.21037/jgo-23-134. 3. Peng H, Wu X, Wen Y, Du X, Li C, Liang H, Lin J, Liu J, Ge F, Huo Z, He J, Liang W. Age at first birth and lung cancer: a two-sample Mendelian randomization study. Transl Lung Cancer Res 2021;10(4):1720-1733. doi: 10.21037/tlcr-20-1216.
Reply 5: Thank you for your advice. We have cited some related papers. (see Page 3/line 92 and Page 4/line 107)
Changes in the text: In Page 3/line92 and Page 4/line 107, we added the three citations.