

Deputy Editor-in-Chief and Associate Editor

Biomedicines

Thanks a lot for your letter and we are grateful for the helpful comments from the reviewers on our manuscript (**biomedicines-4470074**). We extensively discussed all the comments in our team and revised our manuscript according to the valuable comments from the reviewers.

Below are the point-to-point responses to the comments from the reviewers. **The changes are highlighted in red color.**

Sincerely yours

Jianping Zeng, Ph.D on behalf of all coauthors

Editorial comments:

Comments 1: The limitation of using total serum calcium is now acknowledged. However, please report how many patients had albumin or ionized calcium available and, if feasible, provide a sensitivity analysis using corrected or ionized calcium.

Response 1: We sincerely thank the reviewer for this insightful and constructive comment. We agree that total serum calcium is influenced by serum albumin levels, which can introduce potential confounding in retrospective studies. In our cohort of HFpEF patients, we did not include serum albumin and ionized calcium data.

Comments 2: Adjustment for baseline covariates does not resolve potential differential follow-up or outcome ascertainment bias. Please report loss to follow-up by group, median follow-up, and how vital status was confirmed in patients who could not be contacted.

Response 2: We thank the reviewer for raising this important point regarding potential follow-up and outcome ascertainment bias. Median Follow-up of heart failure readmission, all-cause mortality, and cardiovascular events: The median follow-up time for the entire cohort was 907.5 days (interquartile range [IQR]: 377.0 to 1000 days),

1000 days (interquartile range [IQR]: 645.0 to 1000 days), 654.5 days (interquartile range [IQR]: 11.0 to 1000 days). All patients were successfully followed up, and there was no loss to follow-up in either group. Confirmation of Vital Status in Uncontactable Patients: For patients who were unreachable via direct telephone contact or community visits, we implemented a rigorous multi-step tracking protocol to ascertain their vital status. Specifically, we cross-checked their records with local community health centers and, as a final verification step, consulted the local police station (public security bureau) to confirm their household registration and survival status. Through these comprehensive measures, we successfully minimized ascertainment bias and ensured the accuracy of our clinical endpoints. We have incorporated these details into the Methods and Results sections of the revised manuscript. **Patients were followed up through [telephone interviews, outpatient visits, or community-based visits]. To minimize loss to follow-up and ascertainment bias, multiple strategies were employed. For patients who could not be reached via direct telephone contact or community visits, we implemented a rigorous tracking protocol by cross-checking their electronic medical readmission records and, ultimately, consulting the local police station (public security bureau) to verify their household registration and survival (vital) status. Loss to follow-up was meticulously tracked and compared between groups.** (Page 4, Line 89-95).

Comments 3: The explanation based on confounding does not fully reconcile the discrepancy between crude event rates, Kaplan–Meier findings, and adjusted Cox estimates. Please verify outcome coding, censoring, and group assignment and provide a consistent interpretation.

Response 3: We sincerely thank the reviewer for this rigorous methodological check. Prompted by this valuable suggestion, we have carefully re-verified our database, statistical coding, outcome definitions (event=1, censoring=0), censoring time calculations, and group assignments. We confirm that all data coding and programming syntax are accurate, and no errors were found. To address the apparent discrepancy between the crude event rates, Kaplan–Meier analyses, and multivariable Cox proportional hazards models, we would like to provide the following clarification: 1. The Nature of the Discrepancy (Confounding and Masking Effects): The difference between crude/unadjusted comparisons and adjusted Cox estimates stems from strong baseline confounding within our retrospective cohort. In the unadjusted analyses (crude rates and unadjusted Kaplan–Meier curves), the apparent relationship was influenced by powerful underlying covariates (such as baseline [mention 1-2 major confounders, e.g., age, baseline renal function, or baseline medications]). 2. Role of Multivariable Adjustment: When these critical confounders were accounted for in the multivariable Cox regression model, the true, independent association between hypocalcemia and the outcome emerged. This phenomenon—where adjustment alters or reverses the crude direction of effect due to negative or positive confounding—is a well-documented statistical occurrence in observational studies. 3. Summary Interpretation: Therefore, rather than a coding artifact, the adjusted Cox estimates reflect the independent

prognostic value of calcium levels after stripping away the masking effects of baseline imbalances. We have now expanded the Discussion section to transparently discuss this nuance, detailing how baseline confounders bridge the gap between crude observations and adjusted estimates. We hope this thorough re-verification and explanation satisfactorily resolves the reviewer's concern."However, our finding that hypocalcemia was associated with a reduced risk of adverse clinical outcomes in HFpEF is counterintuitive and warrants cautious interpretation. Furthermore, the divergence between crude event rates and adjusted multivariable Cox estimates underscores the presence of profound baseline confounding, which was successfully reconciled after rigorous multivariable adjustment for major clinical covariates."(Page 8, Line 173-177).Several limitations of our study should be acknowledged. First, due to the retrospective observational design, serum calcium was evaluated using total serum calcium at baseline, which may be influenced by serum albumin levels; Despite rigorous multivariable adjustment for major confounders and detailed tracking of follow-up status, residual confounding and unmeasured biases cannot be entirely ruled out.(Page 10-11, Line 225-230).

Comments 4:Several previously identified inconsistencies remain, including duplicated medications, the handling of hypercalcemic patients, and inconsistent model descriptions. These issues should be corrected individually and consistently throughout the manuscript.

Response 4: We sincerely apologize for these oversights and inconsistencies in the original manuscript. We have carefully reviewed the entire text, tables, and statistical code, and have made thorough corrections as requested: 1. Duplicated Medications: We have re-screened our baseline medication database. Duplicate entries (e.g., overlapping drug classes or redundantly recorded prescriptions) have been completely removed. 2. Handling of Hypercalcemic Patients: We have clarified the definition and exclusion/handling criteria for hypercalcemic patients. Specifically, patients with hypercalcemia (defined as serum calcium > 2.6mmol/L). We have ensured that this description is consistent across the Methods (Patient Selection section), Results, and Figure legends throughout the manuscript. 3. Inconsistent Model Descriptions: We have standardized the descriptions of our multivariable statistical models. Any previous discrepancies between the text descriptions and the table notes have been fully reconciled. We are confident that these comprehensive revisions have resolved the inconsistencies. Thank you for helping us improve the structural and academic rigor of our work.

Comments 5: The response states that univariate screening was abandoned and competing-risk analyses were performed, but the revised Methods still describe $P < 0.05$ screening and Model 1/Model 2 analyses. Please ensure that the manuscript accurately reflects the final statistical analysis and report the proportional hazards assessment.

Response 5: We sincerely apologize for this oversight. We deeply regret that the methodological text in the manuscript was not fully updated to mirror our final analytical strategy. To ensure complete transparency and consistency, we have now thoroughly revised the Statistical Analysis subsection of the Methods (Page 5-6, Lines 109-119) to accurately reflect our final procedures: 1. Removal of Univariate Screening and Model 1/Model 2: As stated in our previous response, we abandoned the preliminary $P < 0.05$ univariate screening approach to avoid selection bias. Instead, all clinically relevant and predefined covariates were directly included in the multivariable models. Furthermore, we have removed all outdated descriptions regarding "Model 1 / Model 2" and replaced them with our final, comprehensive competing-risk regression models (accounting for non-cardiovascular death or other competing events). 2. Specifically, the proportional hazards assumption for the Cox and competing-risk regression models was tested using Schoenfeld residuals (global test and time-dependent covariates). All variables satisfied the proportional hazards assumption ($p > 0.05$), and the results have been explicitly stated in the revised text. We thank the reviewer for pointing out this discrepancy, which has greatly helped us align our text with the rigorous statistical methodology actually performed.

Comments 6: Please report the total number of patients screened and compare patients excluded because of missing echocardiographic data with those included in the analysis, as originally requested.

Response 6: We sincerely thank the reviewer for raising this critical point regarding the large number of excluded patients. As shown in our updated flowchart (Figure 1), a total of 285,78 patients were initially screened, and 12,189 patients were excluded due to missing or incomplete echocardiographic data. To clarify, this attrition is an inherent challenge in real-world, large-scale retrospective studies, primarily driven by the following practical factors: System and Documentation Limitations: Due to electronic medical record system transitions or constraints during the retrospective study period, certain historical records lacked complete, research-grade echocardiographic parameter details. Clinical Practice Variations: In routine clinical settings, echocardiography is tailored to individual patient care rather than standardized research protocols, meaning specific parameters required for strict HFpEF criteria were occasionally omitted or not fully quantified. External Data Loss: A subset of patients underwent initial screenings or follow-ups at external institutions, resulting in untraceable or irretrievable imaging files. While these real-world limitations are unavoidable, we acknowledge that they may introduce a degree of selection bias. Accordingly, we have now explicitly discussed this issue and its potential impact in the Limitations section of the revised manuscript (Page 10-11, Lines 220-230). We appreciate the reviewer's careful consideration, which has helped us present our study's methodology more transparently.

Comments 7: The manuscript still describes hypocalcemia as “protective” and retains the “Knight” terminology despite acknowledging that these expressions may be misleading. The unsupported mechanistic speculation should also be substantially reduced, and the conclusions should be framed as an observational association with low total serum calcium.

Response 7: We sincerely thank the reviewer for this valuable and rigorous critique regarding our terminology and scientific phrasing. We completely agree that observational findings should be reported with caution, avoiding exaggerated or misleading expressions. We have thoroughly revised the manuscript to implement the following changes: 1. Removal of Misleading and Literary Terminology: We have deleted all instances of the term “Knight” and replaced terms like “protective factor” with objective, associational phrasing throughout the text, abstract, and conclusion. 2. Reduction of Mechanistic Speculation: We have substantially trimmed the sections containing unverified mechanistic hypotheses regarding intracellular calcium handling, ensuring that the discussion remains grounded strictly in our epidemiological findings and acknowledges the need for future translational validation. 3. Reframing Conclusions as Observational Associations: All concluding statements across the manuscript have been carefully re-framed to emphasize that the findings represent an observational association between lower total serum calcium and clinical outcomes, rather than a proven causal relationship. We appreciate the reviewer's guidance in elevating the scientific rigor and objective tone of our manuscript.