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Peer Review File

Caloric Restriction Improves Glycemic Control via the Adiponectin–Ceramide Axis in Non-obese Men and Women: the CALERIE™ 2 randomized controlled trial

Corresponding Author: Dr Moritz Warmbrunn

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This was a worthwhile exploratory analyses using samples from the previously published CALERIE study, whereby the authors have undertaken additional analyses of serum adiponectin and lipid metabolites including ceramides. The author provides some mechanistic support that improvements in glycaemic control, including insulin sensitivity may be mediated through the increase in adiponectin and the subsequent reductions in several ceramide species. I have some queries which I would like the authors to address:

1. It seems like 27 participants fewer than the original CALERIE study which had 218 participants - given that there were proportionally more participants missing from the CR group than the AL group in this sub-analyses, please provide more details about the missing participants. Could this have affected the original randomisation of this trial?

2. Please specify the number males and females in each group

3. Table 1: The C-peptide data seems identical for CR and AL groups - is this a typo?

4. Line 267: Table S12-S13 is difficult to find from this large file. Please consider separating the source data from non-source data

5. Line 344-345: Given this information did the authors investigate whether there were any sex-specific differences in the mediation effects reported in this study?

6. In addition to CR, what other dietary or lifestyle factors might be influencing adiponectin-ceramide axis that may modulate metabolic outcomes?

Reviewer #2

(Remarks to the Author)
Manuscript Summary:
Based on data from three time points (baseline, 12 months, 24 months) from caloric restriction (CR) intervention trial, the manuscript “Caloric Restriction Improves Glycemic Control via the Adiponectin–Ceramide Axis in Non-obese Men and Women: the CALERIE™ 2 randomized controlled trial” by Moritz V. Warmbrunn and colleagues explores the effects of CR on lipid metabolites and the interplay with biomarkers of metabolic regulation and inflammation. The work relies on post hoc analyses of data from the Calerie 2 randomized controlled trial, a 24-month CR intervention in healthy, non-obese individuals, which reported achieved median caloric intake reduction of approximately 13% along with reductions in fasting insulin, C-peptide, HOMA-IR, AUC insulin and Matsuda index compared with the non-CR control group (2019).

In this secondary analysis of the Calerie 2 trial, the authors assess CR-induced shifts in lipid metabolites by principal coordinate analysis (global shifts) and module preservation analysis within the framework of weighted metabolite co-expression network analysis. One module was not preserved between groups and the cross-sectional associations of this

cluster with markers of insulin (AUC insulin and C-peptide), adiponectin, and inflammation were studied further in mediation analyses.

The study addresses an important topic, the metabolic adaptation to CR, which is very relevant to understand the potential health benefits of reduced energy intake.

Strengths
• The study relies on solid data from repeated collection of biospecimens in a 24-month CR RCT, which allows credible causal conclusions concerning the main effects of the diet intervention on shifts in lipid profiles. The dataset allows cross-sectional linkage between CR-induced metabolite shifts and effects on existing established biomarkers and physiological tests, which were described in the primary and secondary outcome report of the Calerie 2 trial.
• Lipidomics analyses add relevant information because effects on lipid metabolism are a biologically plausible intermediate effect that connects CR to downstream metabolic health outcomes.
• Overall, the language is clear, and the paper is well written.

Weaknesses
• The association studies between lipid metabolites and biomarkers of health and disease rely on cross-sectional analyses. Therefore, the differentiation between mediators, downstream metabolic health effect (as exemplified in Fig. 6), and correlated factors that are not critically involved in biological processes relies on strong assumptions. The authors do not always acknowledge these assumptions explicitly and the biological interpretations tend to employ language that implies clear effect directionality (e.g., “CR significantly reduced TNF-a levels at both time points via changes in triacylglycerols”, “Adiponectin mediates ceramide reduction under caloric restriction”). Some causal conclusions are not supported clearly by the results and must be toned down.
• Some of the modeling choices are debatable and need clearer explanation and justification regarding the biological questions they aim to address. For example, the focus on network preservation statistics as primary approach for lipidomics variable selection needs to be introduced and discussed in the context of specificities of metabolite networks and their sensitivity for diet-induced disturbances. Do we expect that effects of CR on lipid metabolites are restricted to modules with a disturbed coregulation structure? What does that mean biologically? Do we expect that the effect of CR on lipid metabolism is restricted to modification of the modular co-regulation structure in the lipidome that reflects biological pathway organization? Conversely, if we assume that specific metabolites or pathway activities in lipid metabolism can be affected by CR without modifying the modular co-regulation structure, would the selection based on network module preservation statistics be sensitive for such effect? This concern appears particularly relevant because, as shown in Fig. 4A, many of the discarded modules appear to be highly relevant for the metabolic health biomarkers. The variable selection strategy appears to bear the risk of missing relevant effects of CR on lipid metabolism.
• It is unclear how exactly the authors selected specific metabolites for mediation analyses and why they moved from a data-driven multi-variable selection of network modules to an apparently hypothesis-driven single mediator analysis.

Major comments:
The weaknesses should be addressed through clearer description of the methods, additional analyses, and more careful discussion.
• Please explicitly acknowledge the biological/causal assumptions underlying the modeling approaches, especially network analyses and mediation analyses. Move pure interpretation to the discussion. Tone down the causal conclusions concerning mediating roles of the selected lipid metabolites in the relationship between CR and metabolic health throughout the results and discussion. Also, Fig. 6 should be clearly labeled as hypothesis illustration and not as data analysis result; consider moving it up in the MS.
• Motivate and explain the modeling choices and their implications/limitations in more detail. In the main MS, this refers to a clearer and better structured high-level explanation of the specific network analysis methodology and a more nuanced and critical acknowledgement of the hypotheses underlying the specific mediation analyses. Also, provide a clearer description of the network analysis in the methods section that guarantees reproducibility.
• Motivate each single mediation hypothesis. For example, some shifts in lipid metabolites can be mediator of CR effects on insulin resistance (like CR -> lower CER levels -> reduced IR), but in other cases shifts in lipid metabolites may be the consequence of reduced insulin resistance (CR -> reduced IR -> lower TGs).
• The exploration of mediation hypotheses in a multi-dimensional approach would complement the multi-variable selection procedure more organically. For example, the HIMA package could identify main mediators in a multi-variable selection framework.
• The word "pathway" is used too liberally. Analyses did not rely on metabolic pathway annotations. Please avoid the word “pathway” in relation to the analyses results because of the particular meaning of metabolic pathway in molecular biology. Preferably, refer to biological process or similar language.

Specific comments:
- AUC insulin and C-peptide are explored in relation to the lipid metabolites and the results are presented. However, the results of Matsuda index associations are referred to a table in the supplements. Better presentation is needed.
- It is difficult to assess whether all negative results have been presented. Please make sure that all pre-planned analyses are reported as supplementary data.
- There is an overlap between confidence intervals of self-reported caloric intake in the AL and CR group at 24 months. Please discuss how this may affect the outcomes at 24 months.
- Inconsistent text size/font in manuscript
- Need for correction in text in table 1
- Need for explanation of abbreviations earlier in text

- Need for change of color in figure 4 as black * are difficult to see in blue background
- Specify in text that it is HMW adiponectin

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
Caloric Restriction Improves Glycemic Control via the Adiponectin–Ceramide Axis in Non-obese Men and Women: the CALERIE™ 2 randomized controlled trial

Suggestions and Recommendations:
1. line 452-454, Metabolites: "Before analysis, data were scaled and imputed were each biochemical in original scale is re-scaled to have median equal to 1, then, missing values are imputed with the minimum."
The paper does not mention how much data was missing or how many values were absent. Could you clarify if metabolites with a lot of missing values were handled the same way? If yes, could this lead to bias in the analysis?

2. line 460-461, Statistical analysis: "The analysis included only complete cases across selected metabolites Statistical significance of group, time, and interaction effects was evaluated using permutational multivariate analysis of variance (PERMANOVA), with significance assessed via 999 permutations."
It is unclear what "complete case" means in this context. In this analysis, does it refer to the original metabolic data with no missing values, or the metabolic data sets after minimum value imputation?

3. line 466-467, Statistical analysis: "To assess differential longitudinal trajectories between intervention groups, linear mixed-effects models were applied to each metabolite and clinical variable, a robust method that models change over time using all available data and handling missingness via maximum likelihood."
This is a misunderstanding of the statistical method. Mixed-effects model is not a method to address missing data. It can be used in the presence of missing data because it does not require equal number of observation such as MANOVA. But, it may still leads to biased results under certain missing mechanisms.

4. line 469, Statistical analysis: "Analyses were restricted to metabolites with sufficient non-missing data and variability."
line 498, Statistical analysis: "Models with missing data, insufficient variation, or failed model convergence were excluded."
How do you measure sufficient non-missing data and variability, and what are the criteria?

5. line 84-86, Results: "A total of 191 participants with available metabolomic data and functional metabolic outcomes (e.g., oral glucose tolerance test) were included in the final analytical sample from the original CALERIE study."
How were the 191 participants chosen from the original 218 samples in the CALERIE study? Metabolic data seems to have been available for all 218 samples, as seen in Tables 3 and 5 of the published study [1]. Would it be possible to share a flowchart that outlines this selection process?
Reference:
[1] Kraus WE, Bhapkar M, Huffman KM, et al. 2 years of calorie restriction and cardiometabolic risk (CALERIE): exploratory outcomes of a multicentre, phase 2, randomised controlled trial. Lancet Diabetes Endocrinol. 2019;7(9):673-683.

6. line 92, table1: Please remove the P values from the baseline table, as noted in the references, "Such hypothesis testing is superfluous and can mislead investigators and their readers. Rather, comparisons at baseline should be based on consideration of the prognostic strength of the variables measured and the size of any chance imbalances that have occurred."[2], and "Significance testing of baseline differences is not recommended and should not be reported."[3]
Reference:
[2] Moher D, Hopewell S, Schulz KF, et al. CONSORT. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. Int J Surg. 2012;10(1):28-55.
[3] Hopewell S, Chan AW, Collins GS, et al. CONSORT 2025 explanation and elaboration: updated guideline for reporting randomised trials. BMJ. 2025 Apr 14;389:e081124.

7. line 113-114: "A total of 972 lipid-related metabolites were profiled longitudinally" The study included 972 metabolites and many clinical outcomes, leading to a large number of statistical tests. Was the False Discovery Rate (FDR) controlled for? And with so many linear model analyses and mixed model analyses, how can we be confident that the positive results are

real?8. line 169: "To test this hypothesis, we performed causal mediation analysis to disentangle direct and indirect effects of CR on clinical outcomes at 12 months and 24 months."
Adjustment for covariates was not mentioned in the methods of this paper. Since this paper focuses on causal inference rather than just exploratory analysis, it is important to adjust for covariates—even when they are balanced through randomization. It recommends adjusting for key prognostic factors and baseline variables in the main analysis of randomized trials [4]. This approach helps improve the reliability and
robustness of the results. Furthermore, when studying the relationship between metabolites and clinical outcomes, other conditions may not be balanced, which could introduce unknown confounding factors.
Reference:
[4] Holmberg MJ, Andersen LW. Adjustment for Baseline Characteristics in
Randomized Clinical Trials. JAMA. 2022;328(21):2155–2156.

9. line 291-293, Discussion: "Specifically, we identified a causal pathway in which CR increased high-molecular-weight adiponectin, which in turn reduced circulating ceramide species—bioactive lipids mechanistically linked to insulin resistance."
line 300-302, Discussion: "Our findings corroborate prior evidence from animal and human studies indicating that diet and exercise interventions reduce ceramide levels in parallel with improved insulin sensitivity 30."

line 322-325, Discussion: "Together, these findings suggest that ceramides play a direct, causal role in insulin resistance development, a conclusion supported by the findings of our study, where reduced ceramide levels mediated improvements in insulin sensitivity in the CR group, but not in the AL group (Fig. 6)." The findings in this paper are based on just one study and its methods. This study did not control for false-positive results, account for other confounding factors, or perform sensitivity analyses. As a result, its causal claims may be too strong and are not fully convincing. It is recommended that these limitations should be acknowledged in the Discussion section.

10. line 92, table1: Please indicate in the footnotes of Table 1 which distributions of data are represented by SD and which by quartiles.11. line 305: I don't find the descriptions of (E) and (F) in the caption of Figure 5. Please check it.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors seem to have adequately addressed the comments raised by me and the other reviewers which have further improved the manuscript.

Reviewer #2

(Remarks to the Author)
While some of the comments that I had raised during the first revision were addressed, several conceptual issues remain.

1. Applying module preservation analysis to identify lipid metabolites for downstream mediation analyses. The paper by Langfelder and colleagues in 2011 focuses on whether the correlation structure of a network module stays consistent when conditions change, rather than looking at shifts in metabolite levels. This means it can detect structural changes in metabolite relationships but not simple shifts in concentrations (or relative abundances). In other words, even if metabolite levels change, the module would be considered preserved unless the correlation patterns are altered. It appears questionable that the main effect of CR is to rewire the lipid metabolite network; many relevant CR effects would plausibly alter the flux through stable metabolic pathways which would result in a preserved structure of the metabolite network but distinct relative abundances between CR and AL group that affect whole modules. A strong rationale for mediator pre-selection through module preservation analysis is still lacking and limitations of the approach are still not discussed.

2. Mediation analysis. Multi-mediator models are still lacking. At least correlated ceramides should be examined jointly to assess if their role as mediators is mutually independent or not. Confidence intervals for mediation estimates should be reported in the text. Mediation analysis methodology: While bootstrapping improves the reliability of each interval, it does not adjust for the increased likelihood of error across all tests. To control for multiple comparisons the family-wise error rate still needs to be controlled. Adjustment for sex and age is not sufficient to control for potential confounding; it should be tested whether mediation models are robust against adjustment for metrics of adherence, diet composition, physical activity, etc.

3. Discussion of limitations should also include the assumptions underlying the biological (causal) interpretation of mediation analyses and limitations of data and analytical choices.

4. In several instances, the language is still too strong in relation to novelty and causal interpretations of the results. For example, the conclusion at the end of the discussion indicates strong mechanistic evidence, while the mediation models only moderate support for agreement of the data with the tested mediation hypotheses, and any mechanistic interpretation relies on string assumptions.

Reviewer #3

(Remarks to the Author)
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Reviewer #4

(Remarks to the Author)
I have no more comments

Reviewer #5

(Remarks to the Author)
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Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have added multi-mediator models and softened the causal language in the interpretation of the results. The remining limitation, especially related to variable selection based on network module stability, are now acknowledged and discussed. I have no further comments.

Reviewer #3

(Remarks to the Author)
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Reviewer's feedback and Authors' responses

Reviewer #1

1. This was a worthwhile exploratory analyses using samples from the previously published CALERIE study, whereby the authors have undertaken additional analyses of serum adiponectin and lipid metabolites including ceramides. The author provides some mechanistic support that improvements in glycaemic control, including insulin sensitivity may be mediated through the increase in adiponectin and the subsequent reductions in several ceramide species. I have some queries which I would like the authors to address

It seems like 27 participants fewer than the original CALERIE study which had 218 participants - given that there were proportionally more participants missing from the CR group than the AL group in this sub-analyses, please provide more details about the missing participants. Could this have affected the original randomisation of this trial?

Authors' response:
Thank you for this thoughtful comment. In the present analysis, we included only participants with both metabolomic data and functional metabolic outcomes available. As a result, 27 participants from the original CALERIE cohort were excluded.

To assess potential bias, we compared baseline characteristics of the included (n = 191) and excluded (n = 27) participants (Table R1). No significant differences were observed, suggesting that exclusion of these participants is unlikely to have affected the original randomization or the comparability of the study groups.

We note that prior publications from the CALERIE trial have similarly used opportunistic subsamples based on data availability. For example, Waziry et al. (2023, doi:10.1038/s43587-022-00357-y) analyzed 164 participants, and Dorling et al. (2020, doi:10.1007/s00394-020-02361-7) analyzed 188 participants.

Table R1. Baseline characteristics of included CALERIE trial participants (n = 191) and excluded participants (n = 27), as presented in Kraus et al. Lancet 2019 (doi:10.1016/S2213-8587(19)30151-2).

Overall	**Study sample**	**Excluded sample**	**p-value**
	n=191	n=27	
Weight kg (mean (SD))	72.15 (9.23)	69.60 (8.87)	0.179
Fat Mass kg (mean (SD))	25.20 (1.69)	24.85 (1.92)	0.321
Fate Free Mass kg (mean (SD))	48.47 (9.07)	47.33 (8.75)	0.540
Systolic blood pressure mmHg (median [IQR])	113 [106, 121]	109 [103, 117]	0.070
Glucose mg/dL (median [IQR])	86 [82, 91]	84 [80, 88]	0.057
Insulin ulU/m (median [IQR])	5.13 [3.90, 7.21]	4.55 [3.57, 6.96]	0.483

C-peptide pmol/L (median [IQR])	462 [338, 594]	429 [363, 545]	0.634
LDL Cholesterol mg/dL (median [IQR])	102.50 [81.00, 119.75]	98.00 [80.50, 110.50]	0.246
HDL Cholesterol mg/dL (median [IQR])	46 [41, 55]	47 [41, 61]	0.675
Total energy expenditure kcal/day (mean (SD))	2308 [2096, 2640]	2325 [2061, 2540]	0.894

2. Please specify the number males and females in each group

Authors' response:
We have added an additional row to Table 1 reporting the number and percentage of female participants in each group (and, by inference, the number of males).

3. Table 1: The C-peptide data seems identical for CR and AL groups - is this a typo?

Authors' response:
Thank you for pointing this out. The values in Table 1 are correct (this is not a typo) and reflect the similarity between groups at baseline. For clarity, the underlying distribution of C-peptide values (quantiles) by group is shown below:

- **CR group:** q0 = 66, q25 = 363, q50 = 462, q75 = 594, q100 = 825
- **AL group:** q0 = 198, q25 = 330, q50 = 462, q75 = 594, q100 = 924

As shown, the median and upper quantiles are very similar between the CR and AL groups, consistent with the reported means. Thus, the values presented in Table 1 are accurate.

C-peptide pmol/L (median [IQR])	462.00 [363.00, 594.00]	462.00 [330.00, 594.00]	0.796

4. Line 267: Table S12-S13 is difficult to find from this large file. Please consider separating the source data from non-source data

Authors' response:
We apologize for the inconvenience. We have now divided the Supplementary Material into four separate files to improve clarity and ease of navigation:

- **Part 1:** Linear mixed-effects models
- **Part 2:** Module–trait relationships
- **Part 3:** Mediation analysis
- **Part 4:** Module preservation statistics

Tables S12–S13 are now located in the corresponding parts, making them easier to locate.

5. Line 344-345: Given this information did the authors investigate whether there were any sex-specific differences in the mediation effects reported in this study?

Authors' response:
Thank you for this important suggestion. In response, we conducted additional sensitivity analyses including sex and age as covariates in the mediation models for key outcomes (AUC insulin and C-peptide). The results were consistent with our primary analyses: ceramides 18:0, 20:1, and 22:1 remained significant mediators (even when correcting for sex and age) of improved insulin sensitivity in the CR group, with no evidence of mediation in the ad libitum group. Similarly, ceramides 18:0 and 22:0 continued to mediate changes in C-peptide in the CR group only. These patterns were observed both between baseline and 12 months and between baseline and 24 months.

AUC insulin Caloric (BL12M):

CR: Ceramide 18:0 average causal mediated effect (ACME) -0.04[-0.15, -0.02]
AL: Ceramide 18:0 ACME: 0.02 [-0.10, 0.13]
CR: Ceramide 20:1 ACME: -0.02[-0.07, -0.004]
AL: Ceramide 20:1 ACME: -0.02 [-0.09, 0.04]
CR: Ceramide 22:1 ACME: -0.02 [-0.14, -0.006]
AL: Ceramide 22:1 ACME: -0.03 [-0.15, 0.09]

These findings indicate that the mediation effects are robust and not materially influenced by sex or age. We have added a paragraph to the Results section (lines 288–295) under **"Sensitivity analyses"** describing these covariate-adjusted models and referring to Table S16:

'Sensitivity analyses including sex and age as covariates in the mediation models showed that the ceramides identified as mediators of improved insulin sensitivity remained significant after correction for multiple comparison. Ceramides 18:0, 20:1 and 22:1 mediated improvements in insulin sensitivity between baseline and 12 months for AUC insulin in the CR group, but not in the ad libitum group. Similarly, between baseline and 24 months, ceramides 18:0, 20:1 and 22:1 mediated reductions in AUC insulin in the CR group but not in the ad libitum group. These findings suggest that age and sex did not materially affect the mediation analyses (Table S16).'

We also now note in the Discussion (lines 358–359) that adjusting for sex did not meaningfully change the results.

6. In addition to CR, what other dietary or lifestyle factors might be influencing adiponectin-ceramide axis that may modulate metabolic outcomes: AUC insulin and C peptide for BL-12 months and BL-24 months.

Authors' response:

This is an excellent point. In addition to caloric restriction, other lifestyle and dietary factors might influence the adiponectin–ceramide axis and thereby modulate metabolic outcomes. For example, unsaturated fatty acids can upregulate adiponectin expression and reduce ceramide synthesis (Janiszewska et al., Nutrients 2021, doi:10.3390/nu13051394). We have revised the Discussion (lines 376–380) to state:

"Beyond CR, several factors, including dietary fat composition, fibre-rich and low-glycaemic diets, physical activity, and regular sleep patterns, have been shown to favourably modulate the adiponectin–ceramide axis. These factors may partly contribute to the interindividual variability in metabolic responses to CR."

Reviewer #2

1. Major comments: The weaknesses should be addressed through clearer description of the methods, additional analyses, and more careful discussion.
   Please explicitly acknowledge the biological/causal assumptions underlying the modeling approaches, especially network analyses and mediation analyses. Move pure interpretation to the discussion. Tone down the causal conclusions concerning mediating roles of the selected lipid metabolites in the relationship between CR and metabolic health throughout the results and discussion. Also, Fig. 6 should be clearly labeled as hypothesis illustration and not as data analysis result; consider moving it up in the MS.

Authors' response:
We thank the reviewer for this important and constructive comment. In response, we have:

- More clearly described the modeling approaches in the Methods section, including the key biological and causal assumptions underpinning the network and mediation analyses.
- Revised the Results to provide a more neutral, descriptive presentation of the findings and to avoid overstating causal interpretations.
- Shifted more speculative, mechanistic interpretation to the Discussion and explicitly flagged these sections as speculative where appropriate.

We have also revised the legend of Figure 6 to clearly indicate that it represents a hypothetical model (i.e., a conceptual illustration of our working hypothesis) rather than a direct data-analysis result. Because figures must follow the order of appearance in the text according to the journal's formatting requirements, we have retained its position in the manuscript but now clearly label it as a hypothesis-driven schematic.

Examples of changes in wording include:

- "At 12 months, mediation analysis suggests that additional ceramides (16:0, 18:1, 20:0, 22:0, 22:1, 24:1) also contributed to reduced fasting C-peptide levels."

- "Mediation analysis suggested that CR significantly reduced TNF-α levels at both time points via changes in triacylglycerols, but not ceramides, and only in the intervention group."

These revisions aim to better reflect the limitations of causal inference from our data while still conveying the potential mechanistic insights.

2. Motivate and explain the modeling choices and their implications/limitations in more detail. In the main MS, this refers to a clearer and better structured high-level explanation of the specific network analysis methodology and a more nuanced and critical acknowledgement of the hypotheses underlying the specific mediation analyses. Also, provide a clearer description of the network analysis in the methods section that guarantees reproducibility.

Authors' response:
We thank the reviewer for highlighting the need for clearer justification and explanation of our modeling choices. We have expanded and structured the methodological descriptions accordingly.

In brief, we chose weighted metabolite co-expression network analysis (WGCNA) to identify clusters of metabolites that co-vary across participants, aiming to capture coordinated lipidomic responses to CR rather than isolated metabolite changes. This approach is well-suited to uncovering underlying biological or regulatory processes. We also explicitly acknowledge its main limitation, WGCNA is based on linear correlations and may therefore miss important nonlinear relationships, while emphasizing its strength as a data-driven tool for detecting functionally related modules. This text has been added to the Methods section (lines 483–485) as follows:

"This approach is particularly suited to exploring coordinated lipidomic responses to CR. While WGCNA assumes linear correlations and may miss nonlinear dependencies, it enables data-driven detection of functionally related modules.[67]"

For the mediation analyses, we now describe more clearly that these were used to test whether CR-specific metabolite changes were statistically associated with changes in insulin-related outcomes in a manner consistent with a potential mediating role. We explicitly state the key assumptions, most notably, the absence of unmeasured confounding and correct model specification, and discuss these limitations more critically in the Methods and Discussion.

All analyses were conducted using reproducible R scripts (WGCNA v1.73, mediation v4.5.1), and full code and parameter settings are provided in the Supplementary Methods to ensure reproducibility.

3. Motivate each single mediation hypothesis. For example, some shifts in lipid metabolites can be mediator of CR effects on insulin resistance (like CR -> lower CER levels -> reduced

IR), but in other cases shifts in lipid metabolites may be the consequence of reduced insulin resistance (CR -> reduced IR -> lower TGs).

Authors' response:
We appreciate this insightful comment. Our mediation hypotheses were generated through an unbiased, data-driven workflow. First, we applied weighted metabolite co-expression network analysis (WGCNA) to identify coordinated metabolite changes between baseline and 12 months and between baseline and 24 months within the CR group. This approach is specifically designed to detect high-dimensional correlation structures and can capture nonlinear relationships (Zhang & Wong, *Comput Struct Biotechnol J* 2022, DOI: 10.1016/j.csbj.2022.07.018).

Next, we performed module preservation analysis to determine whether these network structures were specific to CR or also present in the AL group. Modules with low preservation were interpreted as CR-specific. Within these CR-specific modules, ceramides were among the most prominently represented metabolites. This prompted the hypothesis that changes in ceramides may lie on the pathway through which CR influences metabolic outcomes. We therefore conducted mediation analyses to formally evaluate this possibility. These analyses suggested that part of the CR effect on insulin-related outcomes was statistically consistent with an indirect pathway via ceramide changes, an effect not observed in the AL group.

Our mechanistic interpretation is also grounded in prior literature: adiponectin has been shown to reduce ceramide levels via adiponectin receptors, which possess ceramidase-like activity (Tanabe et al. *Nature* 2015; Kupchak et al. *Biochemistry* 2009; Holland et al. *Nat Med* 2011). In our study, CR increased HMW adiponectin, the most biologically active isoform, providing a plausible upstream regulator of ceramide reductions. Consistent with the direction of our findings, several studies have demonstrated that lowering ceramide concentrations directly improves insulin sensitivity (Yang et al., *Nat Rev Mol Cell Biol* 2018; de Mello, *Diabetologia* 2009; Majumdar et al., *Endocrine* 2011). Accordingly, in the Discussion and in our hypothetical mechanistic model (Fig. 6), we propose that CR increases adiponectin, which in turn reduces ceramides, likely via ceramidase activation, thereby contributing to improved insulin sensitivity.

To make the logic of our hypotheses transparent, we have added clarifying text to both the Results and Discussion sections of the revised manuscript:

Results section:

"Given the substantial ceramide reductions observed with CR and their potential role in mediating metabolic improvements, we next investigated upstream regulators of ceramide levels in this context. Previous research has suggested that adiponectin, an adipokine primarily secreted by adipose tissue, can lower ceramide concentrations through adiponectin receptors, which share sequence homology with ceramidases.[23–26] On this basis, we hypothesized that CR-induced increases in adiponectin may mediate reductions in plasma ceramides."

Discussion section:

"Mechanistically, we observed that CR-induced increases in high-molecular-weight (HMW) adiponectin preceded ceramide reductions and improvements in glycemic control. This is biologically plausible: HMW adiponectin preferentially binds AdipoR1, which possesses ceramidase activity and promotes intracellular ceramide hydrolysis.[51-53] Although adiponectin receptors share limited homology with canonical ceramidases,[23–26] their functional ability to activate ceramide breakdown supports a mechanistic link between adiponectin and ceramide turnover. Mediation effects were strongest at 12 months, when weight loss was greatest, and diminished by 24 months, possibly reflecting metabolic adaptation or a plateau in adiponectin response. Importantly, these effects were observed in a healthy, non-obese cohort, indicating that CR may exert metabolic benefits beyond traditionally high-risk populations.

To examine indirect pathways, we used causal mediation analysis, which assumes correct specification of the underlying causal model, assumptions that cannot be empirically verified.[54] Thus, support from prior studies, biological plausibility, and the randomized design of our trial are critical for strengthening model robustness and mitigating the risk of type I error.[54] Consistent with our findings, previous work has shown that adiponectin receptors can activate ceramidases[23–26] and that lowering ceramide levels improves insulin sensitivity.[31–34,40,41,45] Taken together, the randomized controlled design and the absence of a mediated effect in the AL group reinforce the specificity of the proposed pathway: CR increases adiponectin, which in turn reduces ceramides, likely via ceramidase activation, thereby contributing to improved glycemic control (Fig. 6).

4. The exploration of mediation hypotheses in a multi-dimensional approach would complement the multi-variable selection procedure more organically. For example, the HIMA package could identify main mediators in a multi-variable selection framework.

Authors' response:
We thank the reviewer for this helpful suggestion. We agree that a multivariable mediation framework, such as that implemented in the HIMA package, could identify key mediators in a more fully data-driven manner while accounting for inter-correlations among lipid species. In this exploratory analysis, we chose to focus on biologically hypothesized mediators derived from the network-preservation approach, but we recognize that high-dimensional mediation methods would be a valuable complement in future work.

Accordingly, we have added the following to the limitations section (lines 394–395 of the revised manuscript):

"While we focused on biologically hypothesized mediators identified through network-preservation analysis, future studies could incorporate multidimensional mediation approaches to more comprehensively identify key mediating metabolites."

5. The word 'pathway' is used too liberally. Analyses did not rely on metabolic pathway annotations. Please avoid the word 'pathway' in relation to the analyses results because

of the particular meaning of metabolic pathway in molecular biology. Preferably, refer to biological process or similar language.

Authors' response:
We appreciate this helpful clarification. In response, we have systematically revised the manuscript to avoid using the term "pathway" when referring to analytical or inferred causal processes. Instead, we now use terms such as "biological process" or "mechanistic process" in these contexts. The term "pathway" is retained only when referring to established metabolic pathways (e.g., GLUT4 translocation).

6. Specific comments: - AUC insulin and C-peptide are explored in relation to the lipid metabolites and the results are presented. However, the results of Matsuda index associations are referred to a table in the supplements. Better presentation is needed.

Authors' response:
Thank you for this helpful comment. We agree that the Matsuda index findings are of interest. However, due to journal-imposed limits on the number of display items, we prioritized figures that most directly support the main narrative, focusing on AUC insulin and C-peptide in the main text.

The associations with the Matsuda index were broadly consistent with these primary outcomes and are therefore presented in detail in the Supplementary Information. Including full graphical displays for all Matsuda index associations would substantially increase the length of an already large Supplementary file (>500 pages). To improve accessibility, we now refer more explicitly in the Results section to the relevant Supplementary table where these findings can be reviewed.

7. It is difficult to assess whether all negative results have been presented. Please make sure that all pre-planned analyses are reported as supplementary data.

Authors' response:
Thank you for this important comment. This work represents a post hoc analysis of existing CALERIE trial data, and the analysis was not pre-registered. Our objective was to explore the effects of CR on glycaemic control and to investigate potential mediating biological processes.

All analyses conducted as part of this exploratory framework, including those yielding null or negative findings, are reported in the Supplementary Material. In the main text, we focus in detail on the adiponectin–ceramide axis because it emerged as the most biologically coherent and statistically robust signal, while the remaining results are fully available in the supplementary tables and figures.

8. There is an overlap between confidence intervals of self-reported caloric intake in the AL and CR group at 24 months. Please discuss how this may affect the outcomes at 24 months.

Authors' response:
Thank you for this thoughtful comment. The apparent overlap at 24 months reflects the **standard error of the mean** (SEM) shown in Figure 1 rather than confidence intervals. Between-group differences in self-reported energy intake were formally tested using linear mixed models. At 24 months, mean self-reported energy intake remained significantly lower in the CR group compared with the AL group (β = –148 kcal/day, SE = 61, p = 0.02; Supplementary Table S1).

Thus, despite some visual overlap in SEM bars, the statistical analysis indicates a sustained, though attenuated, difference in energy intake at 24 months. This reduced separation in caloric intake may partly contribute to the smaller metabolic effects observed at 24 months compared with 12 months.

9. Inconsistent text size/font in manuscript

Authors' response:
Thank you for pointing this out. We have carefully reviewed the manuscript and corrected inconsistencies in font type and text size throughout, including at lines 43, 155, 223, 228, 247, and 280–281.

10. Need for correction in text in table 1

Authors' response:
Thank you for noting this. We have corrected the spacing and text alignment issues in Table 1. These revisions can be seen at line 92 of the revised manuscript.

11. Need for explanation of abbreviations earlier in text

Authors' response:
Thank you for highlighting this. We have revised the manuscript to ensure that all abbreviations are defined at first use.

12. Need for change of color in figure 4 as black * are difficult to see in blue background

Authors' response:
Thank you for this helpful suggestion. We have updated Figure 4 by changing the asterisks to white, which improves visibility against the blue background.

13. Specify in text that it is HMW adiponectin

Authors' response:
Thank you for pointing this out. We have clarified in the text that the adiponectin measured was the high-molecular-weight (HMW) isoform. This correction is reflected at line 350 of the revised manuscript.

Reviewer #4

1. line 452-454, Metabolites: "Before analysis, data were scaled and imputed were each biochemical in original scale is re-scaled to have median equal to 1, then, missing values are imputed with the minimum."

The paper does not mention how much data was missing or how many values were absent. Could you clarify if metabolites with a lot of missing values were handled the same way? If yes, could this lead to bias in the analysis?

Authors' response:
Thank you for raising this important point. We have now clarified the handling of missing metabolite values in the Methods and Supplementary Material.

At the metabolite level, all features were pre-processed using the same scaling and imputation procedure: for each metabolite, raw values were rescaled so that the median equalled 1, and missing values were then imputed with the minimum observed value for that metabolite.

We acknowledge that imputing missing values to the minimum could introduce bias, particularly if missingness is substantial or not completely at random. We have therefore added a statement in the Limitations section noting that this imputation strategy may bias estimates for metabolites with higher levels of missing data and that these results should be interpreted with this caveat in mind.(lines 410-411)

"We also acknowledge that imputing missing values for metabolites can introduce bias."

For clinical parameters maximal likelihood was applied, missing datapoint for clinical parameters are added in Table S17

Variable	n miss BL	pct miss BL	n miss 12 months	pct miss 12 months	n miss 24 months	pct miss 24 months
BMI	0	0	0	0	0	0
Energy expenditure	2	1.04712	3	1.571	3	1.6
Fat free mass	0	0	1	0.524	0	0
Fasting glucose	1	0.52356	2	1.047	0	0
Fat mass	0	0	1	0.524	0	0
HDL	1	0.52356	2	1.047	0	0
IGF-1	2	1.04712	2	1.047	0	0
IGFBP-1	1	0.52356	2	1.047	0	0
Insulin	1	0.52356	2	1.047	0	0

self reported caloric intake	0	0	4	2.094	5	2.6
LDL	1	0.52356	3	1.571	0	0
Leptin	1	0.52356	2	1.047	0	0
Systolic blood pressure	0	0	2	1.047	0	0
T3	1	0.52356	2	1.047	0	0
Triglycerides	1	0.52356	2	1.047	0	0
TNFa	1	0.52356	2	1.047	2	1
Waist circumference	0	0	2	1.047	0	0
Weight	0	0	1	0.524	0	0
AUC glucose	42	21.9895	8	4.188	5	2.6
AUC insulin	57	29.8429	33	17.28	6	3.1

2. line 460-461, Statistical analysis: "The analysis included only complete cases across selected metabolites Statistical significance of group, time, and interaction effects was evaluated using permutational multivariate analysis of variance (PERMANOVA), with significance assessed via 999 permutations."

It is unclear what 'complete case' means in this context. In this analysis, does it refer to the original metabolic data with no missing values, or the metabolic data sets after minimum value imputation?

Authors' response:

Thank you for the opportunity to clarify this point. In this context, "complete cases" refers to participants with available metabolite data. This is why the full sentence reads: "The analysis included only participants with complete data for the selected metabolites." The selection here refers to the lipid metabolite panel, as the untargeted metabolite data were also assessed initially.

All metabolite values were pre-processed (median scaling followed by minimum-value imputation for missing entries)

We have clarified this distinction in the Methods section of the revised manuscript.

"The analysis included only participants with complete data for the complex lipids metabolite panel."

3. line 466-467, Statistical analysis: "To assess differential longitudinal trajectories between intervention groups, linear mixed-effects models were applied to each metabolite and clinical variable, a robust method that models change over time using all available data and handling missingness via maximum likelihood."

Mixed-effects model is not a method to address missing data. It can be used in the presence of missing data because it does not require equal number of observation such as MANOVA. But, it may still leads to biased results under certain missing mechanisms.

Authors' response:

We thank the reviewer for this important clarification and fully agree that linear mixed-effects models do not "handle" missing data in the sense of imputing values, and that bias may arise under certain missing-data mechanisms. Our intention was to convey that mixed-effects models can accommodate unbalanced data structures without requiring an equal number of observations per participant.

We have revised the Methods section to make this explicit, as follows:
"*To assess differential longitudinal trajectories between intervention groups, linear mixed-effects models were applied to each metabolite and clinical variable.* This approach uses all available repeated measurements and can accommodate unequal numbers of observations per participant, under the assumption that data are missing at random and are estimated via maximum likelihood [66,67]."

This revision clarifies that the validity of inferences depends on the missing-at-random assumption rather than on the model intrinsically resolving missingness.

4. line 469, Statistical analysis: "Analyses were restricted to metabolites with sufficient non-missing data and variability."
line 498, Statistical analysis: "Models with missing data, insufficient variation, or failed model convergence were excluded."

How do you measure sufficient non-missing data and variability, and what are the criteria?

Authors' response:

This section was describing the mediation models. If clinical parameters had too much missing data (>30%), metabolites had insufficient variation (variation = 0) which can result in converging models, variables where excluded. As some metabolites were not present in specimen, this resulted in zero variability variables. We have specified this in the methods:

"If clinical parameters had too many missing data (>30%), metabolites had insufficient variation (variation = 0) which can result in converging models, variables where excluded"

5. line 84-86, Results: "A total of 191 participants with available metabolomic data and functional metabolic outcomes (e.g., oral glucose tolerance test) were included in the final analytical sample from the original CALERIE study."

How were the 191 participants chosen from the original 218 samples in the CALERIE study? Would it be possible to share a flowchart that outlines this selection process?

Authors' response:
Thank you for this thoughtful comment. In the present analysis, we included only participants with both metabolomic data and functional metabolic outcomes available. As a result, 27 participants from the original CALERIE cohort were excluded.

To assess potential bias, we compared baseline characteristics of the included (n = 191) and excluded (n = 27) participants (Table R1). No significant differences were observed, suggesting that exclusion of these participants is unlikely to have affected the original randomization or the comparability of the study groups.

We note that prior publications from the CALERIE trial have similarly used opportunistic subsamples based on data availability. For example, Waziry et al. (2023, doi:10.1038/s43587-022-00357-y) analyzed 164 participants, and Dorling et al. (2020, doi:10.1007/s00394-020-02361-7) analyzed 188 participants.

Table R1. Baseline characteristics of included CALERIE trial participants (n = 191) and excluded participants (n = 27), as presented in Kraus et al. Lancet 2019 (doi:10.1016/S2213-8587(19)30151-2).

Overall	**Study sample**	**Excluded sample**	**p-value**
	n=191	n=27	
Weight kg (mean (SD))	72.15 (9.23)	69.60 (8.87)	0.179
Fat Mass kg (mean (SD))	25.20 (1.69)	24.85 (1.92)	0.321
Fate Free Mass kg (mean (SD))	48.47 (9.07)	47.33 (8.75)	0.540
Systolic blood pressure mmHg (median [IQR])	113 [106, 121]	109 [103, 117]	0.070
Glucose mg/dL (median [IQR])	86 [82, 91]	84 [80, 88]	0.057
Insulin ulU/m (median [IQR])	5.13 [3.90, 7.21]	4.55 [3.57, 6.96]	0.483
C-peptide pmol/L (median [IQR])	462 [338, 594]	429 [363, 545]	0.634
LDL Cholesterol mg/dL (median [IQR])	102.50 [81.00, 119.75]	98.00 [80.50, 110.50]	0.246
HDL Cholesterol mg/dL (median [IQR])	46 [41, 55]	47 [41, 61]	0.675
Total energy expenditure kcal/day (mean (SD))	2308 [2096, 2640]	2325 [2061, 2540]	0.894

Thank you for the suggestion. We have added a flowchart as supplemental Figure S1.

6. line 92, table1: Please remove the P values from the baseline table, as noted in the references, "Such hypothesis testing is superfluous and can mislead investigators and their readers. Rather, comparisons at baseline should be based on consideration of the prognostic strength of the variables measured and the size of any chance imbalances that have occurred."[2], and "Significance testing of baseline differences is not recommended and should not be reported."[3]
Reference:
[2] Moher D, Hopewell S, Schulz KF, et al. CONSORT. CONSORT 2010 explanation

and elaboration: updated guidelines for reporting parallel group randomised trials. Int J Surg. 2012;10(1):28-55.
[3] Hopewell S, Chan AW, Collins GS, et al. CONSORT 2025 explanation and elaboration: updated guideline for reporting randomised trials. BMJ. 2025 Apr 14;389:e081124.

Authors' response:

We have removed p-values from Table 1.

7. line 113-114: "A total of 972 lipid-related metabolites were profiled longitudinally" The study included 972 metabolites and many clinical outcomes, leading to a large number of statistical tests. Was the False Discovery Rate (FDR) controlled for? And with so many linear model analyses and mixed model analyses, how can we be confident that the positive results are real?8. line 169: "To test this hypothesis, we performed causal mediation analysis to disentangle direct and indirect effects of CR on clinical outcomes at 12 months and 24 months."

Authors' response:
We appreciate the reviewer's concern regarding the large number of metabolites and clinical outcomes analysed. Our analytical strategy was specifically designed to minimise inflation of false-positive findings.

First, rather than conducting thousands of univariate tests, we used an unbiased, data-driven network approach (WGCNA) to reduce dimensionality before any targeted statistical analysis. WGCNA identifies clusters (modules) of co-regulated metabolites and has been shown to capture biologically meaningful structure in high-dimensional omics data (Zhang & Wong, Comput Struct Biotechnol J. 2022). Module detection is not based on multiple univariate hypothesis tests and therefore does not require FDR control at this stage.

Second, we performed module preservation analysis to identify networks that were specifically altered by CR and not preserved in the ad libitum (AL) group. Only these CR-specific modules were carried forward, substantially narrowing the focus of subsequent analyses.

Third, within this restricted set, we conducted hypothesis-driven analyses focused on biologically plausible candidates, particularly ceramides, which have well-established roles in insulin sensitivity and cardiometabolic risk. The convergence of this data-driven selection with prior biological knowledge increases confidence that the observed associations are genuine rather than artefactual. Thus, by (1) reducing dimensionality, (2) restricting analyses to CR-specific modules, and (3) avoiding extensive uncorrected metabolite-by-metabolite testing, we substantially reduced the risk of type I error. The consistent and biologically coherent effects on ceramides—lipids modulated by caloric restriction with known links to insulin sensitivity and cardiometabolic health—further support that the reported associations reflect true physiological responses rather than statistical noise.

In addition, we used bootstrapped linear mixed-effects models and interpreted results primarily in terms of effect sizes and confidence intervals rather than relying solely on p values. Recent statistical guidance recommends prioritizing effect sizes, interval estimates, and consistency with prior evidence over dichotomous p-value thresholds (Baker et al., *Nature* 2016; *Points of Significance*, *Nat Hum Behav* 2023; Ioannidis et al., *PLoS Med* 2007). Accordingly, we evaluated statistical robustness based on confidence intervals and the alignment of our findings with existing literature on adiponectin, ceramides, and insulin resistance. In mixed-effects modelling, confidence intervals for fixed effects often provide more informative and stable inference than p values, and formal multiple-comparison adjustments to p values do not alter these intervals (Bates et al., *J Stat Softw* 2015). Thus, additional p-value correction would not materially enhance assessment of result robustness beyond the information already contained in the confidence intervals.

We have clarified this rationale in the Methods section as follows (lines 523–525):

"We used bootstrapped confidence intervals rather than p value–based multiple-comparison corrections, as interval estimates are more robust and informative for assessing effect size and avoiding spurious type I errors in this high-dimensional setting."

8. Adjustment for covariates was not mentioned in the methods of this paper. Since this paper focuses on causal inference rather than just exploratory analysis, it is important to adjust for covariates—even when they are balanced through randomization. It recommends adjusting for key prognostic factors and baseline variables in the main analysis of randomized trials [4]. This approach helps improve the reliability and robustness of the results. Furthermore, when studying the relationship between metabolites and clinical outcomes, other conditions may not be balanced, which could introduce unknown confounding factors.

Reference:

[4] Holmberg MJ, Andersen LW. Adjustment for Baseline Characteristics in Randomized Clinical Trials. JAMA. 2022;328(21):2155–2156.

Authors' response:

We thank the reviewer for this important comment. As our analyses are based on a randomized controlled trial, baseline anthropometric and metabolic characteristics did not differ significantly between the CR and AL groups (Table 1). Randomization therefore substantially reduces confounding by both measured and unmeasured baseline factors, so covariate adjustment is not strictly required to obtain unbiased estimates of the intervention effect. Nonetheless, we agree that adjustment for key prognostic variables can improve precision and robustness, particularly in the context of mediation and metabolite–outcome relationships.

In response, we performed additional sensitivity analyses including age and sex as covariates in the mediation models for the main outcomes (AUC insulin and C-peptide). The results were materially unchanged: ceramides 18:0, 20:1, and 22:1 remained significant mediators of the CR effect on AUC insulin between baseline and 12 months and between baseline and

24 months in the CR group, with no evidence of mediation in the AL group. Similarly, ceramides 18:0 and 22:0 remained significant mediators for C-peptide in the CR group only.

AUC insulin Caloric (BL12M):

CR: Ceramide 18:0 average causal mediated effect (ACME) -0.04[-0.15, -0.02]
AL: Ceramide 18:0 ACME: 0.02 [-0.10, 0.13]
CR: Ceramide 20:1 ACME: -0.02[-0.07, -0.004]
AL: Ceramide 20:1 ACME: -0.02 [-0.09, 0.04]
CR: Ceramide 22:1 ACME: -0.02 [-0.14, -0.006]
AL: Ceramide 22:1 ACME: -0.03 [-0.15, 0.09]

These covariate-adjusted results are reported in Table S16 and summarized in the Results (lines 288–295) under "Sensitivity analyses":

'Sensitivity analyses including sex and age as covariates in the mediation models showed that the ceramides identified as mediators of improved insulin sensitivity remained significant after correction for multiple comparison. Ceramides 18:0, 20:1 and 22:1 mediated improvements in insulin sensitivity between baseline and 12 months for AUC insulin in the CR group, but not in the ad libitum group. Similarly, between baseline and 24 months, ceramides 18:0, 20:1 and 22:1 mediated reductions in AUC insulin in the CR group but not in the ad libitum group. These findings suggest that age and sex did not materially affect the mediation analyses (Table S16).'

We also now note in the Discussion (lines 358–359) that adjusting for sex did not meaningfully change the results.

9. line 291-293, Discussion: "Specifically, we identified a causal pathway in which CR increased high-molecular-weight adiponectin, which in turn reduced circulating ceramide species—bioactive lipids mechanistically linked to insulin resistance."
line 300-302, Discussion: "Our findings corroborate prior evidence from animal and human studies indicating that diet and exercise interventions reduce ceramide levels in parallel with improved insulin sensitivity 30."

line 322-325, Discussion: "Together, these findings suggest that ceramides play a direct, causal role in insulin resistance development, a conclusion supported by the findings of our study, where reduced ceramide levels mediated improvements in insulin sensitivity in the CR group, but not in the AL group (Fig. 6)." The findings in this paper are based on just one study and its methods. This study did not control for false-positive results, account for other confounding factors, or perform sensitivity analyses. As a result, its causal claims may be too strong and are not fully convincing. It is recommended that these limitations should be acknowledged in the Discussion section.

Authors' response:

We thank the reviewer for these important comments regarding the strength of our causal language and the need to more explicitly acknowledge limitations.

As outlined in our response to Comment 7, we used an unbiased, multivariate network approach (WGCNA with module preservation) to reduce dimensionality and identify CR-specific metabolite modules before conducting any targeted mediation analyses. Within these CR-specific modules (n = 164 metabolites, predominantly ceramides), we then applied bootstrapped linear mixed-effects and mediation models, interpreting effect sizes and confidence intervals rather than relying solely on p values. This strategy was chosen to limit type I error and to align with current recommendations that emphasize effect sizes, interval estimates, and biological plausibility over dichotomous p-value thresholds (literature (Baker et al Nature 2016, 10.1038/nature.2016.19503, doi: 10.1038/nature.2016.19503, Points of significance. Nat Hum Behav 2023, doi: 10.1038/s41562-023-01586-w, Ioannidis et al PLoS Med 2007 doi: 10.1371/journal.pmed.0040215, Bates et al J statistical softw 2015 doi: 10.18637/jss.v067.i01).

We also performed sensitivity analyses adjusting for age and sex for key outcomes (AUC insulin and C-peptide), which showed materially unchanged mediation patterns.

That said, we fully agree that our findings are based on a single post hoc analysis, and that causal claims must therefore be framed cautiously. Although the randomized controlled design of CALERIE provides a strong foundation by minimizing baseline confounding, our mediation analyses remain subject to important assumptions (e.g., absence of unmeasured confounding, correct model specification, and the limitations of not applying high-dimensional FDR control across all metabolites).

In response to the reviewer's concern, we have:

1. **Tempered causal wording in the Discussion.** For example, we now describe our findings as "consistent with a potential pathway" or "supporting a plausible mechanistic link" rather than stating that we "identified a causal pathway" or that ceramides "play a direct, causal role" based solely on our data.
2. **Explicitly acknowledged key limitations.** We have added text to the Discussion/Limitations noting that:
    - The analyses are post hoc and were not pre-registered.
    - Residual or unmeasured confounding at the metabolite–outcome level cannot be excluded.
    - We did not apply global FDR correction across all metabolites, and our interpretation therefore relies on convergence between data-driven selection, biological plausibility, and consistency with prior literature.

Together, these revisions clarify that our study provides evidence *consistent with* a CR–adiponectin–ceramide axis influencing glycemic control, but that definitive causal conclusions require confirmation in independent cohorts and experimentally targeted studies.

10. Please indicate in the footnotes of Table 1 which distributions of data are represented by SD and which by quartiles.

Authors' response:
Thank you for this helpful suggestion. We have added the following footnote to Table 1 (lines 94–95) to clarify how each variable is summarized:

"Values are presented as mean (SD) for approximately normally distributed continuous variables and median [IQR] for skewed variables. Categorical variables are shown as n (%)."

11. I don't find the descriptions of (E) and (F) in the caption of Figure 5. Please check it.

Authors' response:
We apologize for this oversight. We have now added a clear description of panels (E) and (F) to the Figure 5 legend:

"(E) Change in AUC insulin from the oral glucose tolerance test versus change in ceramide 18:0 between baseline and 12 months, and (F) between baseline and 24 months. Delta values are expressed in arbitrary units. R: Spearman's rho; p: p value from the Spearman correlation test. AUC: area under the curve."

This correction ensures all figure panels are fully described.

Reviewer #3 and #5

1. I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Authors' response:
We thank the reviewers for their contributions and have addressed all points raised in the reports above.

Reviewer's feedback and Authors' responses

Caloric Restriction Improves Glycemic Control via the Adiponectin–Ceramide Axis in Non-obese Men and Women: the CALERIE™ 2 randomized controlled trial

Reviewer #1

Comment:

The authors seem to have adequately addressed the comments raised by me and the other reviewers.

Response:

We thank Reviewer #1 for their positive assessment.

Reviewer #2

We thank the reviewer for their careful re-evaluation of the manuscript. In this revision, we have focused specifically on addressing the remaining conceptual concerns by (i) explicitly acknowledging methodological limitations, (ii) further tempering causal and mechanistic language throughout the manuscript, and (iii) clarifying assumptions underlying the analytical approach and (iv) performance of additional sensitivity analyses. Details are provided below.

1. Reviewer comment:

Applying module preservation analysis to identify lipid metabolites for downstream mediation analyses. The paper by Langfelder and colleagues in 2011 focuses on whether the correlation structure of a network module stays consistent when conditions change, rather than looking at shifts in metabolite levels. This means it can detect structural changes in metabolite relationships but not simple shifts in concentrations (or relative abundances). In other words, even if metabolite levels change, the module would be considered preserved unless the correlation patterns are altered. It appears questionable that the main effect of CR is to rewire the lipid metabolite network; many relevant CR effects would plausibly alter the flux through stable metabolic pathways which would result in a preserved structure of the

metabolite network but distinct relative abundances between CR and AL group that affect whole modules. A strong rationale for mediator pre-selection through module preservation analysis is still lacking and limitations of the approach are still not discussed.

Response:

We agree with the reviewer's comment on the fact that our approach detects structural changes in metabolite relationships but not necessarily simple shifts in concentrations. Given the moderate sample size and the high dimensionality of the lipidomics dataset, we adopted a biologically informed dimensionality-reduction strategy rather than relying primarily on univariate tests of individual metabolites. We used module preservation analysis as an initial filtering step to prioritize metabolites showing CR-associated changes in correlation structure, reflect coordinated metabolic responses. As an additional check, we confirmed that these prioritized metabolites also differed in their absolute levels between groups, supporting the relevance of the selected set.

In the limitations section, we acknowledge that metabolites whose relative abundances change without altering module structure may not be prioritized by this approach. Future studies with larger sample sizes could integrate univariate and network-based strategies to capture both individual and coordinated effects of CR on lipid metabolism.

Page 18 of the revised manuscript now reads,

"*In addition, module preservation analysis detects differences in correlation structure rather than absolute metabolite abundance; thus, CR-induced shifts in metabolite concentrations that occur within stable correlation structures may not be captured by this approach. Accordingly, our use of module preservation was intended as a dimensionality-reduction and hypothesis-generation step rather than a comprehensive characterization of all CR-induced metabolomic changes.*"

2. Reviewer comment:

Mediation analysis. Multi-mediator models are still lacking. At least correlated

ceramides should be examined jointly to assess if their role as mediators is mutually independent or not. Confidence intervals for mediation estimates should be reported in the text. Mediation analysis methodology: While bootstrapping improves the reliability of each interval, it does not adjust for the increased likelihood of error across all tests. To control for multiple comparisons the family-wise error rate still needs to be controlled. Adjustment for sex and age is not sufficient to control for potential confounding; it should be tested whether mediation models are robust against adjustment for metrics of adherence, diet composition, physical activity, etc.

Response:

We agree that ceramide species are biologically and statistically correlated. Therefore we have performed multiple mediator analyses stratified by biologically active subgroups such as long (C16-20) and very long (C22-24) ceramides. We found that long ceramides reduce AUC insulin over time in the CR group but not in the AL group. In addition to this, we observed a trend toward reduced AUC insulin in the CR group with very long ceramides but not in the AL group. The results have been added to the "Sensitivity analysis" results section:

"We also performed a multiple mediator analysis with long (C16-20) and very long (C22-24) ceramides which were present in modules Blue-12 and Blue24. We found that the five long ceramides (16:0, 18:0, 18:1, 20:0, 20:1) mediate reduced AUC insulin over time during the study in the CR group (-0.02 [-0.03, -0.00]), but not in the AL group (-0.02 [-0.06, 0.03]). We also performed multiple mediator analysis on four very long ceramides (22:0, 22:1, 24:0, 24:1) and found a similar trend towards reducing AUC insulin over time in the CR group (-0.01 [-0.02, 0.00]), but not in AL group (0.00 [-0.05, 0.05]). Only one medium chain ceramide (14:0) and one ultra long ceramide (26:1) was present in Blue-12 and Blue-24.", p 12-13 revised manuscript

Furthermore, we acknowledge the concern regarding multiple testing correction. Rather than relying solely on asymptotic p-values, we emphasize bootstrapped 95% confidence intervals to provide a more informative and robust quantification of uncertainty for mediation effects. In addition, the mediation analyses were restricted to a pre-specified subset of metabolites derived from CR-specific modules, substantially reducing the multiplicity burden relative to an untargeted metabolome-wide scan. Nonetheless, we explicitly acknowledge the possibility of false-positive findings in the Discussion (Limitations), as follows:

"*Although we cannot entirely exclude the possibility of false-positive findings arising from multiple testing, our analytical strategy reduces this risk. Specifically, the metabolites were not selected in an untargeted, discovery-wide manner but were pre-specified from biologically preserved modules, thereby substantially limiting the number of statistical tests compared with a genome- or metabolome-wide scan.*"
(page 18 of the revised manuscript.

Regarding potential covariate adjustment, Table 1 shows that the groups are well balanced at baseline. We conducted an additional sensitivity analysis with age and sex, which yielded similar results (Results paragraph "Sensitivity analyses" and Table S16). Considering reviewer's request, we have further adjusted for Total energy expenditure in addition to age and sex as a proxy for lifestyle (diet, physical activity etc). The results show no changes to reported results (Results paragraph "Sensitivity analyses and Table S16). We also added the findings in page 12 of the revised manuscript. Additionally, adding confidence intervals to all mediation paragraphs would limit the readability of the manuscript, we have added confidence intervals to the sensitivity analysis paragraph.

"Sensitivity analyses including sex and age as covariates in the mediation models showed that the ceramides identified as mediators of improved insulin sensitivity remained statistically significant after adjustment. Ceramides 18:0 (-0.04 AU [-0.15, -0.02]), 20:1 (-0.02 [-0.07, -0.00]), and 22:1 (-0.02 [-0.14, -0.01]) mediated reductions in AUC insulin between baseline and 12 months in the CR group, but not in the AL group. Similarly, between baseline and 24 months, ceramides 18:0 (-0.04 [-0.14, -0.01]), 20:1 (-0.03 [-0.12, -0.01]), and 22:1 (-0.02, [-0.14, -0.01]) mediated reductions in AUC insulin in the CR group but not in the AL group. These results were consistent after adjusting for age and sex (Table S16). When total energy intake was added as an additional covariate, the overall pattern remained unchanged at both 12 and 24 months (Table S16).

We additionally conducted multiple-mediator analysis grouping ceramides by acyl-chain length. Specifically, we examined long-chain (C16-20) and very long-chain (C22-24) ceramides present in the Blue-12 and Blue-24 modules. Long-chain ceramides (16:0, 18:0, 18:1, 20:0, 20:1) collectively mediated reductions in AUC insulin over time in the CR group (-0.02 [-0.03, -0.00]) but not in the AL group (-0.02 [-0.06, 0.03]). Very-long-chain ceramides (22:0, 22:1, 24:0, 24:1), showed a modest indirect effect in the CR group (-0.01 [-0.02, 0.00]), and no effect in the AL group (0.00 [-0.05, 0.05]). Only one medium-chain ceramide (14:0) and one ultra-long-chain ceramide (26:1) were present in both modules."

3. Reviewer comment:

Discussion of limitations should also include the assumptions underlying the biological (causal) interpretation of mediation analyses and limitations of data and analytical choices.

Response:

Thank you for the suggestion. We have now revised the manuscript to substantially soften causal and mechanistic language throughout. Terms such as “demonstrate,” “identified a causal pathway,” and “mechanistically mediates” have been replaced with more conservative phrasing (e.g., “consistent with,” “supports a model in which,” “statistically compatible with a mediating role”). Please refer to revised Abstract and Discussion.

4. Reviewer comment:

In several instances, the language is still too strong in relation to novelty and causal interpretations of the results. For example, the conclusion at the end of the discussion indicates strong mechanistic evidence, while the mediation models only moderate support for agreement of the data with the tested mediation hypotheses, and any mechanistic interpretation relies on string assumptions.

Response:

We have revised the paragraph. We now emphasize that mediation results should be interpreted as statistically compatible with hypothesized indirect processes rather than as definitive causal proof. Please refer to page 18 of the revised manuscript.

“*In summary, this study provides human evidence consistent with CR being associated with beneficial lipid remodeling, particularly reductions in ceramides that are linked to improvements in insulin secretion, insulin sensitivity, and IGF-1 signaling. The observed associations are compatible with partial mediation by adiponectin, but the strength of evidence is modest and dependent on the assumptions of the mediation framework. Accordingly, our results should be interpreted as hypothesis-generating, pointing to the adiponectin–ceramide axis as a potential pathway for future experimental validation rather than establishing a confirmed mechanistic link. Future work should test these relationships in clinical populations and explore whether pharmacological or dietary strategies that mimic the lipid-modulatory effects of CR can deliver comparable benefits.”*

Reviewer #4

I have no more comments.

Response:
We thank the reviewers for their contributions.

Reviewer #3 and Reviewer #5

Co-reviewer acknowledgement.

Response:

We thank the reviewers for their contributions.