nature portfolio

Peer Review File

Lifespan Trajectories of Serum Sphingolipids in a Clinically Healthy Swiss Population, Towards Precision Medicine in Cardiometabolic Health Assessment

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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 study investigates age-associated variations in sphingolipid profiles and establishes reference values across age groups, aiming to advance their clinical utility as biomarkers for cardiometabolic disorders. The work is scientifically valuable, methodologically transparent, and clearly presented, with strong potential to inform future research and clinical applications. Below are suggestions to strengthen the manuscript further:

1.The cross-sectional design appropriately identifies associations between age and sphingolipid levels but inherently limits causal interpretation. While the directed acyclic graph (DAG) posits age as a causal driver of sphingolipid changes, this assumption requires explicit justification. For instance:
- What evidence supports the unidirectionality of the relationship (age → sphingolipids) over reverse causation (e.g., sphingolipid-driven aging)?
- The inclusion of fitness, physical activity, and body fat as upstream causal factors for age in the DAG is unconventional. Could the authors clarify the rationale for this structure, ideally with references to theoretical or empirical frameworks?

2.The age-stratified reference values for sphingolipids represent a key contribution. To enhance accessibility and impact, consider adding a summary figure to the main text, contingent on journal figure limits. Tabular data in supplements could then complement this visual synthesis.

3.The conclusion that sphingolipid ratios "inform cardiometabolic health" appears overextended given the mixed trends observed (e.g., some ratios increase with age, others decrease; Lines 300–305), which also contradicts ‘ratio independent of age’ claim by the authors. The authors might wish to add some statistics to the P-spline curves to inform if the relationship is non-flat or not.

Reviewer #2

(Remarks to the Author)
In the manuscript COMMSMED-25-0309-T by Carrard et al. entitled “Lifespan Trajectories and Reference Values of the Serum Sphingolipidome in a Clinically Healthy Population: Towards Precision Medicine in Cardiometabolic Health Assessment” authors quantitatively measured 26 sphingolipids and observed that concentrations of multiple sphingolipids increase with age and emphasized the need in age-specific reference intervals. The manuscript is well written and interesting, while the method used for analysis of the sphingolipids and its performance are incompletely described. Due to the physicochemical properties, lipids are notoriously challenging analytes for development of quantitative LC-MS methods; in order to support data on analysis of the clinical study samples, detailed description of the LC-MS/MS method, as well as information on the standardization and the methods’ performance should be provided.

Below are detailed comments on the manuscript.

Methods

Section 2.3. List LOQ and imprecision for the commercial assays used for testing the study samples.
Line 147. Provide vendor and catalog number for the tube type used for the sample's storage.
Lines 169 and 173. Information on the glycated hemoglobin test is listed twice.
Section 2.4. Sphingolipid quantification.
• Establishing reference values for clinical diagnostic biomarkers requires the use of validated methods. A full description of the method, detailed information on the method validation and the methods' performance should be provided. If the method was not fully validated and standardized the determined concentration distributions should not be referred to as population reference values.
• Use of certified reference materials for preparing calibration standards is essential for ensuring the accuracy and reliability of analytical methods. Were standards of the lipids obtained from a commercial company and were certificates of analysis (COA) provided for the standards? Its need to include in the manuscript source and catalog numbers of the standards used for preparing the working calibration standards. If COAs were not available, it is necessary to provide information on the procedures used to establish accurate concentrations of the lipids.
• Include description of preparation of the stock and working standards of the analytes and the internal standards (solvents used, concentration, storage conditions, handling and storage stability).
• Include list of vendors, catalog item numbers and specifications for all critical reagents and supplies used in the method (standards, internal standards, solvents, reagents, tubes, vials, 96 well plates, etc.).
• What was the matrix for preparing the working calibration standards?
• Describe preparation of the working calibration standards, concentrations of the calibrators, frequency of performing calibration and analysis of quality control samples.
• Describe preparation and use of quality control materials analyzed by the method?
• How was the specificity of analysis assessed? Were multiple mass transitions monitored for the targeted sphingolipids?
• Describe the process of data analysis, criteria used to assess acceptability for the batches and the patient samples. Were interferences with the targeted sphingolipids observed in in the sample analysis? What was the frequency of the interferences?
• Method performance. The following method performance characteristics should be provided: imprecision, accuracy, limit of quantification, limit of detection, analytical measurement intervals, strategy for assessment of specificity, method accuracy, assessment of ion suppression, comparison to methods of other laboratories.
• Include example chromatograms for one of the calibration standards and a representative patient sample.
• Mass transitions of the internal standards, as well as retention times of the targeted sphingolipids and internal standards should be provided.
• Considering that stabile isotope labeled analogs for many of the analyzed sphingolipids were not available, how ion suppression was assessed and mitigated for the sphingolipids which did not have stabile isotope labeled analog internal standards?
• Lipids tend to irreversibly absorb in surfaces (labware, pipette tips, etc). Were adsorptive losses evaluated during the method development and validation? How the adsorptive losses were mitigated.
If the method was not fully validated and standardized, statements that the manuscript provides reference values are inappropriate.

Results
Lines 269-277. Were the associations described statistically significant? Provide p-values corresponding to the listed associations.

Limitations
If the method for measuring sphingolipids was not fully validated and standardized, this is the major limitation for this study and that needs to be stated as a limitation.

Figures and Tables
It is unclear what caption corresponds to what figure, label Figures and Tables included in the manuscript.

Reviewer #3

(Remarks to the Author)
Brief Summary:
Here the authors measured 26 sphingolipid species using tandem HPLC-MS in the serum of 522 clinincally healthy individuals to assess the effect of age on circulating sphingoliid concentrations and to generate reference ranges for the aforementioned species to enable more accurate interpretation of clinical sphingolipid measurements in the context of cardiovascular disease risk scoring.

The authors report that 17 sphingolipid species were positively associated with advanging age (including the ceramides used in the CERT1 and 2 scores); 8 species were significantly higher in males, 4 species were significantly higher in females, 3 glycosphingolipids demonstrated significantly lower levels with statin use and 2 ceramides were associated with increased body fat. The authors presented age-specific reference ranges and quantile curves of each sphingolipid over the course of a lifespan.

Overall Impression:
The scientific basis motivating this work is robust - indeed sphingolipid reference values have not so far been systematically calculated in healthy human subjects over the course of their life expectancy. Whilst the cross-sectional study desgin does

present limitations in that it cannot conclude anything more than association, it is nevertheless appropriate for the research question at hand and the authors do not make inappropriate claims or conclusions from their data. The minutiae of the study design are appropriate and well thought out. The level of detail in the methodology pertaining to data collection and statistical analysis are suffiecient in my opionion to facilitate reproducibility of the results. On general inspection, the supplied code used in statistical formatting and analysis seems appropriate.

The results of the authors analyses are interesting and for the most part novel. The authors presentation of age-specific sphingolipid reference ranges will be of interest and value to scientists with an interest in lipidomics as well as the broader field of cardiovascular disease. Results and described and displayed well and the discussion and interpretation of these results is appropriate and insightful. The figures and tables in both the main text and the supplement are easy to read and interpret with apppriate information provided in the figure legends. What stands out about this work in particular is that the results have been analysed and summarised in a way that makes them accessible and easily utilised by other academics in the field both clinical and non-clinical. All the STROBE criteria have been met in this report.

The authors appropriately acknowledge that whilst such results may be generalisable in Switzerland, they may not fit so well diverse populations in other countries. Nevertheless, I believe this doesn't negate the utility of this work in providing a starting point from which Sphingolipid reference ranges for more diverse populations can be generated. This study did not comment on participant enthnicity and this perhaps would have been interesting to comment on, not least because some indication of the ethnic makeup of the study population may allow readers to evaluate the generalisabilty of these results to the populations they encounter in their own clinical and academic practice.

Overall, I think this is an excellent study. The concept is novel, the execution is robust, and the results push forward the respective field, filling an important gap in the implementation of Sphingolipid-based cardiovascular risk scoring. My commendations to the authors on their high quality work.

Specific comments:

1. The authors consider a number of important confounding factors on sphingolipid including age and sex but not ethnicity. Are data on participant ethnicity available for inclusion in the participant demographics table as this will help readers determine the applicability of these results to populations in their own localities. Potentially it may even be interesting to identify if there are any trends in circulating sphingolipid species between different ethnicities though I appreciate this may be best explored in a separate publication.

2. Consider ensuring consistency in the expression of numerical values either as digits or as fully-written prose, such as in the following examples:
o Line 248: ‘Eighty-eight per cent of females (n=220) and 83% of males’
o Line 249: ‘Nine per cent of females (n=22) and 13% of males... while four per cent of females’

Otherwise I have no other specific comments - this is a well designed and implemented study and a well-prepared manuscript.

Reviewer #4

(Remarks to the Author)
In this manuscript (COMMSMED-25-0309-T), the authors measured 26 sphingolipids across a healthy cohort from Switzeralnd (n=522). This cross-sectional, population-based study aimed to establish age-specific reference quantiles for 26 sphingolipid species in healthy adults and to investigate their associations with chronological age across the lifespan.
1. Since this study involved healthy participants from Switzerland, it is important to reflect this in the manuscript title.
2. Too many references grouped in a few sentences (e.g., (4–8) or (7, 50)). Distribute references more evenly and consider highlighting landmark studies to support major claims.
3. The term "sphingolipidome" is not accurate for referring to 26 sphingolipids. Please replace it with “targeted measurement of 26 sphingolipid species.”
4. The gap in knowledge that this study addresses is implied but not directly stated. Add a sentence such as:“However, normative age-related trajectories of circulating sphingolipids in healthy individuals remain poorly characterized.”
5. Current aim statement: “This cross-sectional population-based study aimed to model age-specific sphingolipid quantile curves and assess associations between circulating sphingolipids and age.” Can be replaced with a more precise statement like:“This cross-sectional, population-based study aimed to establish age-specific reference quantiles for 26 sphingolipid species in healthy adults and to investigate their associations with chronological age across the lifespan.”
6. Some sentences are overly long or redundant. Example: “Cer(d18:1/18:0), characterised by its long fatty acyl chain, has been considered a marker of poor cardiometabolic health. Elevated levels of Cer(d18:1/18:0) are thought to impair mitochondrial function...” . Condense repetitive phrases. Use shorter sentences where possible for clarity.
7. Repetition of long chemical names (e.g., Cer(d18:1/24:0)) without visual aids may hinder readability.Use short codes (e.g., Cer16:0) after first mention. Include a figure legend or abbreviation table for easy reference. Avoid over-reliance on supplements.
8. Several biochemical terms (e.g., HexCer, HexSph) are not briefly defined for general readers. Add a one-line explanation or include a glossary in supplementary material for non-specialist readers.
9. Some statistical results (β coefficients, p-values, CI ranges) are omitted from the main text but are said to be in supplementary tables. Present key statistics for significant findings in the main text to support claims and allow the reader to

evaluate the strength of associations. Avoid over-reliance on supplements.
10. The results are presented in a highly descriptive and fragmented manner, lacking integration or reference back to study objectives/hypotheses.Explicitly state the study's main hypotheses at the beginning of the results section and connect each major finding back to these hypotheses.
11. Reduction from 522 to 487 participants for association analysis is briefly mentioned. Clearly explain the reason and nature of the missing data and assess whether the excluded data could bias results.
12. Explore if associations differ by medication use, BMI categories, or other health status markers. Assess robustness of associations by removing participants on statins or antihypertensives.
13. Test interactions between age and sex or between VO2peak and lipid levels.
14. The statement that sphingolipid accumulation might indicate "normal ageing" vs. "incipient disease" needs cautious interpretation. Clarify that longitudinal data are needed to draw causal inferences and consider including caveats.
15. The association of VO2peak with Cer1P(d18:1/16:0) and SM (d18:1/12:0) is described as novel but left largely unexplored. Even speculative biological interpretations or hypotheses would strengthen this section.
16. The repeated reference formatting for ceramides becomes heavy and may be condensed. For example, the full chemical notation could be written once per paragraph with abbreviations thereafter.
17. In disucssion, authors could briefly highlight possible mechanisms linking sphingolipids to ageing and mitochondrial function. How changes in specific ceramides or ratios relate to cardiometabolic risk.Possible biological mechanisms or implications of observed age-related patterns.Discuss how findings can guide future research—e.g., could sphingolipid profiles be used in preventive screening for age-related metabolic decline? How changes in specific ceramides or ratios relate to cardiometabolic risk.
18. Although some confounders are considered (e.g., statin use, VO2peak), there is no mention of how multivariable models were built or whether collinearity was assessed.
19. Model adjustment strategy. Variable selection rationale. Whether interaction terms (e.g., age × sex) were tested and if not, why.
20. The conclusion that “the data provided little evidence of association between sphingolipids and total daily physical activity” is vague and not quantified. Provide statistical metrics for this null finding. Also, clarify whether smoking history was adjusted for in regression models given its known lipidomic impact.
21. Discuss how findings compare to previously published sphingolipid reference values across ethnicities or cohorts such as in the following study (PMID: 38561799; DOI: 10.1186/s12944-024-02080-6)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have reviewed the author's response to the comments and am content with the response. No additional comments.

Reviewer #2

(Remarks to the Author)
In the manuscript COMMSMED-25-0309-T by Carrard et al. entitled “Lifespan Trajectories of Serum Sphingolipids in a Clinically Healthy Swiss Population: Towards Precision Medicine in Cardiometabolic Health Assessment” authors quantitatively measured 26 sphingolipids and observed that concentrations of multiple sphingolipids increase with age and emphasized the need in age-specific reference intervals. The manuscript is well written and presents an interesting study; the study population, the participant information, and statistical analysis methods are appropriate. However, there are some concerns related to the LC-MS method used for analyzing lipids in the study samples, which may question the observations and the conclusions of the study. Below are detailed comments on the manuscript.
• Matrix effects are one of the primary sources of interference in mass spectrometry-based methods, which are typically manifest as signal suppression or enhancement. The most effective strategy to account for, and compensate for these effects is the use of stable isotope-labeled analogs of the target analytes as internal standards. In the method used in this study, 26 lipids were measured, while 10 internal standards were used. Internal standards correct for matrix effects in corresponding structural analogs, while may not compensate for the matrix effects for the remaining 16 lipids analyzed in the study. As a result, there is uncertainty regarding the accuracy of the measured concentrations.
• While it is common in lipidomics research to quantify many lipids using only a few internal standards, this practice is unacceptable in clinical research, where data accuracy is important. At a minimum, this methodological limitation should be clearly acknowledged in the manuscript’s limitations section to ensure readers are aware of the potential for erroneous findings.
• Conclusions presented in the abstract emphasize the need for age-specific reference values for the measured lipids. However, due to methodological limitations, age-specific reference intervals cannot be reliably established using data from this study.
Supplemental table S2:
• It is not stated what mass transitions were used for quantification?
• It is not stated what internal standards were used for what analyte?
• For some analytes there are 2, for some 3 mass transitions listed in the table, but it is not stated how were multiple mass transitions used in the assay and in the data analysis?

• Was the ratio of mass transitions evaluated during the data analysis? What was the tolerance for the ratio of mass transitions? If the ratios were assessed, what percentage of results was removed from consideration due to the presence of suspected interferences.

Supplemental table S5:
• What was the matrix of the sample used for evaluation of the method's accuracy?
• What were concentrations of the targeted lipids in the sample?
• Provide description of preparation of samples used in the experiment (lipids have limited solubility and may not dissolve in the sample matrix).
• What was the number of replicates?
• Were the samples prepared on each day of experiment or were some samples reinjected on different days?

Supplemental table S6:
• Provide description of preparation of samples used in the experiment.
• How were the lipid containing samples prepared?
• Were the samples prepared on each day of experiment or were same samples reinjected on different days?
• Precision should be evaluated at multiple concentrations within measurement range of the method.
• What was the number of replicates?
• There are 2-7 significant digits used for listed concentrations, considering the methodological issues, it is misleading to use more than 2 or 3 significant digits in presenting the lipid concentrations.

Supplemental table S7:
• See above comment related to the number of significant digits.
• Matrix of the NIST SRM 1950 sample is not identical to human serum/plasma sample.
• Were matrix matched controls (serum/plasma) analyzed along with the patient samples? Quality control samples containing multiple concentrations of the analytes of interest spanning the measurement range of the assay should be included in every batch of samples. The quality controls samples should be prepared in the same matrix as the patient samples analyzed in the study.
• If matrix-matched controls were not analyzed alongside the study samples and their performance in the method was not assessed, this raises concerns about the reliability of the analytical results. Without proper quality control, it is impossible to evaluate between runs stability of the instrument performance, and acceptability of the results from each batch. This should be addressed in the manuscript, either by including relevant control data or by stating this as the limitation, to inform readers of the potential impact on data accuracy and interpretation.
Supplemental tables S12-40:
• The units for the concentrations shown in the tables are not specified.
• The sample size (N) for each age group is not provided. If the sample size is limited, it could be more appropriate to group participants into broader age categories (e.g., 5-year intervals).

Supplementary Figures 1S to S26. The example chromatograms shown in the figures suggest a suboptimal performance of the method
• Peak intensity in some of the mass transitions is comparable to noise (e.g. Fig 7)
• Presence of split peaks in some of the mass transitions (e.g. Figs 9, 22)
• Different peak width (Figs 9, 13, 14, 21, 22, 25) in two mas transitions corresponding to the same analyte (suggests presence of interferences).
• Different retention times in different mass transitions corresponding to the same analyte (Figs 12, 20, 22).
• Considering that these performance issues were observed in calibrators prepared in methanol, it is likely that the methods' performance during analysis of the study samples was also suboptimal. Based on the above, the accuracy of the reported quantitative results is questionable. This limitation should be acknowledged in the manuscript to ensure transparency and to inform readers of the potential impact on data interpretation.

The data analysis process
• For transparency, the manuscript should list all the details of the data analysis, including the qualitative criteria which were used to accept or reject the results.
• In response to the comment from the original review regarding the data analysis process, the authors stated: "All observed interferences in Cal0 (across 11 of the 26 measured species)", suggesting that calibrators prepared in methanol contained impurities which produced peaks in the mass transitions of the targeted lipids. Considering much greater complexity of the human plasma samples as compared to the methanolic standards, this above suggests that interference in mass transitions was likely a frequent occurrence in during the analysis of plasma samples, raising concerns about accuracy of the concentrations measured in the study samples.

Reviewer #3

(Remarks to the Author)
The authors have clearly made some very comprehensive changes to the manuscript and i believe these are sufficient to make this article acceptable for publication.

Reviewer #4

(Remarks to the Author)
The authors fulfilled all my comments. No further comments from my side.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors adequately addressed my prior comments; the revised manuscript is interesting and well written.

Dear editor, dear reviewers,

We would like to thank you for your positive and insightful comments. All comments have been addressed point-by-point (below) and in the revised manuscript via track changes.

As kindly suggested by the editor, we highlighted the study's novelty in the discussion section. It now reads (see page 15, lines 362-369):

> "The novelty of this study lies in being the first to quantify a panel of 26 sphingolipid species in the serum of clinically healthy and well-characterised adults across the lifespan using a targeted liquid chromatography-tandem mass spectrometry approach (Figure 5). Since all included participants were free of manifest clinical conditions, the confounding effect of cardiometabolic diseases on lipid metabolism is assumed to be small (1). The association analyses confirm that age was an essential determinant for the levels of 17 of 26 circulating sphingolipids. This finding was complemented by age-specific sphingolipid quantile curves, which descriptively demonstrated a global accumulation of circulating sphingolipids with age in females and males."

Finally, we corrected a few linguistic typos, which are highlighted in the revised manuscript via track changes.

We hope you agree that this revised version is now suitable for publication.

Yours sincerely,
The authors

Reviewer #1

This study investigates age-associated variations in sphingolipid profiles and establishes reference values across age groups, aiming to advance their clinical utility as biomarkers for cardiometabolic disorders. The work is scientifically valuable, methodologically transparent, and clearly presented, with strong potential to inform future research and clinical applications.
Thank you for the positive feedback.

Below are suggestions to strengthen the manuscript further:

1. The cross-sectional design appropriately identifies associations between age and sphingolipid levels but inherently limits causal interpretation. While the directed acyclic graph (DAG) posits age as a causal driver of sphingolipid changes, this assumption requires explicit justification. For instance:
- What evidence supports the unidirectionality of the relationship (age → sphingolipids) over reverse causation (e.g., sphingolipid-driven aging)?
Thank you for this important comment. We fully agree that the cross-sectional nature of our study limits causal inference and that our analysis can only establish associations, not causation.

Our aim was "to assess the associations between circulating sphingolipids and chronological age in healthy adults across the lifespan, and to establish age-specific sphingolipid quantile curves." Accordingly, our DAG was constructed under the assumption that age influences sphingolipid levels, which aligns with our goal of estimating age-related variation in sphingolipid concentrations.

This assumption is supported by robust evidence demonstrating that sphingolipids accumulate in aged skeletal muscle and that pharmacological or genetic inhibition of sphingolipid synthesis can mitigate age-related sarcopenia in mice (2). In humans from the UK Biobank and Helsinki Birth Cohort Study, genetic variants that reduce sphingolipid biosynthesis were associated with improved physical fitness in older individuals (2). In contrast, variants linked to enhanced sphingolipid synthesis were associated with reduced fitness (2). In vitro, targeting the same pathway in aged human muscle cells restored mitochondrial function and proteostasis (3).

These findings support the notion that sphingolipids accumulate with advancing age and that inhibiting sphingolipid synthesis can restore muscle function in both mice and humans. However, it remains challenging to determine whether age-related processes drive sphingolipid accumulation or whether accumulating sphingolipids actively contribute to the ageing process. Given the multifactorial nature of ageing, it is unlikely that sphingolipid metabolism alone can account for its progression (4). Nonetheless, we acknowledge that some sphingolipid species may play an active role in modulating biological ageing, and that the relationship between age and sphingolipids could be bidirectional.

Our DAG should therefore be viewed as a simplified causal structure to guide covariate selection for statistical adjustment. It does not preclude reverse or reciprocal biological effects. Modelling the reverse pathway (sphingolipids → age) would have implied a different research

question: how well sphingolipids predict age. It would have resulted in different coefficient estimates and model interpretations. To conclude, our modelling approach aligns with the study's stated aim and is supported by robust scientific evidence.

To better reflect the above in the manuscript, we added the following sentences to the legend of the DAG (see Supplementary Figure 27):

> "This DAG should be viewed as a simplified causal structure to guide covariate selection for statistical adjustment. It does not preclude reverse or reciprocal biological effects".

Finally, the limitation section already addressed the fact that associations and not causality can be established due to the cross-sectional nature of the study (see page 20, lines 489-490):

> "Furthermore, this cross-sectional study establishes associations, rather than causality, between sphingolipids and clinical phenotypes".

We hope this clarifies your concern.

- The inclusion of fitness, physical activity, and body fat as upstream causal factors for age in the DAG is unconventional. Could the authors clarify the rationale for this structure, ideally with references to theoretical or empirical frameworks?

Thank you for this valuable comment. We acknowledge that positioning fitness, physical activity, and body fat as factors potentially influencing age in the DAG is unconventional. Our intention was not to imply a direct causal influence on chronological age, but rather to reflect their role in influencing ageing and health trajectories. As sport and exercise physicians and scientists, we are particularly attuned to the role of physical activity and body composition in promoting healthy ageing. An extensive body of evidence demonstrates that regular physical activity, a higher cardiorespiratory fitness level, and a lower body fat mass positively impact ageing throughout life.

We actually cited references in the manuscript and the legend of the DAG to justify the choice of including these variables as having a potential impact on ageing. It reads (page 10, lines 233-235):

> "Regressions were adjusted for the following confounders: total daily physical activity (5), cardiorespiratory fitness (6), and body fat percentage (7) , which were identified by drawing a causal-directed acyclic graph (DAG, see Supplementary Figure 1) using DAGitty (8)".

The reference (5) is a systematic review that identifies physical activity as one of the determinants of healthy ageing. The reference (6) is a milestone original study demonstrating that cardiorespiratory fitness was inversely related to mortality risk across the spectrum of age in 750,302 U.S. veterans aged 30 to 95. The reference (7) is a review highlighting the

associations between body fat mass and ageing and how excess body fat contributes to biological ageing through inflammageing.

We added additional milestone studies as references to further support that total daily physical activity, cardiorespiratory fitness, and body fat percentage can reasonably be considered potential confounding variables in the present case.

Concretely, we added the work of You et al., published in 2024 in npj Aging (9). This study demonstrated the positive influence of physical activity on DNA methylation-predicted epigenetic clocks, emphasising the role of physical activity in slowing biological ageing and reducing age-related health risks. Regarding cardiorespiratory fitness, we added the citation of a systematic review published in 2020 in the Journal of Sport Sciences (10). The review demonstrated the positive and significant relationship between cardiorespiratory fitness and telomere length, emphasising the importance of cardiorespiratory fitness for healthy ageing. Finally, we added the citation of a review published in April 2025 in the European Heart Journal, highlighting the negative impact of body fat and obesity on cardiovascular ageing (11).

We hope this clarifies your concern.

2. The age-stratified reference values for sphingolipids represent a key contribution. To enhance accessibility and impact, consider adding a summary figure to the main text, contingent on journal figure limits. Tabular data in supplements could then complement this visual synthesis.
Thank you for this important comment. As suggested, we added a figure (see Figure 5), summarising how the age-specific sphingolipid quantile curves were generated.

3. The conclusion that sphingolipid ratios "inform cardiometabolic health" appears overextended given the mixed trends observed (e.g., some ratios increase with age, others decrease; Lines 300–305), which also contradicts 'ratio independent of age' claim by the authors. The authors might wish to add some statistics to the P-spline curves to inform if the relationship is non-flat or not.
Thank you for this insightful comment. We agree that the original wording regarding the findings of the ceramide ratios may have been misleading. We also acknowledge the potential confusion between the two analytical approaches used: multiple linear regressions (inferential) and GAMLSS models (descriptive).

First, we clarify that the age-specific sphingolipid quantile curves were modelled using GAMLSS. These models serve a descriptive purpose by visualising the distribution of lipid levels across age in males and females, without adjusting for covariates. As such, they are not intended for formal hypothesis testing, and adding statistical tests (e.g., significance testing of P-splines) to these curves would be inappropriate.

The multiple linear regression models assessed statistical associations between age and sphingolipids, including their ratios. These models incorporated potential confounders

identified via a DAG. Accordingly, our interpretations of p-values and confidence intervals pertain exclusively to these regression analyses, not the GAMLSS-derived quantile curves.

Regarding the specific point on sphingolipid ratios, none of the ratios showed statistically significant associations with age in the regression models. To avoid overinterpretation, we revised the relevant text to reflect the GAMLSS output's descriptive nature and ensure a clear distinction between descriptive trends and inferential results.

Revisions made:

- Abstract (page 2, line 38):
  Descriptive age- and sex-specific sphingolipid quantile curves were modelled using generalised additive models for location, scale, and shape.

- Methods (page 11, line 248):
  Descriptive age-specific sphingolipid quantile curves were modelled for females and males separately using generalised additive models for location, scale, and shape (GAMLSS, R package version 5.4-12) (12).

- Results, section 3.4 (page 14, line 343):
  Descriptive age-specific sphingolipid quantile curves were modelled separately for females and males for all 26 sphingolipid species.

- Discussion (page 17, lines 407):
  "Descriptive modelling using GAMLSS showed that the Cer16:0/Cer24:0 and Cer24:1/Cer24:0 ratios tended to decline with age in both sexes (Figure 4A, B, E, and F), while Cer18:0/Cer24:0 showed a slight increase in males (Figure 4D) and a slight decrease in females (Figure 4C)".

- Conclusion (page 21, lines 501):
  "Descriptive, age-specific quantile curves complemented this finding, showing a global accumulation of circulating sphingolipids in both sexes".

We also confirm that the following conclusion sentence is statistically supported, given the absence of significant age associations in the regression models (page 21, lines 504-505):

> " Notably, ratios of deleterious to neutral sphingolipids were independent of age".

Thus, the subsequent sentence is posed as a hypothesis and does not contradict our results (page 21, lines 505-507):

> "This may suggest that while overall sphingolipid accumulation is linked to ageing, these ratios could more closely reflect current cardiometabolic status".

Please note that both sentences were combined as follows to address several comments (6 and 10 notably) of the fourth reviewer:

> "Notably, ratios of deleterious to neutral sphingolipids were independent of age. This may suggest that while overall sphingolipid accumulation is linked to ageing, these ratios could more closely reflect current cardiometabolic status. Nonetheless, this observation must be interpreted cautiously, as causal inferences cannot be drawn from cross-sectional data".

We hope this clarifies the intent of the analyses and addresses your concern comprehensively.

Reviewer #2

In the manuscript COMMSMED-25-0309-T by Carrard et al. entitled "Lifespan Trajectories and Reference Values of the Serum Sphingolipidome in a Clinically Healthy Population: Towards Precision Medicine in Cardiometabolic Health Assessment" authors quantitatively measured 26 sphingolipids and observed that concentrations of multiple sphingolipids increase with age and emphasized the need in age-specific reference intervals. The manuscript is well written and interesting, while the method used for analysis of the sphingolipids and its performance are incompletely described. Due to the physicochemical properties, lipids are notoriously challenging analytes for development of quantitative LC-MS methods; in order to support data on analysis of the clinical study samples, detailed description of the LC-MS/MS method, as well as information on the standardization and the methods' performance should be provided.

Thank you for your careful review and for highlighting the importance of a comprehensive description of the analytical methodology. We fully acknowledge that the robustness and reproducibility of quantitative lipidomics data, particularly for sphingolipids, depend on detailed reporting of LC-MS/MS procedures, standardisation approaches, and performance metrics. We appreciate your observation and agree that further clarification and detail regarding our analytical method, standardisation procedures, and method performance are essential to support the validity and reproducibility of our findings. In our revised manuscript, we have expanded the relevant sections to provide this information and address your specific comments point by point below.

We hope these revisions satisfactorily address your concern.

Below are detailed comments on the manuscript.

Methods

Section 2.3. List LOQ and imprecision for the commercial assays used for testing the study samples

We added the following sentence in section 2.3 (page 8, 180-181):

> "The limit of quantification and the imprecision of the methods used are indicated in Supplementary Table 1".

We decided to present this information in a new Supplementary Table 1 to avoid overloading the main manuscript. This Supplementary Table 1 is reproduced below:

Analyte	**Method**	**Limit of quantification**	**Imprecision (reported as coefficient of variation between days)**	**Reference**
HDL-C	Enzymatic reagents (DiaSys,	3 mg/dL (0.078 mmol/L)	2.3% at 28 mg/dL (0.724 mmol/L) 1.9% at 56 mg/dL (1.448 mmol/L)	(13, 14)

	Holzheim, Germany) Olympus AU680 automatic analyser (Beckman Coulter, Brea, CA, USA)			
LDL-C	Enzymatic reagents (DiaSys, Holzheim, Germany) Olympus AU680 automatic analyser (Beckman Coulter, Brea, CA, USA)	4 mg/dL (0.103 mmol/L)	2.1% at 91 mg/dL (2.353 mmol/L) 1.8% at 153 mg/dL (3.957 mmol/L)	(13, 15)
Triglycerides	Enzymatic reagents (DiaSys, Holzheim, Germany) Olympus AU680 automatic analyser (Beckman Coulter, Brea, CA, USA)	0,5 mg/dL (0.006 mmol/L)	1.7% at 76 mg/dL (0.858 mmol/L) 1.2% at 230 mg/dL (2.597 mmol/L)	(13, 16)
HbA1c	D-10 (Bio-Rad, Hercules, CA, USA)	3.8 %	< 1.3 %	(17)
Creatinine	DiaSys (Holzheim, Germany)	8.84 µmol/L	3.63% at 71.7 µmol/L 0.87% at 141.4 µmol/L	(18)
Hs CRP	DiaSys, (Holzheim, Germany)	0.3 mg/L	3.35% at 0.38 mg/L 0.97% at 2.20 mg/L	(19)
NT-proBNP	Architect, (Abbott, IL, United States)	7.9 pg/mL	<5% (140 - 5,000 pg/mL)	(20)

Supplementary Table 1: Limits of quantification and imprecision of the methods used for biochemical analysis. The limit of quantification values was based on manufacturer

specifications, while the coefficient of variation was determined in-house during the measurement period.

In addition, we realised that the methods used to measure creatinine and calculate eGFR were missing. Therefore, we added the following two sentences (page 8, lines 174-177):

> "Creatinine was measured using the Jaffé method with reagents and a calibrator from DiaSys (Holzheim, Germany). eGFR was calculated with the CKD EPI formula using the ckd_epi function of the R package transplantr (21)".

Line 147. Provide vendor and catalog number for the tube type used for the sample's storage.
The aliquots used were "sterile screw cap tubes SCT-050-C-S (Axygen Scientific, California, USA). We adapted the sentence consequently as follows (page 7, lines 149-151):

> "Serum samples were gently shaken for 30 min, centrifuged (3000 g; 10 min; 20–23 °C), aliquoted (sterile screw cap tubes SCT-050-C-S, Axygen Scientific, California, USA), and stored at −80 °C."

Lines 169 and 173. Information on the glycated hemoglobin test is listed twice.
Thank you for pointing out this duplicate. The Olympus AU680 automatic analyser was also mentioned twice. Consequently, we removed these sentences to avoid duplicates (see page 8, lines 173-174).

Section 2.4. Sphingolipid quantification.
• Establishing reference values for clinical diagnostic biomarkers requires the use of validated methods. A full description of the method, detailed information on the method validation and the methods' performance should be provided. If the method was not fully validated and standardized the determined concentration distributions should not be referred to as population reference values.
Thank you for this important comment. We fully agree that establishing clinical reference values requires fully validated and standardised analytical methods. In response to your suggestion, we added the following sentence to the section on sphingolipid quantification (see page 10, lines 221-224):

> "A detailed description of the analytical conditions and method validation results—including accuracy, precision, range of calibration curves, background and matrix effect assessment, and representative chromatograms—is provided in Supplementary Tables 2 to 7."

These new Supplementary Tables correspond to the following:

> ***Supplementary Table 2:*** *Multiple Reaction Monitoring (MRM) method for Sphingolipid Quantification, including sphingolipid names, precursor and product ions, retention*

times, and MS settings. The transitions for internal standards are listed below, in a separate table. Remark: Water loss is commonly observed in-source

Supplementary Table 3: *List of measured sphingolipids (with high precision) and their corresponding chemical names, abbreviations, suppliers, catalogue numbers for all standards, and associated internal standards (used for quantification).*

Supplementary Table 4: *Calibration Curve Concentrations for Commercially Available Sphingolipid Standards (concentration values in nM for calibration levels 0–10 used in quantitative assays).*

Supplementary Table 5: *Intra- and Inter-Day Accuracy of Sphingolipid Quantification (percent accuracy at low and high concentrations across three separate days). Accuracy and precision data for Cer(d18:2/24:0) and Cer(d18:2/24:1) are not available, as these species were acquired using separate calibration curves and spike-in standards.*

Supplementary Table 5: *Intra- and Inter-Day Accuracy of Sphingolipid Quantification (percent accuracy at low and high concentration levels across three separate days). Remark: Accuracy data for Cer(d18:2/24:0) and Cer(d18:2/24:1) are not available, as these species were added to the method later, and the data acquired using separate calibration curves.*

Supplementary Table 6: *Intra- and Inter-Day Precision of Sphingolipid Quantification evaluated in External Plasma Quality Control Material: precision data from replicate analyses of NIST SRM 1950 plasma. Remark: Precision data for Cer(d18:2/24:0) and Cer(d18:2/24:1) are not available, as these species were added later.*

Supplementary Table 7: *Between-Batch Reproducibility evaluated on NIST SRM 1950 Plasma Material analyzed in every batch as external QC (comparison of quantified values over four months of data acquisition).*

In addition, we added the Supplementary Figures 1 to 26, which display comparisons of extracted ion chromatograms for Calibrator Level 9 and a randomly selected participant sample for each species as requested.

That said, we acknowledge that full validation according to clinical laboratory criteria is currently not feasible for all measured species, due to the lack of authentic internal standards and certified reference materials. We have therefore revised the manuscript to avoid using the

term “reference values,” which may imply diagnostic readiness. Instead, we now refer to the reported ranges as “age-specific values”.

• Use of certified reference materials for preparing calibration standards is essential for ensuring the accuracy and reliability of analytical methods. Were standards of the lipids obtained from a commercial company and were certificates of analysis (COA) provided for the standards? Its need to include in the manuscript source and catalog numbers of the standards used for preparing the working calibration standards. If COAs were not available, it is necessary to provide information on the procedures used to establish accurate concentrations of the lipids.
Well-established chemical companies (e.g., Sigma-Aldrich, Fisher Scientific, Cayman Chemical) provide a Certificate of Analysis (COA) for each lot or batch of the chemical standards used. We have included Supplementary Table 3, which lists the corresponding catalogue numbers for each standard and internal standard used in the preparation of the calibrators and ISTD mixture. While the COAs are not included in the supplementary material, they are available upon request.

• Include description of preparation of the stock and working standards of the analytes and the internal standards (solvents used, concentration, storage conditions, handling and storage stability).
We have added a detailed description of the preparation of stock solutions, calibrators, and the internal standard (ISTD) mixture, including the solvents used and storage conditions (see pages 8-9, lines 187-194):

> “The initial standard mixture of sphingolipids was prepared in argon-purged methanol. This mixture was further diluted to match previously reported – biologically relevant concentration ranges for selected species in human plasma and tissues (22). The concentrations in the highest-level calibrator ranged from 250 nM to 5000 nM depending on species (see Table S4). Nine additional calibration points were prepared by serial dilutions of this highest-level calibrator using argon-purged methanol.
> The stock internal standard mixture was also prepared in argon-purged methanol, with concentrations ranging from 3750 nM to 10,000 nM, depending on the molecular species. Before sample spiking, the internal standard mixture was diluted 1:35 with methanol.”

However, we acknowledge that information on the long-term stability of the standards is currently not available, as this has not been systematically evaluated.

• Include list of vendors, catalog item numbers and specifications for all critical reagents and supplies used in the method (standards, internal standards, solvents, reagents, tubes, vials, 96 well plates, etc.).
The requested information, including vendors, catalogue numbers, and specifications for all critical reagents and materials used in the method, is now provided in Supplementary Table 2-7 and in the Method section.

• What was the matrix for preparing the working calibration standards?

The calibration standard mixture was prepared in neat solvent—100% argon-purged methanol—as no commercially available depleted matrix currently exists for sphingolipid analysis. This approach represents the best available option given current methodological constraints.

• Describe the preparation of the working calibration standards, concentrations of the calibrators, frequency of performing calibration and analysis of quality control samples.

The preparation of the standard and internal standard stock solutions and mixtures is described in our response above and the manuscript (pages 8-9, lines 187-194). The concentrations of the calibration standards are provided in Supplementary Table 4. One calibration curve was analysed within each 96-well plate. As an external quality control material, NIST 1950 plasma reference material was analysed twice within each sample batch. This was done primarily to allow for the harmonization of reported concentration values across different studies using different methodologies, if necessary, in the future.

All measured concentrations fell within the range of the calibration curves, except for the species listed below, which were extrapolated:

- SM(d18:1/16:0)
- SM(d18:1/18:1)
- SM(d18:1/18:0)
- SM(d18:1/24:1)
- SM(d18:1/24:0)
- Cer(d18:1/24:1)

• Describe preparation and use of quality control materials analyzed by the method?

As noted above, no certified reference materials are currently available for comprehensive sphingolipid measurement. Therefore, we used NIST 1950 plasma as an external quality control (QC) material. This QC sample was analysed twice within each sample batch essentially to allow for the harmonization of reported concentration values across different studies using different methodologies, if necessary, in the future.

• How was the specificity of analysis assessed? Were multiple mass transitions monitored for the targeted sphingolipids?

The transitions were optimised through the analysis of pure chemical standards under the same analytical conditions. In most cases, one transition was sufficient; a second qualifier transition was used only when necessary. The complete list of optimized transitions per sphingolipid species is provided in Supplementary Table 2.

• Describe the process of data analysis, criteria used to assess acceptability for the batches and the patient samples. Were interferences with the targeted sphingolipids observed in the sample analysis? What was the frequency of the interferences?

Our main criteria included measurement accuracy and precision. Concentrations were reported only for species with a coefficient of variation (CV) from inter-day analysis below 20%. The same threshold was applied for measurement accuracy. Interferences, if any, were subtracted

using the extracted blank or Cal0 sample, which was prepared in the same manner as the plasma samples. All observed interferences in Cal0 (across 11 of the 26 measured species) have been subtracted from the calculated concentrations in samples using blank offset.
Please note that participants should not be referred to as 'patients,' as they were classified as clinically healthy. The assessment of the matrix effect is discussed below.

• Method performance. The following method performance characteristics should be provided: imprecision, accuracy, limit of quantification, limit of detection, analytical measurement intervals, strategy for assessment of specificity, method accuracy, assessment of ion suppression, comparison to methods of other laboratories.
All available method performance data are now provided in Supplementary Tables 2–7, including imprecision, accuracy, range of calibration curves, etc. The strategy used to assess specificity is outlined in our response above. We always used multiple transitions (at least one quantifier and one qualifier). Please note that the LOD and LOQ were not evaluated, as they are highly dependent on the matrix and matrix effects, which can vary significantly between samples collected from different individuals.
To support external validity, we also participated in a recently published international ring trial coordinated by the International Lipidomics Society, involving 30 laboratories worldwide and recently published in Nature Communications (22). The concentrations of four ceramide species included in the CERT1 score (Cer16:0, Cer18:0, Cer24:0, and Cer24:1) measured using our method were in concordance with the consensus values reported in that study. Cross-comparison was not performed for other sphingolipid species due to the lack of available data.

• Include example chromatograms for one of the calibration standards and a representative patient sample.
Thank you for this suggestion. Example chromatograms for one of the calibration standards and a representative sample from a clinically healthy participant are included in Supplementary Figures 1 to 26.

• Mass transitions of the internal standards, as well as retention times of the targeted sphingolipids and internal standards should be provided.
All information related to the scheduled MRM method, including Q1/Q3 mass transitions and retention times for both the targeted sphingolipids and internal standards, is now provided in Supplementary Table 2.

• Considering that stabile isotope labeled analogs for many of the analyzed sphingolipids were not available, how ion suppression was assessed and mitigated for the sphingolipids which did not have stabile isotope labeled analog internal standards?
Matrix effects can be directly evaluated only for sphingolipid species with available authentic stable isotope-labelled internal standards. For other species, correction for matrix effects and subsequent quantification were performed using surrogate internal standards selected based on structural similarity, particularly fatty acyl chain composition, and retention time (RT). This approach follows commonly accepted practice in targeted lipidomics when authentic labelled standards are not available.

• Lipids tend to irreversibly absorb in surfaces (labware, pipette tips, etc). Were adsorptive losses evaluated during the method development and validation? How the adsorptive losses were mitigated.

Thank you for this observation. We have not evaluated potential adsorptive losses; however, measurement accuracy was assessed through recovery assays of spiked standards, which provided very good results as presented in Supplementary Table 5. These recovery data suggest that any potential adsorptive losses were minimal or effectively mitigated under our experimental conditions.

If the method was not fully validated and standardized, statements that the manuscript provides reference values are inappropriate.

We agree that the term "reference values" implies a level of clinical validation and standardisation that exceeds the current scope of our method, particularly given the absence of certified reference materials and isotope-labelled standards for all measured species. To avoid any misunderstanding, we have revised the manuscript throughout and now refer to the reported concentrations as "age-specific values," which more accurately reflect the nature of our data.

Results

Lines 269-277. Were the associations described statistically significant? Provide p-values corresponding to the listed associations.

Yes, all reported associations were statistically significant ($p \leq 0.05$) and remained significant after correction for multiple testing using the Benjamini–Hochberg (BH) method (adjusted $p \leq 0.05$) across all models (23).

As detailed in Supplementary Table 9, we reported β coefficients, 95% confidence intervals, and both raw and adjusted p-values for the associations between each sphingolipid species (dependent variables) and the independent variables: age, sex, age × sex interaction, total daily physical activity, cardiorespiratory fitness, body fat percentage, and statin intake.

Given the length of Supplementary Table 9 (seven pages), incorporating it into the main manuscript was not feasible. In response to this comment and to Reviewer 4's comments 9 and 20, we have included a new Table 3 in the main text, summarising all statistically significant associations. Supplementary Table 9 is retained as a comprehensive reference.

We hope this revision satisfactorily addresses your concern.

Limitations

If the method for measuring sphingolipids was not fully validated and standardized, this is the major limitation for this study and that needs to be stated as a limitation.

Thank you for this important point. We agree that the development of clinical reference values requires full compliance with laboratory medicine standards, including the use of certified reference materials and authentic internal standards for all analytes. Although our targeted LC-MS/MS method demonstrated robust analytical performance, through recovery assays, inter-

and intra-day precision, and inter-laboratory comparability for selected ceramides (22), authentic internal standards and certified reference materials are not available for all sphingolipid species. As a result, we refer to the reported concentrations as "age-specific values" in a clinically healthy population, rather than formal reference values. This limitation has been explicitly acknowledged in the revised manuscript (see page 20, lines 482-488).

> "One limitation of this study is that, despite robust analytical performance of the targeted LC-MS/MS method, demonstrated through recovery assays, inter- and intra-day precision, and inter-laboratory comparability for selected ceramides (22), authentic internal standards and certified reference materials are not available for all sphingolipid species. This prevents full compliance with the criteria required to establish clinical reference values. The reported concentrations are therefore referred to as age-specific values in a clinically healthy population, rather than formal reference intervals".

Figures and Tables

It is unclear what caption corresponds to what figure, label Figures and Tables included in the manuscript.

Thank you for this helpful comment. You are correct that the order of the figures was previously inconsistent. We have now revised the manuscript so that all the Figures and Tables appear in the correct chronological order.

In line with the journal's formatting guidelines, all main Figure and Table captions are provided together at the end of the manuscript (see pages 30–36). Each caption is provided directly beneath the corresponding figure or table in the supplementary material for the Supplementary Figures and Tables.

We hope this revision and clarification satisfactorily address your concern.

Reviewer #3

Brief Summary:

Here the authors measured 26 sphingolipid species using tandem HPLC-MS in the serum of 522 clinincally healthy individuals to assess the effect of age on circulating sphingoliid concentrations and to generate reference ranges for the aforementioned species to enable more accurate interpretation of clinical sphingolipid measurements in the context of cardiovascular disease risk scoring.

The authors report that 17 sphingolipid species were positively associated with advanging age (including the ceramides used in the CERT1 and 2 scores); 8 species were significantly higher in males, 4 species were significantly higher in females, 3 glycosphingolipids demonstrated significantly lower levels with statin use and 2 ceramides were associated with increased body fat. The authors presented age-specific reference ranges and quantile curves of each sphingolipid over the course of a lifespan.

Overall Impression:

The scientific basis motivating this work is robust - indeed sphingolipid reference values have not so far been systematically calculated in healthy human subjects over the course of their life expectancy. Whilst the cross-sectional study desgin does present limitations in that it cannot conclude anything more than association, it is nevertheless appropriate for the research question at hand and the authors do not make inappropriate claims or conclusions from their data. The minutiae of the study design are appropriate and well thought out. The level of detail in the methodology pertaining to data collection and statistical analysis are suffiecient in my opionion to facilitate reproducibility of the results. On general inspection, the supplied code used in statistical formatting and analysis seems appropriate.

The results of the authors analyses are interesting and for the most part novel. The authors presentation of age-specific sphingolipid reference ranges will be of interest and value to scientists with an interest in lipidomics as well as the broader field of cardiovascular disease. Results and described and displayed well and the discussion and interpretation of these results is appropriate and insightful. The figures and tables in both the main text and the supplement are easy to read and interpret with apppriate information provided in the figure legends. What stands out about this work in particular is that the results have been analysed and summarised in a way that makes them accessible and easily utilised by other academics in the field both clinical and non-clinical. All the STROBE criteria have been met in this report.

The authors appropriately acknowledge that whilst such results may be generalisable in Switzerland, they may not fit so well diverse populations in other countries. Nevertheless, I believe this doesn't negate the utility of this work in providing a starting point from which Sphingolipid reference ranges for more diverse populations can be generated. This study did not comment on participant enthnicity and this perhaps would have been interesting to comment on, not least because some indication of the ethnic makeup of the study population may allow readers to evaluate the generalisabilty of these results to the populations they encounter in their own clinical and academic practice.

Overall, I think this is an excellent study. The concept is novel, the execution is robust, and the results push forward the respective field, filling an important gap in the implementation of Sphingolipid-based cardiovascular risk scoring. My commendations to the authors on their high-quality work.
Thank you very much for this positive feedback.

Specific comments:

1. The authors consider a number of important confounding factors on sphingolipid including age and sex but not ethnicity. Are data on participant ethnicity available for inclusion in the participant demographics table as this will help readers determine the applicability of these results to populations in their own localities. Potentially it may even be interesting to identify if there are any trends in circulating sphingolipid species between different ethnicities though I appreciate this may be best explored in a separate publication.
We did not collect data on ethnicity in the COmPLETE study. Conversely to what is done in the USA (24), ethnicity is not a commonly collected variable in Europe (25).
Importantly, the United States National Academies of Sciences, Engineering, and Medicine have recently published recommendations to "rethink and redesign the use of race and ethnicity in biomedical research" (24, 26). In these recommendations, they defined ethnicity as "a socially and politically constructed term used to describe people from a similar national or regional background who share common cultural, historical, and social experiences (24)." These recommendations further mentioned that "races and ethnicities are not useful proxies for biology because there is no genetic, or biological, basis for race. The Human Genome Project found in 2003 that humans were 99.9 per cent identical to each other at the DNA level. Moreover, genetic variation overlaps across racial and ethnic groups instead of creating distinct clusters. A single racial category used in social and political contexts encompasses people with diverse genetic features. Combining these individuals into one group for scientific analyses can lead to oversimplification, misinterpretation, and inaccurate science. Moreover, the use of race and ethnicity in this scientific context can distract from deeper investigations into the true drivers of disease (e.g., genetics, environmental exposures)."
Other authors agreed that "ethnicity" does not have a strong biological basis, referring to the social and cultural background of a person rather than to their genotype and should therefore not be used anymore as surrogates for genotypes (27).

Therefore, not using ethnicity as a variable to investigate differences in sphingolipid levels appears to be in line with the latest recommendations of the United States National Academies of Sciences, Engineering, and Medicine. That being written, the vast majority of the included participants would have been classified as Caucasian from an ethnic perspective. Since ethnicity is now defined as "a socially and politically constructed term used to describe people from a similar national or regional background who share common cultural, historical, and social experiences", describing the participants as inhabitants of the Basel area (Switzerland) is scientifically sounder and more precise than labelling them Caucasian.

2. Consider ensuring consistency in the expression of numerical values either as digits or as fully-written prose, such as in the following examples:
Line 248: ‘Eighty-eight per cent of females (n=220) and 83% of males’
Line 249: ‘Nine per cent of females (n=22) and 13% of males... while four per cent of females’
Thank you for this comment. We now consistently express all numerical values using digits (also known as numerals). The only exceptions we made are numbers representing counts of something (e.g., four ceramides, or eight participants) if the count is smaller than or equal to ten (10) or serves as the first word of a sentence, as is usually done in scientific manuscripts. We are happy to further adapt it according to the wishes of Communications Medicine.

Otherwise I have no other specific comments - this is a well designed and implemented study and a well-prepared manuscript.
Thank you for this positive feedback.

Reviewer #4

In this manuscript (COMMSMED-25-0309-T), the authors measured 26 sphingolipids across a healthy cohort from Switzerland (n=522). This cross-sectional, population-based study aimed to establish age-specific reference quantiles for 26 sphingolipid species in healthy adults and to investigate their associations with chronological age across the lifespan.

1. Since this study involved healthy participants from Switzerland, it is important to reflect this in the manuscript title.
   We rewrote the manuscript title as follows: "Lifespan Trajectories of Serum Sphingolipids in a Clinically Healthy Swiss Population: Towards Precision Medicine in Cardiometabolic Health Assessment".
   Please note that we have replaced "sphingolipidome" with "sphingolipids" as per your comment 3 below.

2. Too many references grouped in a few sentences (e.g., (4–8) or (7, 50)). Distribute references more evenly and consider highlighting landmark studies to support major claims.
   As suggested, we removed references and now focus on landmark studies to support our claims.
   Concretely, we removed references 4 to 7 when referring to the superiority of ceramides versus cholesterol and triglycerides to predict cardiovascular diseases. We kept the landmark study of Laaksonen et al. (28). It now reads (see page 4, lines 72-76):

   > "Nevertheless, these scores outperformed traditional tools, such as cholesterol (28), triglycerides (28), the Framingham score (29), the SMART score (30), the TIMI score (30), the ASCVD score (31), or the 2019 SCORE of the European Society of Cardiology (32) in stratifying cardiovascular risk in primary (29, 33, 34) and secondary prevention (28, 30, 31, 35)".

   In section 4.2, we removed the reference 50 but kept the landmark study by Hilvo et al. (30). We rewrote the affected paragraph of section 4.2 according to your comment 17, which now reads (page 17, lines 397-401):

   > "Beyond absolute concentrations, the relative abundance of deleterious versus neutral ceramides has emerged as a clinically relevant indicator of cardiometabolic risk. Cer16:0, Cer18:0, and Cer24:1 are considered cardiometabolically deleterious, whereas Cer24:0 is regarded cardiometabolically neutral and used as a common denominator for comparing the levels of different ceramide species (30)".

   Please note that the reference numbering in this point-by-point reply differs from that in the main manuscript, as not all references from the manuscript are included in this response.

3. The term "sphingolipidome" is not accurate for referring to 26 sphingolipids. Please replace it with “targeted measurement of 26 sphingolipid species.”
   Thank you for this comment. We agree that the term “sphingolipidome” may have overstated the scope of our analysis. Accordingly, we have replaced it throughout the manuscript with the term “sphingolipids.”
   While we considered the phrasing “targeted measurement of 26 sphingolipid species,” it was too lengthy and not contextually appropriate in all instances. Therefore, we opted for the more concise and accurate term “sphingolipids,” which aligns better with the manuscript’s readability and focus.

4. The gap in knowledge that this study addresses is implied but not directly stated. Add a sentence such as: “However, normative age-related trajectories of circulating sphingolipids in healthy individuals remain poorly characterized.”
   We added the suggested sentence to the last paragraph of the introduction. It now reads as follows (pages 5-6, lines 106 -115):

   > “Determining trajectories and age-specific values of circulating sphingolipid levels in healthy and clinical populations is essential before sphingolipid profiling can be implemented. However, normative age-related trajectories of circulating sphingolipids in healthy individuals remain poorly characterised. Establishing age-specific values across the lifespan in individuals free of manifest diseases will enable researchers and clinicians to distinguish more accurately between age-related and pathological changes. This cross-sectional, population-based study aimed to assess the associations between circulating sphingolipids, chronological age, and sex in healthy adults across the lifespan, and to establish age- and sex-specific sphingolipid quantile curves. It was hypothesised that circulating sphingolipid levels would increase with age in females and males”.

5. Current aim statement: “This cross-sectional population-based study aimed to model age-specific sphingolipid quantile curves and assess associations between circulating sphingolipids and age.” Can be replaced with a more precise statement like: “This cross-sectional, population-based study aimed to establish age-specific reference quantiles for 26 sphingolipid species in healthy adults and to investigate their associations with chronological age across the lifespan.”
   Thank you for this helpful suggestion. We revised the aim statement accordingly. The updated sentence now reads (pages 5-6, lines 111–115):

   > “This cross-sectional, population-based study aimed to assess the associations between circulating sphingolipids, chronological age, and sex in healthy adults across the lifespan, and to establish age- and sex-specific sphingolipid quantile curves. It was hypothesised that circulating sphingolipid levels would increase with age in females and males”.

We chose to explicitly state the aim of assessing sex differences and to include the corresponding hypotheses. We also opted not to specify the number of sphingolipids (n = 26) in the aim statement, as such detail is more appropriate in the Results section. This aligns with standard practice, where the number of analytes and participants is typically introduced alongside descriptive data. Indeed, the sphingolipid panel was obtained using a targeted LC-MS/MS approach designed to provide the most comprehensive coverage of circulating sphingolipids.

6. Some sentences are overly long or redundant. Example: "Cer(d18:1/18:0), characterised by its long fatty acyl chain, has been considered a marker of poor cardiometabolic health. Elevated levels of Cer(d18:1/18:0) are thought to impair mitochondrial function...". Condense repetitive phrases. Use shorter sentences where possible for clarity.

Thank you for this constructive comment. We agree that the original section was overly wordy and have revised it for clarity and conciseness. In addition, this section 4.4 was expanded according to your comment 15. The updated version now reads as follows (pages 18–20, lines 445–479):

> **"VO_2peak, a robust health marker, associates differently with sphingolipids**
> VO_2peak, the gold-standard measure of cardiorespiratory fitness, is a strong predictor of health and is inversely associated with numerous chronic diseases (36). Improvements in VO_2peak reduce morbidity from non-communicable diseases and lower all-cause mortality (36). This makes VO_2peak a valuable benchmark for evaluating emerging blood-based biomarkers. In addition, assessing VO_2peak requires time, specialised equipment, and trained personnel (36). Identifying a blood-based surrogate of VO_2peak could facilitate large-scale implementation in clinical and epidemiological settings.
> In this study, Cer18:0 and the ratio of Cer18:0/Cer24:0 were significantly and negatively associated with VO_2peak. This aligns with findings from a systematic review that identified metabolic signatures of cardiorespiratory fitness (37). Cer18:0, a long-chain ceramide, is a known marker of poor cardiometabolic health (30). Its accumulation has been linked to mitochondrial dysfunction, insulin resistance, and lipotoxicity (38). Given that VO_2peak reflects maximal oxygen uptake and mitochondrial efficiency (36), impaired mitochondrial function may underlie the observed inverse relationship with Cer18:0.
> The ratio of Cer18:0 to Cer24:0 further emphasises this relationship. Cer24:0 has been suggested to exert less harmful or neutral cardiometabolic effects than shorter-chain ceramides (30). A higher Cer18:0/Cer24:0 ratio may thus indicate a relative dominance of deleterious ceramide species, reflecting a lower cardiorespiratory fitness.
> Lastly, VO_2peak was significantly and positively associated with Cer1P16:0 and SM12:0. These findings may reflect exercise-induced adaptation. Phosphorylated ceramides, such as ceramide-1-phosphate (Cer1P), exert biological effects that are functionally opposed to those of unphosphorylated ceramides. Cer1P16:0 has been shown to promote cell survival and exert anti-inflammatory effects (39, 40). It mediates inflammatory responses by stimulating cytosolic phospholipase A_2, releasing arachidonic acid and prostaglandin synthesis (40). Through these mechanisms, elevated Cer1P16:0 levels are thought to support vascular integrity and

endothelial function, consistent with the enhanced cardiac output and vasodilatory capacity observed in individuals with higher VO_2peak.
Although the specific function of SM12:0 remains poorly characterised, short-chain sphingomyelins are known to modulate membrane fluidity and contribute to the organisation of lipid rafts involved in signal transduction (41). It can therefore be hypothesised that elevated circulating levels of SM12:0 reflect subtle alterations in membrane composition or lipid-mediated signalling efficiency associated with aerobic training adaptations, potentially explaining its positive association with VO_2peak. This interpretation remains speculative and warrants further mechanistic investigation".

We hope this clarification satisfactorily addresses your concern.

7. Repetition of long chemical names (e.g., Cer(d18:1/24:0)) without visual aids may hinder readability. Use short codes (e.g., Cer16:0) after first mention. Include a figure legend or abbreviation table for easy reference. Avoid over-reliance on supplements.
   Thank you for this important comment. We now use short codes instead of the full sphingolipid species names consistently throughout the manuscript. The short codes and their corresponding species, subclass and main class names according to the LIPID MAPS classification system are defined in a new Table 2, which replaces the former Supplementary Table 3.
   Please note that three sphingolipid species could not be further abbreviated without losing critical chemical information (Cer(m18:12/2:0), Cer(d18:2/24:0) and Cer(d18:2/24:1)).

   We hope this satisfactorily addresses your concern.

8. Several biochemical terms (e.g., HexCer, HexSph) are not briefly defined for general readers. Add a one-line explanation or include a glossary in supplementary material for non-specialist readers.
   Thank you for this important comment. As mentioned in the previous comment, we added a new Table 2, which defines the short codes used and their corresponding species, subclass, and main class names according to the LIPID MAPS classification system. The legend of Table 2 defines all biochemical terms in plain English. Table 2 replaces the former Supplementary Table 3.

   We hope this satisfactorily addresses your concern.

9. Some statistical results (β coefficients, p-values, CI ranges) are omitted from the main text but are said to be in supplementary tables. Present key statistics for significant findings in the main text to support claims and allow the reader to evaluate the strength of associations. Avoid over-reliance on supplements.
   Thank you for this comment.
   Given the length of Supplementary Table 9 (seven pages), incorporating it into the main manuscript was not feasible. In response to this comment and a similar comment from

Reviewer 1, we added a new Table 3 to the main manuscript, summarising all statistically significant associations. Table 3 supports our key claims and improves readability, reducing reliance on supplementary material.

Supplementary Table 9 is retained as a comprehensive reference. Indeed, it contains β coefficients, 95% confidence intervals, and both raw and adjusted p-values for the associations between each sphingolipid species (dependent variables) and the independent variables: age, sex, age × sex interaction, total daily physical activity, cardiorespiratory fitness, body fat percentage, and statin intake.

We hope this revision satisfactorily addresses your concern.

10. The results are presented in a highly descriptive and fragmented manner, lacking integration or reference back to study objectives/hypotheses. Explicitly state the study's main hypotheses at the beginning of the results section and connect each major finding back to these hypotheses.

Thank you for this valuable comment. In response, we have revised the entire Results section. The study's aim and hypotheses are now clearly stated at the beginning of the section. Additionally, we improved the structure and flow of the results to ensure that each significant finding is explicitly linked to the prespecified hypotheses. The revised section now reads as follows (see pages 11–15, lines 258–359):

"Results

This study aimed to characterise age-related changes in circulating sphingolipid levels and establish age- and sex-specific values in healthy adults across the lifespan. It was hypothesised that circulating levels of sphingolipid species would increase with age (i) in females and males (ii).

Participants' characteristics

A total of 522 participants (251 females and 271 males) were included in the study (Table 1). Participants were distributed across all pre-defined age categories (20–29, 30–39, 40–49, 50–59, 60–69, 70–79, and 80–100 years), with at least 66 individuals in each group (Supplementary Table 8) (1). Mean values for BMI, blood pressure, HbA1c, HDL-C and triglycerides were within physiological ranges, while LDL-C values were slightly elevated in both sexes (mean (SD): 3.24 (0.77) mmol/L in females; 3.11 (0.69) mmol/L in males). NT-proBNP and eGFR values were consistent with previously established age-specific reference ranges for healthy Western European populations (42, 43).

Most participants (88% of females and 83% of males) did not report any medication use. Antihypertensive drug use was reported by 9% of females and 13% of males, while statin use was reported by 4% and 5%, respectively. Four out of five participants were lifelong non-smokers; the remaining were former smokers who had quit at least ten years ago. Mean VO_2peak was 31.4 (8.6) ml/min/kg in females and 38.2 (10.5) ml/min/kg in males. Mean daily moderate-to-vigorous physical activity was 171 (62) minutes in females and 170 (63) minutes in males.

Due to missing data on key covariates, 487 participants were included in the association analysis, while all 522 participants were used to model age- and sex-specific

sphingolipid quantile curves (see Figure 1). The missingness resulted from random technical issues or documentation oversights and was best characterised as missing completely at random.

*** insert Table 1 near here ***
*** insert Figure 1 near here ***

Associations between sphingolipids, age, sex, and confounders

Using a targeted liquid chromatography-tandem mass spectrometry approach, 26 sphingolipid species were quantified in sera of all 522 participants (251 females and 271 males). These 26 sphingolipids comprised 14 ceramides, five glycosphingolipids, four sphingomyelins, and three sphingoid bases (Table 2).

Consistent with hypothesis (i), 17 sphingolipid species were significantly and positively associated with age (Figure 2, Table 3). These sphingolipids comprise 11 ceramides, including the four ceramide species composing the CERT1 and CERT2 scores (i.e. Cer16:0, Cer18:0, Cer24:0 and Cer24:1), three glycosphingolipids and three sphingomyelins. Ceramide ratios used in CERT scores were not significantly associated with age (Supplementary Table 9).

In support of hypothesis (ii), none of the sphingolipids was statistically significantly associated with the interaction between age and sex, meaning there was no evidence for sex-specific differences in age trajectories (Supplementary Table 9). However, eight sphingolipid species and the Cer16:0/Cer24:0 ratio were significantly higher in females than in males (Figure 2, Table 3). These included four sphingomyelins, two ceramides, and one glycosphingolipid (HexCer18:0). Conversely, Sph(d18:1), Cer24:1, Cer18:0, and Cer22:0 showed lower levels in females.

Finally, CerP16:0 and SM12:0 were significantly and positively associated with VO_2peak, whereas Cer18:0 and the Cer18:0/Cer24:0 ratio showed significant negative associations (Figure 2, Table 3). Additional statistically significant associations were observed: three glycosphingolipids were negatively associated with statin intake; two ceramides were positively associated with body fat percentage (Figure 2, Table 3). No statistically significant associations were found with total daily physical activity (Supplementary Table 9).

Due to the large number of tested associations, complete results cannot be shown in tabular format in the main text. Instead, an overview of all associations is provided in graphical form in Figure 2. Associations that remained statistically significant after BH adjustment are summarised in Table 3. Detailed results, including β coefficients, 95% confidence intervals, raw and BH-adjusted p-values for all sphingolipid species, are provided in Supplementary Table 9.

*** insert Figure 2, Tables 2 and 3 near here ***

Alternative sets of multiple linear regression models

Two alternative sets of multiple linear regression models were examined to assess the robustness of the primary findings. In the first set, BMI replaced body fat percentage as an independent variable. In the second set, 77 participants (including 31 females) who reported taking at least one medication (Table 1) were excluded, resulting in a subsample of 445 medication-free participants.

The results from both alternative models were globally consistent with those of the primary analysis. Notably, the ceramide species included in the CERT1 and CERT2 scores (Cer16:0, Cer18:0, Cer24:0, and Cer24:1) remained significantly and positively

associated with age (Supplementary Figures 28 and 29; Supplementary Tables 10 and 11). Likewise, none of the ceramide ratios showed a significant association with age (Supplementary Figures 28 and 29; Supplementary Tables 10 and 11).
As in the primary models, no sphingolipid species showed a statistically significant association with the interaction between age and sex (Supplementary Figures 28 and 29; Supplementary Tables 10 and 11). VO_2peak was consistently and positively associated with CerP16:0 and SM12:0, and negatively associated with Cer18:0 across both alternative models (Supplementary Figures 28 and 29; Supplementary Tables 10 and 11). The Cer18:0/Cer24:0 ratio was also negatively associated with VO_2peak in both models, reaching statistical significance only in the BMI-adjusted model (β coefficient: -0.289; 95% confidence intervals: -0.435 to -0.144; BH-adjusted p-value: ≤ 0.01).
Overall, the alternative models confirmed the main findings of the primary analysis, reinforcing their robustness.

Sphingolipid quantile curves along the lifespan
Descriptive age-specific sphingolipid quantile curves were modelled separately for females and males for all 26 sphingolipid species. The four ceramide species entering the ceramide scores, Cer16:0, Cer18:0, Cer24:0 and Cer24:1, showed increasing serum levels with age in females and males (Figure 3, A to H). Cer24:1 showed a markedly increased variance in females after 80 years (Figure 3, G).

*** insert Figure 3 near here ***

Concerning the three ceramide ratios included in the CERT1 score, a decrease along the lifespan was observed for the ratios Cer16:0/Cer24:0 and Cer24:1/Cer24:0 in both sexes (Figure 4, A, B, E and F). Conversely, the ratio Cer18:0/Cer24:0 showed an increase along the lifespan in males (Figure 4, D) and a very slight decrease in females (Figure 4, C).

*** insert Figure 4 near here ***

An increase in serum levels with age was observed in 22 of the 26 sphingolipid species (Supplementary Figures 30 to 35). The only exceptions were Hex2Cer16:0, which decreased with age in females, and Cer(d18:2/24:1), whose 50th percentile remained stable across the lifespan in females. Age- and sex-specific values for all 26 sphingolipids in females and males between 20 and 91 are provided in Supplementary Tables 12 to 40".

We hope this revision satisfactorily addresses your concern.

11. Reduction from 522 to 487 participants for association analysis is briefly mentioned. Clearly explain the reason and nature of the missing data and assess whether the excluded data could bias results.
Thank you for this critical comment. As illustrated in Figure 1, the reduction from 522 to 487 participants was due to missing data for key covariates included in the regression models:

- Physical activity data (n = 24)
- Body composition data (n = 6)

- Sampling time data (n = 5)

These variables were identified for inclusion in the multiple linear regression models based on a causal-directed acyclic graph (Supplementary Figure 27). Total daily physical activity (5, 9) and body fat percentage (7, 11) were considered potential confounders of the relationship between age and circulating sphingolipids. Sampling time (44) was included to reduce outcome variation and improve the precision of the estimated associations with age (45).

Since these data were missing in only 6.7% of participants (35/522), we chose not to impute them and instead excluded the affected cases from the association analyses.

The missing data arose from random technical issues (physical activity, body composition) or human oversight during documentation (sampling time). Therefore, the missingness is best characterised as missing completely at random (MCAR). While no formal statistical test can confirm this assumption, MCAR data are not expected to introduce bias into parameter estimates. We thus consider the impact of this exclusion on our findings to be minimal.

To better reflect this, we adapted the following paragraph of the result section (see page 12, lines 280-283):

> "Due to missing data on key covariates, 487 participants were included in the association analysis, while all 522 participants were used to model age- and sex-specific sphingolipid quantile curves (see Figure 1). The missingness resulted from random technical issues or documentation oversights and was best characterised as missing completely at random".

We hope this revision satisfactorily addresses your concern.

12. Explore if associations differ by medication use, BMI categories, or other health status markers. Assess robustness of associations by removing participants on statins or antihypertensives.

Thank you for these thoughtful suggestions. First, we would like to emphasise that statin intake was already included as an independent variable in all multiple linear regression models (see page 10, lines 233–239):

> "Regressions were adjusted for the following confounders: total daily physical activity (5, 9), cardiorespiratory fitness (6, 10), and body fat percentage (7, 11) identified by drawing a causal-directed acyclic graph (DAG, see Supplementary Figure 1) using DAGitty (8). Based on the DAG, sex (46), the interaction between age and sex (46), statin intake (47), sampling time (44) and fasting time (48) were also included in the regression models to reduce the outcome variation and improve the precision of the association between age and sphingolipids (45)".

In response to your suggestion, we computed two additional sets of multiple linear regression models to assess the robustness of our findings.

In the first one, we replaced the independent variable "body fat percentage" with the independent variable "body mass index" (BMI). From a physiological perspective, including both variables simultaneously would be redundant and would introduce multicollinearity. In addition, this would introduce unnecessary multicollinearity to the models. Furthermore, we chose to retain BMI as a continuous variable, in line with current methodological recommendations, as categorising continuous variables can lead to substantial loss of information and statistical power (49, 50).

In the second one, we removed all participants who were taking any medications as suggested.

Globally, both alternative sets of models provided findings similar to those of the original set of multiple linear regressions, highlighting the robustness of our findings.

We added a mention of these alternative models in the method section (pages 10-11, lines 239-242):

> "During the review process, the authors were advised to perform two additional sets of multiple linear regressions. In the first, body mass index (BMI) was included as an independent variable instead of body fat percentage. In the second, all participants taking any medications were excluded from the analysis".

The R code was adapted accordingly. It now indicates where and how it should be adapted to compute these two alternative models. As a reminder, the R code is available at https://github.com/JustinCarrard/Lifespan-Trajectories-of-Serum-Sphingolipid-Levels-in-a-Healthy-Swiss-Population-The-COmPLETE-Study.

Likewise, we added a new section in the results, dedicated to these alternative models (see pages 13-14, 319-340):

> **"Alternative sets of multiple linear regression models**
> Two alternative sets of multiple linear regression models were examined to assess the robustness of the primary findings. In the first set, BMI replaced body fat percentage as an independent variable. In the second set, 77 participants (including 31 females) who reported taking at least one medication (Table 1) were excluded, resulting in a subsample of 445 medication-free participants.
> The results from both alternative models were globally consistent with those of the primary analysis. Notably, the ceramide species included in the CERT1 and CERT2 scores (Cer16:0, Cer18:0, Cer24:0, and Cer24:1) remained significantly and positively associated with age (Supplementary Tables 10 and 11). Likewise,

none of the ceramide ratios showed a significant association with age (Supplementary Tables 10 and 11).
As in the primary models, no sphingolipid species showed a statistically significant association with the interaction between age and sex (Supplementary Tables 10 and 11). VO_2peak was consistently and positively associated with CerP16:0 and SM12:0, and negatively associated with Cer18:0 across both alternative models (Supplementary Tables 10 and 11). The Cer18:0/Cer24:0 ratio was also negatively associated with VO_2peak in both models, reaching statistical significance only in the BMI-adjusted model (β coefficient: -0.289; 95% confidence intervals: -0.435 to -0.144; BH-adjusted p-value: ≤ 0.01).
Overall, the alternative models confirmed the main findings of the primary analysis, reinforcing their robustness".

We hope to have convinced you about the robustness of our findings.

13. Test interactions between age and sex or between VO2peak and lipid levels.

Thank you for this comment. As indicated in section "2.5 Statistical analysis", the interaction between age and sex was already included in the regression models (see page 10, lines 233-239):

> "Regressions were adjusted for the following confounders: total daily physical activity (5, 9), cardiorespiratory fitness (6, 10), and body fat percentage (7, 11) identified by drawing a causal-directed acyclic graph (DAG, see Supplementary Figure 27) using DAGitty (8). Based on the DAG, sex (46), the interaction between age and sex (46), statin intake (47), sampling time (44) and fasting time (48) were also included in the regression models to reduce the outcome variation and improve the precision of the association between age and sphingolipids (45)".

Associations between sphingolipids and the interaction of age x sex were already presented in Figure 2. Full numerical findings are displayed in Supplementary Table 9, as none of the associations were statistically significant. Further, this was evoked in the result section (page 11, lines 266-267):

> "Notably, there was no evidence that the shape of the age trajectory differed between males and females for any sphingolipid".

However, we acknowledge that this statement might not have been clear enough. Therefore, we rewrote it as follows (page 13, lines 297-299):

> "In support of hypothesis (ii), none of the sphingolipids was statistically significantly associated with the interaction between age and sex, meaning there was no evidence for sex-specific differences in age trajectories (Supplementary Table 9)".

Regarding the interaction between VO_2peak and sphingolipid levels, it is essential to realise that sphingolipids serve as a dependent variable (also called an outcome) in the current form of the multiple linear regression models. At the same time, VO_2peak serve as an independent variable (also called a predictor). As an interaction term can only be created between two independent variables, adding an interaction term between VO_2peak and sphingolipid levels does not make sense.
However, we did assess the association between VO_2peak and sphingolipid levels, as mentioned in the section "2.5 Statistical analysis" (see page 10, lines 233-236):

> "Regressions were adjusted for the following confounders: total daily physical activity (5, 9), cardiorespiratory fitness (6, 10), and body fat percentage (7, 11) identified by drawing a causal-directed acyclic graph (DAG, see Supplementary Figure 27) using DAGitty (8)".

Results of this association analysis are then reported in the section "3.2. Associations between sphingolipids, age, and confounders" (see page 13, lines 304-306):

> "Finally, CerP16:0 and SM12:0 were significantly and positively associated with VO_2peak, whereas Cer18:0 and the Cer18:0/Cer24:0 ratio showed significant negative associations (Figure 2, Table 3)".

Again, the complete numerical findings (statistically significant and non-statistically significant) are reported in Supplementary Table 9.

Finally, section 4.4 (pages 18-20, lines 445-479) "VO_2peak, a robust health marker, associates differently with sphingolipids" discusses the associations between VO_2peak and sphingolipid levels.

We hope this clarification satisfactorily addresses your concern.

14. The statement that sphingolipid accumulation might indicate "normal ageing" vs. "incipient disease" needs cautious interpretation. Clarify that longitudinal data are needed to draw causal inferences and consider including caveats.

Thank you for this thoughtful comment. We understand the importance of exercising caution when interpreting potential causal relationships from cross-sectional data. We assumed that this comment refers to the following section:

Discussion, page 15, lines 361-364:

> "It could be hypothesised that serum sphingolipid accumulation reflects the normal ageing process, while ratios between deleterious and neutral species provide insights into the biological significance of this accumulation and, ultimately, into the actual cardiometabolic health".

Section 4.2 was rewritten according to your comment 17. We now better acknowledge the limitations of causal interpretation in a cross-sectional design (pages 16-17, lines 389 – 419):

“Clinically healthy aged adults showed favourable ceramide ratios

The four ceramide species entering the CERT1 and CERT2 scores, Cer16:0, Cer18:0, Cer24:0, and Cer24:1, were associated with age, showing increasing serum levels across the lifespan in females and males. From a mechanistic perspective, sphingolipids, particularly ceramides, have been implicated in cellular ageing processes, including mitochondrial dysfunction, oxidative stress, inflammation, and cellular senescence (51). Ceramides such as Cer18:0 have been shown to impair oxidative phosphorylation and promote mitochondrial outer membrane permeabilisation, contributing to biological ageing and cardiometabolic decline (52).

Beyond absolute concentrations, the relative abundance of deleterious versus neutral ceramides has emerged as a clinically relevant indicator of cardiometabolic risk. Cer16:0, Cer18:0, and Cer24:1 are considered cardiometabolically deleterious, whereas Cer24:0 is regarded cardiometabolically neutral and used as a common denominator for comparing the levels of different ceramide species (30). Higher ratios of deleterious to neutral ceramides have been consistently linked to increased risk of cardiovascular events, insulin resistance, and mortality in diverse populations (28, 53). This likely reflects a shift in sphingolipid metabolism toward species that impair vascular function, promote inflammation, or trigger lipotoxic stress (38).

Interestingly, in the present study, there was little evidence that the ceramide ratios used in CERT1 and CERT2 (Cer16:0/Cer24:0, Cer18:0/Cer24:0, and Cer24:1/Cer24:0) were associated with age. Descriptive modelling using GAMLSS showed that the Cer16:0/Cer24:0 and Cer24:1/Cer24:0 ratios tended to decline with age in both sexes (Figure 4A, B, E, and F), while Cer18:0/Cer24:0 showed a slight increase in males (Figure 4D) and a slight decrease in females (Figure 4C). These findings suggest that although absolute ceramide levels rise with age, the relative abundance of risk-associated species does not, implying a preserved or potentially improved sphingolipid profile in clinically healthy older adults.

This distinction highlights the potential utility of sphingolipid profiling, particularly ceramide ratios, for differentiating normal ageing trajectories from early metabolic dysregulation. However, longitudinal data are needed to determine whether changes in specific ceramide species or their ratios predict future cardiometabolic disease, functional decline, or mortality. If validated, sphingolipid-based panels could serve as preventive screening tools for detecting subclinical cardiometabolic dysfunction associated with ageing before clinical symptoms emerge”.

Further, your present comment likely relates to the following sentence of the conclusion (page 19, lines 444-446):

> "Notably, ratios of cardiometabolic deleterious to neutral sphingolipids were independent of age, suggesting that while sphingolipid accumulation may reflect biological ageing, their ratios may better indicate current cardiometabolic health".

We rewrote the conclusion to better acknowledge the limitations of causal interpretation in a cross-sectional design (page 21, lines 504-507):

> "Notably, ratios of deleterious to neutral sphingolipids were independent of age. This may suggest that while overall sphingolipid accumulation is linked to ageing, these ratios could more closely reflect current cardiometabolic status. Nonetheless, this observation must be interpreted cautiously, as causal inferences cannot be drawn from cross-sectional data".

Finally, we would like to emphasise that this cautious approach is already echoed in the following sections:

Discussion (page 16, lines 383–386):

> "Even though the confounding effect of cardiometabolic diseases is assumed to be small in clinically healthy individuals, the present cross-sectional study cannot definitively elucidate whether the observed serum sphingolipid accumulation reflects biological ageing per se or is the first sign of developing cardiometabolic diseases".

Limitations (page 20, lines 489-490):

> "Furthermore, this cross-sectional study establishes associations, rather than causality, between sphingolipids and clinical phenotypes".

We hope that these clarifications sufficiently address your concern.

15. The association of VO2peak with Cer1P(d18:1/16:0) and SM (d18:1/12:0) is described as novel but left largely unexplored. Even speculative biological interpretations or hypotheses would strengthen this section.

Thank you for this critical and constructive comment. We acknowledge that our initial interpretation was overly succinct and did not adequately explore the potential biological relevance of the associations observed, particularly with Cer1P16:0. We have expanded this section to include plausible hypotheses, supported by recent literature. The revised paragraph (pages 19-20, lines 464-479) reads as follows:

> "Lastly, VO_2peak was significantly and positively associated with Cer1P16:0 and SM12:0. These findings may reflect exercise-induced adaptation. Phosphorylated ceramides, such as ceramide-1-phosphate (Cer1P), exert

biological effects that are functionally opposed to those of unphosphorylated ceramides. Cer1P16:0 has been shown to promote cell survival and exert anti-inflammatory effects (39, 40). It mediates inflammatory responses by stimulating cytosolic phospholipase A_2, releasing arachidonic acid and prostaglandin synthesis (40). Through these mechanisms, elevated Cer1P16:0 levels are thought to support vascular integrity and endothelial function, consistent with the enhanced cardiac output and vasodilatory capacity observed in individuals with higher VO_2peak.

Although the specific function of SM12:0 remains poorly characterised, short-chain sphingomyelins are known to modulate membrane fluidity and contribute to the organisation of lipid rafts involved in signal transduction (41). It can therefore be hypothesised that elevated circulating levels of SM12:0 reflect subtle alterations in membrane composition or lipid-mediated signalling efficiency associated with aerobic training adaptations, potentially explaining its positive association with VO_2peak. This interpretation remains speculative and warrants further mechanistic investigation".

We hope this satisfactorily addresses your concern.

16. The repeated reference formatting for ceramides becomes heavy and may be condensed. For example, the full chemical notation could be written once per paragraph with abbreviations thereafter.

Thank you for this important comment. As mentioned in our response to comments 7 and 8, we now consistently use short codes instead of the full sphingolipid species names throughout the manuscript. The short codes and their corresponding species, subclass, and main class names according to the LIPID MAPS classification system are defined in a new Table 2, which replaces the former Supplementary Table 3.

However, please note that three sphingolipid species could not be further abbreviated without losing critical chemical information (Cer(m18:12/2:0), Cer(d18:2/24:0) and Cer(d18:2/24:1)).

We hope this satisfactorily addresses your concern.

17. In the discussion, authors could briefly highlight possible mechanisms linking sphingolipids to ageing and mitochondrial function. How changes in specific ceramides or ratios relate to cardiometabolic risk. Possible biological mechanisms or implications of observed age-related patterns. Discuss how findings can guide future research—e.g., could sphingolipid profiles be used in preventive screening for age-related metabolic decline? How changes in specific ceramides or ratios relate to cardiometabolic risk.

Thank you for this insightful comment, which helped clarify and expand the discussion on the biological relevance of our findings. We rewrote the section "4.2. Clinically healthy aged adults showed favourable ceramide ratios" to specifically address your points (pages 16-17, lines 389–419):

“Clinically healthy aged adults showed favourable ceramide ratios

The four ceramide species entering the CERT1 and CERT2 scores, Cer16:0, Cer18:0, Cer24:0, and Cer24:1, were associated with age, showing increasing serum levels across the lifespan in females and males. From a mechanistic perspective, sphingolipids, particularly ceramides, have been implicated in cellular ageing processes, including mitochondrial dysfunction, oxidative stress, inflammation, and cellular senescence (51). Ceramides such as Cer18:0 have been shown to impair oxidative phosphorylation and promote mitochondrial outer membrane permeabilisation, contributing to biological ageing and cardiometabolic decline (52).

Beyond absolute concentrations, the relative abundance of deleterious versus neutral ceramides has emerged as a clinically relevant indicator of cardiometabolic risk. Cer16:0, Cer18:0, and Cer24:1 are considered cardiometabolically deleterious, whereas Cer24:0 is regarded cardiometabolically neutral and used as a common denominator for comparing the levels of different ceramide species (30). Higher ratios of deleterious to neutral ceramides have been consistently linked to increased risk of cardiovascular events, insulin resistance, and mortality in diverse populations (28, 53). This likely reflects a shift in sphingolipid metabolism toward species that impair vascular function, promote inflammation, or trigger lipotoxic stress (38).

Interestingly, in the present study, there was little evidence that the ceramide ratios used in CERT1 and CERT2 (Cer16:0/Cer24:0, Cer18:0/Cer24:0, and Cer24:1/Cer24:0) were associated with age. Descriptive modelling using GAMLSS showed that the Cer16:0/Cer24:0 and Cer24:1/Cer24:0 ratios tended to decline with age in both sexes (Figure 4A, B, E, and F), while Cer18:0/Cer24:0 showed a slight increase in males (Figure 4D) and a slight decrease in females (Figure 4C). These findings suggest that although absolute ceramide levels rise with age, the relative abundance of risk-associated species does not, implying a preserved or potentially improved sphingolipid profile in clinically healthy older adults.

This distinction highlights the potential utility of sphingolipid profiling, particularly ceramide ratios, for differentiating normal ageing trajectories from early metabolic dysregulation. However, longitudinal data are needed to determine whether changes in specific ceramide species or their ratios predict future cardiometabolic disease, functional decline, or mortality. If validated, sphingolipid-based panels could serve as preventive screening tools for detecting subclinical cardiometabolic dysfunction associated with ageing before clinical symptoms emerge.”

We hope these additions address your concerns and provide a more comprehensive interpretation of our findings.

18. Although some confounders are considered (e.g., statin use, VO2peak), there is no mention of how multivariable models were built or whether collinearity was assessed.
To identify confounders for inclusion in the multivariable models, we constructed a directed acyclic graph (DAG) using the online tool DAGitty (8, 54). DAGs offer a transparent framework for identifying confounding variables that require conditioning (i.e. adjustment) when estimating causal associations (54). This approach makes our assumptions explicit and facilitates critical appraisal pre- and post-publication (54).

The structure of our DAG was informed by existing literature and domain knowledge; references supporting these choices are listed in the legend of Supplementary Figure 27. Based on this DAG, we adjusted all regression models for the following covariates: total daily physical activity, cardiorespiratory fitness (VO_2peak), body fat percentage, sex, the interaction between age and sex, statin use, sampling time, and fasting duration. These variables were included a priori and not selected through data-driven variable selection.

We assessed multicollinearity among independent variables by calculating the variance inflation factor (VIF) for each multivariable model. This procedure is explicitly documented in the R code (lines 232–237), available at: https://github.com/JustinCarrard/Lifespan-Trajectories-of-Serum-Sphingolipid-Levels-in-a-Healthy-Swiss-Population-The-COmPLETE-Study

```
# check for multicollinearity
vif <- lapply(my_lms, function(model) car::vif(model, type = "predictor"))
vif
```

Problematic multicollinearity leads to unstable coefficients and large confidence intervals (55, 56). The VIF quantifies how much larger the standard errors and, consequently, confidence intervals become compared to a situation with no collinearity. Some degree of correlation between predictors is expected in any real-world data. The relevant question is whether the amount of inevitable multicollinearity in the data hinders a valuable interpretation of the estimates.

In our study, none of the independent variables exhibited problematic multicollinearity. All GVIF and GVIF^(1/(2*Df)) values were well below commonly accepted thresholds (e.g., 5 or 10) (55, 56), indicating stable coefficient estimates.

We trust this addresses the concern adequately.

19. Model adjustment strategy. Variable selection rationale. Whether interaction terms (e.g., age × sex) were tested and if not, why.
Our model adjustment strategy was guided by a directed acyclic graph (DAG) constructed using DAGitty (8, 54), as described in our response to Comment 18. DAGs provide a theory-driven approach to identifying variables that require adjustment to

estimate causal associations. The structure of our DAG was informed by prior literature and expert knowledge; relevant references are provided in the legend of Supplementary Figure 27.

Accordingly, we included the following covariates in all regression models: total daily physical activity, cardiorespiratory fitness (VO_2peak), body fat percentage, sex, the interaction term age × sex, statin use, sampling time, and fasting duration.

As noted in Section 2.5 ("Statistical analysis", page 10, lines 235–239), the interaction between age and sex was explicitly tested and included in all models:

> Based on the DAG, sex (46), the interaction between age and sex (46), statin intake (47), sampling time (44) and fasting time (48) were also included in the regression models to reduce the outcome variation and improve the precision of the association between age and sphingolipids (45)".

Although this interaction term was modelled, none of the associations between sphingolipids and age × sex reached statistical significance (see Supplementary Table 9). This was reported in Figure 2 and in the Results section (page 11, lines 266–267):

> "Notably, there was no evidence that the shape of the age trajectory differed between males and females for any sphingolipid".

However, we acknowledge that this statement might not have been clear enough. Therefore, we clarified the phrasing for better reader understanding (page 13, lines 297–299):

> "In support of hypothesis (ii), none of the sphingolipids was statistically significantly associated with the interaction between age and sex, meaning there was no evidence for sex-specific differences in age trajectories (Supplementary Table 9)".

We hope this clarification fully addresses your comment.

20. The conclusion that "the data provided little evidence of association between sphingolipids and total daily physical activity" is vague and not quantified. Provide statistical metrics for this null finding. Also, clarify whether smoking history was adjusted for in regression models given its known lipidomic impact.
Provide stats in the main document
Thank you for this comment. We rewrote the statement as follows (see page 13, lines 308-309):

> "No statistically significant associations were found with total daily physical activity (Supplementary Table 9)".

Following your suggestions and those from Reviewer 1, we added a new Table 3 to the main manuscript, summarising all statistically significant associations. Table 3 supports our key claims and reduces reliance on supplementary material, improving readability.

Further, the Supplementary Table 9 reported the full results for all regression models (including β coefficients, 95% confidence intervals, raw and adjusted p-values for associations between each sphingolipid species and the independent variables: age, sex, age × sex interaction, total daily physical activity, cardiorespiratory fitness, body fat percentage, and statin intake).
Please consider that Supplementary Table 9 is seven pages long, making it impossible to include it in the main document.

All participants were either never smokers (n = 418) or had quit smoking at least 10 years before the study (n = 104). Smoking has well-established effects on circulating lipids, notably decreasing HDL-cholesterol and increasing triglyceride levels (57, 58). However, multiple studies have demonstrated that these lipid alterations essentially reverse within weeks to months after cessation, with HDL levels increasing significantly during the first months (59) and stabilising within one year (60). From an epidemiological perspective, large prospective cohorts indicate that former smokers no longer exhibit elevated cardiovascular risk compared to never-smokers after 10–15 years of abstinence (61, 62). Taken together, these data suggest it is unlikely that smoking history had a residual impact on sphingolipid profiles in this clinically healthy population. Therefore, smoking history was accounted for by study design and was not included as a covariate in the regression models.

We hope this satisfactorily addresses your concern.

21. Discuss how findings compare to previously published sphingolipid reference values across ethnicities or cohorts such as in the following study (PMID: 38561799; DOI: 10.1186/s12944-024-02080-6)
Thank you for highlighting this study. While we recognise its relevance, direct comparisons with our findings are limited by methodological differences. Specifically, the authors did not establish sex-specific or age-stratified reference values, instead reporting only median, 2.5th, and 97.5th percentiles for five ceramide species and their ratios across the entire cohort. Furthermore, the study was restricted to participants aged 6 to 17 years, precluding meaningful comparison with our adult cohort. Given these limitations, we do not consider formal statistical comparisons between the two datasets appropriate. Nevertheless, as suggested, we now acknowledge this study in our discussion to reflect its contribution to the emerging field of ceramide reference values (page 18, lines 427–433):

> "One of the few available attempts to generate age-specific values for ceramides comes from a study in Emirati children and adolescents (aged 6 to 17 years), which reported percentiles for five plasma ceramide species (Cer16:0, Cer18:0,

Cer22:0, Cer24:0, and Cer24:1) and selected ratios (63). However, the ranges were not stratified by sex or narrower age bands, limiting their interpretability. Moreover, because that cohort did not include individuals over 17, comparisons with the present study are challenging. It seems, however, that children and adolescent Emiratis tend to display lower levels of plasma ceramides than Swiss adults in their twenties."

We hope this satisfactorily addresses your suggestion.

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22.

Dear editor, dear reviewer,

We would like to thank you for reviewing our manuscript a second time.

All comments have been carefully addressed point by point (see below) and incorporated into the revised manuscript using track changes.

We hope the revised version meets your expectations and is now suitable for publication.

Sincerely,

The authors

Reviewer #2 (Remarks to the Author):
In the manuscript COMMSMED-25-0309-T by Carrard et al. entitled "Lifespan Trajectories of Serum Sphingolipids in a Clinically Healthy Swiss Population: Towards Precision Medicine in Cardiometabolic Health Assessment" authors quantitatively measured 26 sphingolipids and observed that concentrations of multiple sphingolipids increase with age and emphasized the need in age-specific reference intervals. The manuscript is well written and presents an interesting study; the study population, the participant information, and statistical analysis methods are appropriate. However, there are some concerns related to the LC-MS method used for analyzing lipids in the study samples, which may question the observations and the conclusions of the study. Below are detailed comments on the manuscript.

Thank you for your comments. We have carefully addressed all your comments regarding the LC-MS method below and incorporated the necessary changes into the revised manuscript using Track Changes.

1. Matrix effects are one of the primary sources of interference in mass spectrometry-based methods, which are typically manifest as signal suppression or enhancement. The most effective strategy to account for, and compensate for these effects is the use of stable isotope-labeled analogs of the target analytes as internal standards. In the method used in this study, 26 lipids were measured, while 10 internal standards were used. Internal standards correct for matrix effects in corresponding structural analogs, while may not compensate for the matrix effects for the remaining 16 lipids analyzed in the study. As a result, there is uncertainty regarding the accuracy of the measured concentrations.

We agree that it is not possible to accurately correct for matrix effects or determine absolute concentrations using surrogate internal standards. At present, however, there is no alternative, as only a limited number of stable isotope-labelled internal standards are commercially available, given the structural diversity of endogenous sphingolipids. This limitation is well known and, as the reviewer highlighted below, widely accepted in the lipidomics community.
However, we agree that a clearer distinction should be made between sphingolipids for which absolute concentrations could be established (i.e., 7 species for which authentic internal standards were available) and those for which only estimated concentrations could be reported based on surrogate internal standards (n = 14). For the 7 species for which authentic internal

standards were available (Cer16:0, Cer18:0, Cer24:0, Cer24:1, SM18:1, Sph18:1 and Sph1P18:1), we reliably established age- and sex-specific concentration distributions across the lifespan (20–90 years). In this regard, it is important to note that the four ceramide species incorporated into the ceramide scores belong to these 7 species.

To better acknowledge this undeniable methodological limitation, we adapted the limitations as follows (pages 21-22, lines 500-516):

> "One limitation of this study is that, despite the robust analytical performance of the targeted LC-MS/MS method, demonstrated through recovery assays, inter- and intra-day precision, and inter-laboratory comparability, for selected ceramides (62), authentic internal standards, and certified reference materials are not available for all sphingolipid species. This limitation may introduce some uncertainty in the absolute quantification of specific lipids and thus prevents full compliance with the stringent criteria required to establish clinical reference values. Consequently, the reported concentrations are referred to as age-specific values in a clinically healthy population rather than formal reference intervals.
> Specifically, for the subset of sphingolipid species (n = 7), i.e. Cer16:0, Cer18:0, Cer24:0, Cer24:1, SM18:1, Sph18:1 and Sph1P18:1, absolute concentrations could be reliably quantified using authentic, stable-isotope-labelled internal standards and multipoint calibration curves. It is important to note that the ceramides used in the ceramide-based risk scores are included in these seven species. For the remaining sphingolipid species (n = 14), it was not possible to accurately correct for matrix effects due to the lack of stable isotope-labelled internal standards. Therefore, concentrations were estimated using surrogate internal standards, a practice widely accepted in lipidomics. The authentic internal standards are listed in Supplementary Table 3".

In addition, following your comments regarding the chromatograms (see comments 25–28) and to ensure the robustness of the reported results, we decided to remove five sphingolipid species from the manuscript for which ambiguities in species identification could not be fully excluded (i.e. Cer(d18:0/22:0); Cer(d18:1/18:1); Cer(d18:1/22:0); Cer(d18:2/24:0); and Cer(d18:2/24:1)). We hope that this additional precaution will satisfactorily address your concern and further strengthen confidence in the study findings. This modification required rerunning the three sets of multiple linear regression analyses, as p-values across all models were adjusted jointly using the Benjamini–Hochberg procedure, which depends on the total number of tested species. All results were updated accordingly; however, the overall findings remained unchanged, except for minimal differences in adjusted p-values.
In contrast, this reanalysis was not required for the descriptive age-specific sphingolipid quantile curves, which were modelled using generalised additive models for location, scale, and shape, as these analyses are descriptive and do not rely on multiple-testing correction.

We hope this clarification satisfactorily addresses your concern about the potential impact of limited internal standards on data accuracy.

2. While it is common in lipidomics research to quantify many lipids using only a few internal standards, this practice is unacceptable in clinical research, where data accuracy is important. At a minimum, this methodological limitation should be clearly acknowledged in the manuscript's limitations section to ensure readers are aware of the potential for erroneous findings.

Thank you for your comment, which we agree with.
We already acknowledged the following in the limitations (p. 21, lines 488-494):

> "One limitation of this study is that, despite robust analytical performance of the targeted LC-MS/MS method, demonstrated through recovery assays, inter- and intra-day precision, and inter-laboratory comparability for selected ceramides (62), authentic internal standards and certified reference materials are not available for all sphingolipid species. This prevents full compliance with the criteria required to establish clinical reference values. The reported concentrations are therefore referred to as age-specific values in a clinically healthy population, rather than formal reference intervals".

However, to even better acknowledge your point, we rewrote the limitations as previously highlighted (pages 21-22, lines 500-516):

> "One limitation of this study is that, despite the robust analytical performance of the targeted LC-MS/MS method, demonstrated through recovery assays, inter- and intra-day precision, and inter-laboratory comparability, for selected ceramides (62), authentic internal standards, and certified reference materials are not available for all sphingolipid species. This limitation may introduce some uncertainty in the absolute quantification of specific lipids and thus prevents full compliance with the stringent criteria required to establish clinical reference values. Consequently, the reported concentrations are referred to as age-specific values in a clinically healthy population rather than formal reference intervals.
> Specifically, for the subset of sphingolipid species (n = 7), i.e. Cer16:0, Cer18:0, Cer24:0, Cer24:1, SM18:1, Sph18:1 and Sph1P18:1, absolute concentrations could be reliably quantified using authentic, stable-isotope-labelled internal standards and multipoint calibration curves. It is important to note that the ceramides used in the ceramide-based risk scores are included in these seven species. For the remaining sphingolipid species (n = 14), it was not possible to accurately correct for matrix effects due to the lack of stable isotope-labelled internal standards. Therefore, concentrations were estimated using surrogate internal standards, a practice widely accepted in lipidomics. The authentic internal standards are listed in Supplementary Table 3."

Finally, we would like to emphasise that the present work represents a translational research study, rather than an assay intended for immediate implementation in routine clinical practice. Beyond describing age- and sex-related patterns in sphingolipid concentrations, this study aims to characterise baseline biological variability in an apparently healthy population, which is a necessary prerequisite for any future clinical application. In this context, our findings also highlight current analytical and standardisation gaps, particularly the limited availability of

stable isotope-labelled internal standards and certified reference materials, which must be addressed before sphingolipid profiling can be reliably translated into routine clinical use.

We hope this clarification addresses your concern about the potential impact of limited internal standards on data accuracy.

3. Conclusions presented in the abstract emphasize the need for age-specific reference values for the measured lipids. However, due to methodological limitations, age-specific reference intervals cannot be reliably established using data from this study.

We agree that establishing formal reference intervals will require further methodological advances, particularly regarding the availability of authentic internal standards.

Our manuscript, however, does not claim to have derived such intervals. Instead, we present descriptive percentile values to illustrate age- and sex-related variation in sphingolipid concentrations among clinically healthy individuals.

The statement in the abstract, "highlighting the need for age-specific values", refers to the clinical interpretation of our findings, and not to the methodological establishment of reference intervals.

To avoid any potential ambiguity, we have slightly revised the concluding sentence to read (page 4, lines 55–57):

> "Serum sphingolipid levels depended on age, underscoring the need for age-specific interpretation when assessing cardiometabolic risk using sphingolipid-based scores."

We hope this clarification satisfactorily addresses your concern.

Supplemental table S2:

4. It is not stated what mass transitions were used for quantification?

The mass transitions were originally reported in the columns "Precursor Ion" and "Product Ion." However, we acknowledge that we did not explicitly specify which transitions were used for quantification (quantifiers) and which were used for confirmation of lipid annotation (qualifiers). We have now clarified this by specifying the quantifier and qualifier transitions for each analyte in Table S2 (column F).

5. It is not stated what internal standards were used for what analyte?

The internal standards associated with each analyte are specified in Table S3, which was added during the first round of revision.

6. For some analytes there are 2, for some 3 mass transitions listed in the table, but it is not stated how were multiple mass transitions used in the assay and in the data analysis?

We have clarified this point in the Methods section (page 11, lines 222-224) by adding the following statement:

"The transition with the highest intensity and specificity was used as the quantifier, whereas the remaining one or two transitions were used as qualifiers to increase confidence in lipid annotation."

In addition, information on quantifier and qualifier transitions for each analyte is provided in Table S2, as highlighted above (see response to comment 4).

7. Was the ratio of mass transitions evaluated during the data analysis? What was the tolerance for the ratio of mass transitions? If the ratios were assessed, what percentage of results was removed from consideration due to the presence of suspected interferences.

We have assessed the qualifier-to-quantifier ratios for the reported sphingolipid species. A tolerance of 30% was applied, defined as the acceptable deviation when the ratio for a pure standard was compared to the ratio from the endogenous compound measured. This was added to Section 2.4 Sphingolipid analysis (see page 11, lines 22-228):

"Sphingolipid species were retained for downstream analysis only if all predefined qualitative criteria were fulfilled, including consistent retention times across transitions, acceptable quantifier-to-qualifier ion ratios relative to pure standards (±30 %), and sufficient signal intensity above background".

No results were excluded based on this criterion, except for Cer d18:1/18:1, as specified in the reply to your question 25 below. As highlighted in our reply to your question 1, to ensure the robustness of the reported results, we decided to remove four other sphingolipid species from the manuscript for which ambiguities in species identification could not be fully excluded (i.e. Cer(d18:0/22:0); Cer(d18:1/22:0); Cer(d18:2/24:0); and Cer(d18:2/24:1)). We hope that this additional precaution will satisfactorily address your concern and further strengthen confidence in the study findings.

Supplemental table S5:

8. What was the matrix of the sample used for evaluation of the method's accuracy?

Method accuracy was evaluated using 5% BSA in water spiked at high and low concentration levels. The same matrix was used in the ceramide ring trial recently published in *Nature Communications* (https://www.nature.com/articles/s41467-024-52087-x), as it represents the best compromise given the unavailability of a plasma matrix depleted of sphingolipids.

9. What were concentrations of the targeted lipids in the sample?

The concentrations of the targeted lipids were defined by spiking calibration solutions into a 5% BSA matrix. The low-concentration level was prepared by adding 30 µL of Cal6 to 570 µL of 5% BSA in water, while the high-concentration level was prepared by adding 30 µL of Cal10 to 570 µL of 5% BSA in water (Tables S4 and S6). The resulting concentrations correspond to the defined low- and high-level calibration ranges used for method validation.

10. Provide description of preparation of samples used in the experiment (lipids have limited solubility and may not dissolve in the sample matrix).

Sample preparation, including the handling of lipid solubility, is described in Section 2.4 (Sphingolipid analysis, pages 9–10, lines 188–232) and in our response to Comment 9 above. Briefly, calibration and spike samples were prepared in a protein-containing matrix (5% BSA) to mimic serum conditions and ensure adequate solubilisation of lipids prior to extraction. The robustness of this preparation is supported by the observed accuracy and recovery results, indicating efficient extraction across the tested concentration range.

11. What was the number of replicates?

Six independently prepared replicates per concentration level were analysed each day over three consecutive days (i.e., 18 measurements per concentration level).

12. Were the samples prepared on each day of experiment or were some samples reinjected on different days

Replicates were freshly prepared and extracted on each experimental day, with no reinjection of previously prepared samples, as shown in Table S5.

Supplemental table S6:

13. Provide description of preparation of samples used in the experiment.

Samples used for the precision evaluation were prepared using NIST SRM 1950 plasma reference material, as described in the Materials and Methods section (pages 9–10, lines 189–202). For clarity, the relevant procedure is summarised below:

> "Serum samples, calibrators, and quality controls (QCs, 25µL) were extracted by the addition of 100µL of methanol spiked with internal standards in 96 deep-well plates (Waters, Milford, MA, USA) using a Bravo automated liquid-handling platform (Agilent Technologies, Santa Clara, California, USA). The initial standard mixture of sphingolipids was prepared in argon-purged methanol. The standard mixture was further diluted to match previously reported concentration ranges for selected metabolites in human plasma and tissues (30). The concentrations in the highest-level calibrator spanned from 250 nM to 5000 nM. The subsequent 11 points of the calibration curve were prepared by serial dilutions of this highest calibrator using argon-purged methanol. The stock internal standard mixture was prepared in argon-purged methanol with concentrations ranging from 3750 nM to 10000 nM, depending on the molecular species. The stock internal standard mixture was diluted 1/35 times with methanol before the sample spike.
> The extracts were shaken (10 min at 1000 rpm) and centrifuged externally (15 min at 2700g at 15˚C) (Hermle Z 326 K, Gosheim, Germany). The resulting supernatant was transferred using the liquid handler into a new 96-well plate (Thermo Scientific, San Jose, CA, United States)".

14. How were the lipid containing samples prepared?

Lipid-containing samples were prepared using the same extraction and sample preparation protocol described above (see response to Comments, 9, 10, 13 and Section 2.4, Sphingolipid analysis, pages 9–10, lines 189–202).

15. Were the samples prepared on each day of experiment or were same samples reinjected on different days?

As mentioned above, samples were freshly prepared on each experimental day (i.e. independently extracted) and analysed over three different days; no samples were reinjected on subsequent days.

16. Precision should be evaluated at multiple concentrations within measurement range of the method.

Precision was evaluated using NIST SRM 1950 plasma reference material which is the best strategy, as it mimics the complex plasma matrix. The sphingolipid species for which concentrations are reported were present in this reference material at levels spanning the concentration range measured by the method and observed in the analysed study population. Precision was therefore assessed across relevant concentration levels within the method's measurement range.

17. What was the number of replicates?

Ten independently analysed replicates were included, as detailed in Table S6.

18. There are 2-7 significant digits used for listed concentrations, considering the methodological issues, it is misleading to use more than 2 or 3 significant digits in presenting the lipid concentrations.

Thank you for this comment. We changed it to give two significant digits for all concentrations contained in Tables 6 and 7. Please note that Tables 2-5 did not show any concentration.
We hope that this modification will satisfactorily address your concern.

<u>Supplemental table S7:</u>

19. See above comment related to the number of significant digits.

Thank you for this comment. We changed it to give two significant digits for all concentrations contained in Tables 6 and 7. Please note that Tables 2-5 showed no concentration.
We hope that this modification will satisfactorily address your concern.

20. Matrix of the NIST SRM 1950 sample is not identical to human serum/plasma sample.

We fully agree that the matrix of NIST SRM 1950 is not identical to that of native human serum or plasma. However, this reference material provides a well-characterised, complex plasma matrix that is widely used for analytical validation purposes. Importantly, the sphingolipid species for which concentrations are reported in this study are all present in NIST SRM 1950 within the concentration range measured by the method and observed in the analysed study population. Therefore, the use of this reference material is appropriate for evaluating analytical performance in the context of the present study.

21. Were matrix matched controls (serum/plasma) analyzed along with the patient samples? Quality control samples containing multiple concentrations of the analytes of interest spanning the measurement range of the assay should be included in every batch of

samples. The quality controls samples should be prepared in the same matrix as the patient samples analyzed in the study.

Matrix-matched quality control samples were included using NIST SRM 1950 plasma reference material, which provides a well-characterised plasma matrix. This material was used to monitor analytical precision for sphingolipid measurements at concentrations spanning the measurement range of the method and corresponding to those observed in the analysed study population. Quality control samples were analysed alongside study samples, which is now specified in Section 2.4 Sphingolipid analysis (page 11, lines 233-238):

> "For quality control purposes, NIST SRM 1950 plasma reference material was analysed as a matrix-matched control at concentrations spanning the measurement range of the assay and was included alongside study samples to monitor analytical precision and performance. These quality control samples were used to monitor run-to-run analytical stability and batch acceptability throughout the analytical sequence".

We hope this clarification satisfactorily addresses your concern.

22. If matrix-matched controls were not analyzed alongside the study samples and their performance in the method was not assessed, this raises concerns about the reliability of the analytical results. Without proper quality control, it is impossible to evaluate between runs stability of the instrument performance, and acceptability of the results from each batch. This should be addressed in the manuscript, either by including relevant control data or by stating this as the limitation, to inform readers of the potential impact on data accuracy and interpretation.

We agree that appropriate quality control is essential to assess analytical reliability and instrument performance. In the present study, matrix-matched quality control samples based on NIST SRM 1950 plasma reference material were analysed to monitor analytical precision and performance. This reference material provides a complex plasma matrix, and all sphingolipid species for which concentrations are reported were present within the concentration range measured by the method and observed in the analysed study population.

Quality control measurements demonstrated stable analytical performance across the analysed concentration range, as detailed in the method validation results provided in the Supplementary Tables. These data support the reliability of the reported measurements. Nevertheless, we acknowledge that future studies aiming at routine clinical implementation would benefit from the inclusion of population-specific internal quality control samples analysed systematically across batches.

<u>Supplemental tables S12-40:</u>

23. The units for the concentrations shown in the tables are not specified.

We respectfully disagree with this comment. The units for all concentrations presented in Tables S12–S40 are clearly indicated in the third row of each table, as illustrated below:

Cer18:0										
Sex	**Females**					**Males**				
Age (yrs)	**3 P (nM)**	**15 P (nM)**	**50 P (nM)**	**85 P (nM)**	**97 P (nM)**	**3 P (nM)**	**15 P (nM)**	**50 P (nM)**	**85 P (nM)**	**97 P (nM)**

20	25.97	39.54	58.82	93.74	115.14	19.32	34.13	58.03	86.02	121.61
21	26.27	39.94	59.33	94.44	115.95	19.63	34.56	58.59	86.68	122.36
22	26.57	40.34	59.86	95.15	116.76	19.95	34.99	59.15	87.35	123.12

As shown, all concentrations are expressed in **nanomoles per litre (nM)**.

24. The sample size (N) for each age group is not provided. If the sample size is limited, it could be more appropriate to group participants into broader age categories (e.g., 5-year intervals).

The sample size for each decade of age is provided in Supplementary Table 8 ("Participant characteristics by sex and decade").

To model concentration percentiles, we used *generalised additive models for location, scale, and shape* (GAMLSS, R package version 5.4-12), which are particularly well suited for computing continuous reference values (even though we do not refer to reference values in our case) (27-29). GAMLSS enables simultaneous modelling of the mean (location), variability (scale), and shape (e.g., skewness, kurtosis) of the data (27-29). This approach produces smooth centile curves that describe how concentrations change across the lifespan, providing bidirectional interpretation (i.e., determining which percentile a measurement for a given age falls into and vice versa).

Age was modelled as a continuous variable across the entire sample (n = 522), without subdivision into discrete categories. Therefore, the total sample size, not per-age group counts, is the relevant determinant of model stability.

Importantly, major reference values have been developed using GAMLSS, including those from the Global Lung Function Initiative (30, 31), some of which relied on smaller sample sizes than ours (32, 33). Our group has also successfully applied the same methodology to generate reference values for cardiopulmonary exercise testing and muscular strength using the COmPLETE-Health cohort, which were well accepted in the sports science and medicine community (20, 21).

Supplementary Figures 1S to S26: The example chromatograms shown in the figures suggest a suboptimal performance of the method

25. Peak intensity in some of the mass transitions is comparable to noise (e.g. Fig 7)

This corresponds to a low-intensity peak, for which the analyte's presence was confirmed by a qualifier ion. However, due to low signal intensity, the quantifier-to-qualifier ion ratio fell outside the predefined acceptance criteria relative to the pure standard, resulting in increased measurement uncertainty. For this reason, we have removed this sphingolipid species (Cer d18:1/18:1) from the reported results.

We hope this clarification satisfactorily addresses your concern.

26. Presence of split peaks in some of the mass transitions (e.g. Figs 9, 22)

We decided to remove the sphingolipid species Cer d18:1/22:0 from the report because an incorrect transition was inadvertently applied during automated quantification (622.6 → 604.6 instead of 622.6 → 264.3), resulting in split peaks and ambiguous signal interpretation. In addition, the closely eluting dihydroceramide Cer d18:0/22:0 was also removed, as it cannot be reliably distinguished from DeoxyCer m18:1/22:0 due to insufficient chromatographic resolution on the C8 column.

We hope this clarification satisfactorily addresses your concern.

27. Different peak width (Figs 9, 13, 14, 21, 22, 25) in two mas transitions corresponding to the same analyte (suggests presence of interferences).

For the sphingolipid species presented in Figures 9, 14, 21, 22, and 25, we agree that potential interferences cannot be entirely excluded. However, for these species, the quantifier-to-qualifier ion ratios remained within the predefined acceptance criteria, supporting that the predominant signal originates from the targeted analyte and that quantification is reliable.

In contrast, the species shown in Figure 13 (Cer d18:2/24:0) was removed from the reported results. In this case, the quantifier-to-qualifier ratio could not be reliably evaluated because the qualifier ion signal was below the limit of detection. As a result, the specificity of the annotation could not be ensured, and this species was therefore excluded from the final dataset.

We hope this clarification satisfactorily addresses your concern.

28. Different retention times in different mass transitions corresponding to the same analyte (Figs 12, 20, 22).

The sphingolipid species shown in Figure 12 (Cer d18:2/24:1) was removed from the reported results due to inconsistent retention times between the corresponding mass transitions, preventing confident confirmation of annotation specificity.

In contrast, for the species shown in Figures 20 and 22, the quantifier-to-qualifier ion ratios met the predefined acceptance criteria, and both precision and accuracy were within acceptable limits. Although minor interferences cannot be entirely excluded, these species were therefore retained and annotated with high confidence.

29. Considering that these performance issues were observed in calibrators prepared in methanol, it is likely that the methods' performance during analysis of the study samples was also suboptimal. Based on the above, the accuracy of the reported quantitative results is questionable. This limitation should be acknowledged in the manuscript to ensure transparency and to inform readers of the potential impact on data interpretation.

We agree that analytical performance issues observed at the level of individual transitions warrant careful consideration. In response to these concerns, and as detailed above, we adopted a conservative approach and removed five sphingolipid species for which annotation specificity or chromatographic performance could not be confirmed with high confidence, thereby limiting the reported results to species meeting predefined acceptance criteria for retention time consistency, quantifier-to-qualifier ion ratios, and signal quality.

Importantly, extracted ion chromatograms were evaluated for both calibrators and participant samples, allowing matrix-dependent effects to be assessed directly. No conclusions were drawn from calibrator data alone. The remaining quantitative results are therefore based exclusively on sphingolipid species that demonstrated robust analytical performance across both calibration standards and study samples.

These considerations, together with the resulting limitations on absolute quantification for selected species, are now explicitly acknowledged in the Limitations section of the manuscript to ensure transparency and to inform interpretation of the findings. In addition, we have clarified this conservative reporting strategy in Section 2.4 (Sphingolipid analysis, see page 11, lines 238-240), where we now explicitly state that only sphingolipid species fulfilling predefined qualitative and quantitative criteria in both calibration standards and plasma samples were retained for analysis.

The data analysis process

30. For transparency, the manuscript should list all the details of the data analysis, including the qualitative criteria which were used to accept or reject the results.

We have now explicitly expanded Section 2.4, Sphingolipid analysis (see pages 9-10), to include a detailed description of the data processing workflow and qualitative acceptance criteria. This includes the predefined criteria used to retain or exclude sphingolipid species from downstream analyses: retention-time consistency across transitions, acceptable quantifier-to-qualifier ion ratios (±30% relative to pure standards), sufficient signal intensity above background, and confirmation of these criteria in both calibration standards and plasma samples.

31. In response to the comment from the original review regarding the data analysis process, the authors stated: “All observed interferences in Cal0 (across 11 of the 26 measured species)”, suggesting that calibrators prepared in methanol contained impurities which produced peaks in the mass transitions of the targeted lipids. Considering much greater complexity of the human plasma samples as compared to the methanolic standards, this above suggests that interference in mass transitions was likely a frequent occurrence in during the analysis of plasma samples, raising concerns about accuracy of the concentrations measured in the study samples.

We agree that the presence of background signals and potential interferences warrants careful consideration, particularly when analysing complex biological matrices such as human plasma. In lipidomics, low-level background signals can arise from multiple sources (e.g. solvents, plastics, tubing, or reagents), which is why blank subtraction was performed systematically and conservatively in the present study.

Importantly, analytical performance was not evaluated solely on the basis of calibrators. Extracted ion chromatograms were examined in both calibration standards and participant samples, and species annotation and quantification were accepted only when predefined qualitative criteria were met, including retention time consistency, quantifier-to-qualifier ion ratios, and signal quality. These criteria were further supported by the use of stable-isotope-labelled internal standards and by NIST SRM 1950 plasma reference material as an external quality control.

As detailed above, sphingolipid species for which annotation specificity or chromatographic performance could not be confirmed with sufficient confidence were conservatively excluded from the reported results. The remaining quantitative data, therefore, reflect species that demonstrated robust analytical performance in both standards and plasma samples.

Finally, we emphasise that the present work represents a translational research study rather than an assay intended for immediate routine clinical implementation. Beyond describing age- and sex-related patterns in sphingolipid concentrations, the study aims to characterise baseline biological variability in an apparently healthy population and to identify current analytical and standardisation gaps, particularly the limited availability of certified reference materials, that must be addressed before sphingolipid profiling can be reliably implemented in clinical routine.