Brief Communication

MOLECULAR METABOLISM

Non-alcoholic fatty liver disease is characterised by a reduced polyunsaturated fatty acid transport via free fatty acids and high-density lipoproteins (HDL)

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ABSTRACT

Background and objectives: Non-alcoholic fatty liver disease (NAFLD) develops due to impaired hepatic lipid fluxes and is a risk factor for chronic liver disease and atherosclerosis. Lipidomic studies consistently reported characteristic hepatic/VLDL "lipid signatures" in NAFLD; whole plasma traits are more debated. Surprisingly, the HDL lipid composition by mass spectrometry has not been characterised across the NAFLD spectrum, despite HDL being a possible source of hepatic lipids delivered from peripheral tissues alongside free fatty acids (FFA). This study characterises the HDL lipidomic signature in NAFLD, and its correlation with metabolic and liver disease markers.

Methods: We used liquid chromatography-mass spectrometry to determine the whole serum and HDL lipidomic profile in 89 biopsy-proven NAFLD patients and 20 sex and age-matched controls.

Results: In the whole serum of NAFLD versus controls, we report a depletion in polyunsaturated (PUFA) phospholipids (PL) and FFA; with PUFA PL being also lower in HDL, and negatively correlated with BMI, insulin resistance, triglycerides, and hepatocyte ballooning. In the HDL of the NAFLD group we also describe higher saturated ceramides, which positively correlate with insulin resistance and transaminases.

Conclusion: NAFLD features lower serum lipid species containing polyunsaturated fatty acids; the most affected lipid fractions are FFA and (HDL) phospholipids; our data suggest a possible defect in the transfer of PUFA from peripheral tissues to the liver in NAFLD. Mechanistic studies are required to explore the biological implications of our findings addressing if HDL composition can influence liver metabolism and damage, thus contributing to NAFLD pathophysiology.

Keywords Non-alcoholic fatty liver disease (NAFLD); Obesity; Lipoprotein metabolism; Lipidomics; LC-MS

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) develops due to an impairment in systemic/hepatic metabolism leading to hepatic lipid accumulation and, eventually lipotoxicity [1]. From a histological perspective, NAFLD is a continuum of presentations ranging from "simple" steatosis (NAFL; intrahepatic fat deposition in more than 5% of hepatocytes) to steatohepatitis (NASH; steatosis in the presence of inflammation and ballooning), fibrosis, and cirrhosis, which can ultimately evolve to hepatocellular carcinoma (HCC) [2].

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NAFLD has reached pandemic proportions with a global prevalence of 24% in the general population, thus being a public health priority [3]. NAFLD is strongly associated with features of the Metabolic Syndrome (MetS), including obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM), mixed dyslipidaemia [low high-density lipoprotein cholesterol (HDL-C) and increased very low-density lipoprotein triglycerides (VLDL-TG)], which partly explain the elevated cardiovascular disease (CVD) risk [4]. The latter represents the leading cause of death in these patients, and recent studies suggest that NALFD could be an independent risk factor for atherosclerosis and CVD outcomes [5]. However, the mechanisms linking both conditions are still debated [4]. Over the last decade, lipidomics has helped to understand the NAFLD pathophysiology, pointing to specific changes in lipid metabolism that can serve as candidate biomarkers [6]. Specifically, lipidomic studies of liver tissues have reported a lipid imbalance alongside the NAFLD spectrum characterised by elevated levels of saturated fatty acids (SFA) and reduced levels of phospholipids (PL) and polyunsaturated fatty acids (PUFA) [7]. Major players expected to contribute to these lipidomic changes are: 1) adipose tissue (AT) [the foremost source of fatty acids (FA) to the liver]; 2) hepatic *de novo* lipogenesis (DNL), enhanced by hyperinsulinaemia and increased (refined) carbohydrate intake; 3) dietary fat intake [1,8,9]. Over the years, studies have described differences in whole serum/plasma lipids, mainly focusing on TG fatty acid remodelling alongside circulating candidate biomarkers. The latter has been prompted by the risks and costs associated with liver biopsy procedures. Compared to healthy controls, NAFLD patients displayed an increased level of SFA and monounsaturated fatty acids (MUFA) and reduced PUFA within the TG fraction of the whole plasma [10–13], partly explained by the enhanced DNL [14]. However, apart from these findings focusing on TG, whole plasma lipidomic studies have reported conflicting results regarding the abundance and composition of other lipid classes [13,15–17]. Several factors might contribute to the discrepancies observed in the circulating lipidome of NAFLD patients: 1) differences in inclusion criteria among studies (such as sex, dyslipidaemia, ethnicity, and dietary habits); 2) while TG composition reflects more direct changes in liver metabolism (being enriched in VLDL) and TG/VLDL metabolism is particularly stressed under the metabolic pressure of obesity/MetS/NAFLD [1], other lipids are abundant in multiple lipoprotein fractions including low-density lipoproteins (LDL) and HDL, thus rendering the biological interpretation of results generated in a complex matrix, such as whole plasma, more challenging.

The use of isolated lipoprotein fractions (lipoprotein lipidomics) can provide a higher granularity than whole plasma lipidomics, allowing a more detailed study of biological processes such as lipoprotein remodelling and organ-to-organ exchange/crosstalk.

This study aims to investigate the quantitative and qualitative lipidomic differences in patients across the NAFLD spectrum. Given the association of NAFLD with cardiovascular risk (CVR) and considering the mounting evidence associating HDL reduction with (central) obesity/IR/NAFLD [18–20], we posited that HDL may play a role in NAFLD acting as a possible source of hepatic lipids delivered from peripheral tissues to the liver and that, together with FFA, differences in HDL composition might be associated to NAFLD development and/or progression. With this proposition, we studied the lipidome of the whole serum and HDL (obtained through fast protein liquid chromatography) by liquid chromatography coupled with mass spectrometry in healthy and biopsy-confirmed NAFLD participants.

2. METHODS

2.1. Ethics and the BioNASH study cohort

Eighty-nine patients with biopsy-proven NAFLD (patients with alternate diagnoses, aetiologies, and kidney dysfunction were excluded) and 20 healthy volunteers were involved in this study. Patients were recruited by the NASH Service at Cambridge University Hospitals NHS Foundation Trust, whereas healthy volunteers were recruited either by the NIHR Cambridge BioResource (http://www.cambridgebioresource.org.uk) or by the NHS Blood and Transplant Unit, Cambridge, UK. Participant enrolment was approved by NHS Research Ethics Committees (REC 06/Q0106/70; 12/EE/0040; 17/EE/0389). Study protocols followed the principles of the Declaration of Helsinki, and all participants gave written informed consent.

Liver biopsies were scored by an experienced liver pathologist for steatosis (0–3), ballooning (0–2), inflammation (0–2), and fibrosis (0–4) and were classified according to the Kleiner score [21] and classified into NAFL and NASH following the same algorithm proposed by Bedossa and colleagues [22]. In healthy controls, where liver biopsy was not clinically indicated, they were selected on the basis of the predicted absence of NAFLD according to the non-invasive score proposed by Kotronen et al. [based on: presence/absence of metabolic syndrome, T2DM, and levels of insulin, aspartate aminotransferase (AST) and AST/alanine aminotransferase (ALT)] [23].

Sample collection and processing for serum and lipoprotein lipidomics have been previously described [20] and are here reported in supplementary materials.

2.2. Statistical analyses

Data are shown as mean $\pm$ standard deviation unless otherwise specified. Normality was visually assessed from plots of the data (skewness/kurtosis) obtained with the lm function in R, and logarithmic transformations were applied to non-normally distributed data. Comparisons of clinical data between healthy and NAFLD patients were assessed using three-way and two-way ANOVA controlling for sex and the presence of T2DM, followed by the Tukey HSD post hoc test to estimate the statistical significance among groups. Regarding categorical variables, a chi-square test was adopted. Whole serum lipidomic data were analysed using three-way ANOVA controlling for sex, presence of T2DM and interaction between sex, T2DM and disease state, followed by the Tukey HSD post hoc test to estimate the statistical significance among groups. A p-value <0.05 was considered significant. However, when lipids were investigated as independent hits, multiple testing correction [Benjamini-Hochberg procedure to control the False Discovery Rate (FDR)] was applied as specified in the legend to tables. Lipoprotein lipidomics data, where participants were only males, were analysed using two-way ANOVA controlling for the presence of T2DM and the interaction between disease state and T2DM, followed by the Tukey HSD post hoc test to estimate the statistical significance among groups. A p-value <0.05 was considered significant. As with whole serum, when lipids were considered as an independent unit, FDR was reported along with the raw p-value. To assess the power of this study, whole serum and lipoprotein lipidomics, we performed a Post Hoc Power Analysis with G*power software. Variables with an effect size (f) below 0.3 (whole serum) and 0.5 (lipoproteins) fell below an acceptable power level of 0.7, therefore being potentially exposed to type 2 error. All the significant variables had an optimal

power (>0.8). Univariate correlations were carried out using the Pearson Correlation Coefficient. Statistical analysis and graphs were performed with R version 4.2.1.

2.3. Other experimental procedures

Detailed experimental procedures are described in the supplementary files.

3. RESULTS

3.1. Clinical characteristics and whole serum lipidomic profile of healthy and NAFLD patients

This study involved 109 participants, including 20 healthy volunteers (age and sex-matched), 36 NAFL, and 53 NASH (Supplementary Table 1). The patients in the NAFLD spectrum displayed significantly higher BMI, insulin resistance (as assessed by the Homeostasis Model Assessment 2 of Insulin Resistance HOMA2-IR), and mixed dyslipidaemia (higher TG and lower HDL-C), along with increased liver enzymes (ALT, AST), while LDL-C and total cholesterol were not significantly different across the groups (Supplementary Table 1). NASH patients displayed a worse metabolic profile (significantly higher glucose, insulin, HOMA2-IR, AST) compared to NAFL, despite similar BMI (Supplementary Table 1).

Lipids were analysed as total lipid class (sum of each lipid measured), and according to their acyl chain saturation levels (saturated, monounsaturated and polyunsaturated), as fatty acids saturation level imbalances have been described in the livers of NAFLD patients and are involved in the pathophysiology of NAFLD.

The whole serum lipidomic analysis revealed a quantitative and qualitative depletion of several phospholipid classes [phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), and sphingomyelins (SM)] in the NAFLD (both NAFL and NASH) groups as compared to the controls (Figure 1 A-G). The lower levels of total PC and PE were mainly driven by a depletion in their PUFA component, while PG were markedly lower irrespectively of their acyl chain saturation levels (Figure 1B,D,F). Lower SM levels in NAFLD groups were attributable to a reduction in MUFA and PUFA components (Figure 1H). Furthermore, we observed a significant sex effect on most of the PL measured (Figure 1), in line with known differences in the lipoprotein metabolism among sexes [24,25]. While between NAFLD and controls total free fatty acids (FFA) were not significantly different (Figure 1I), NAFLD patients showed markedly higher saturated FFA, coupled with a depletion in polyunsaturated FFA (Figure 1J). Similar results were found in the triglyceride fraction (Supplementary Fig. 1a,b), likely reflecting the known activation of DNL programs previously described in NAFLD [11,14,26,27]. Within the lysophosphatidylcholines (LPC), the only

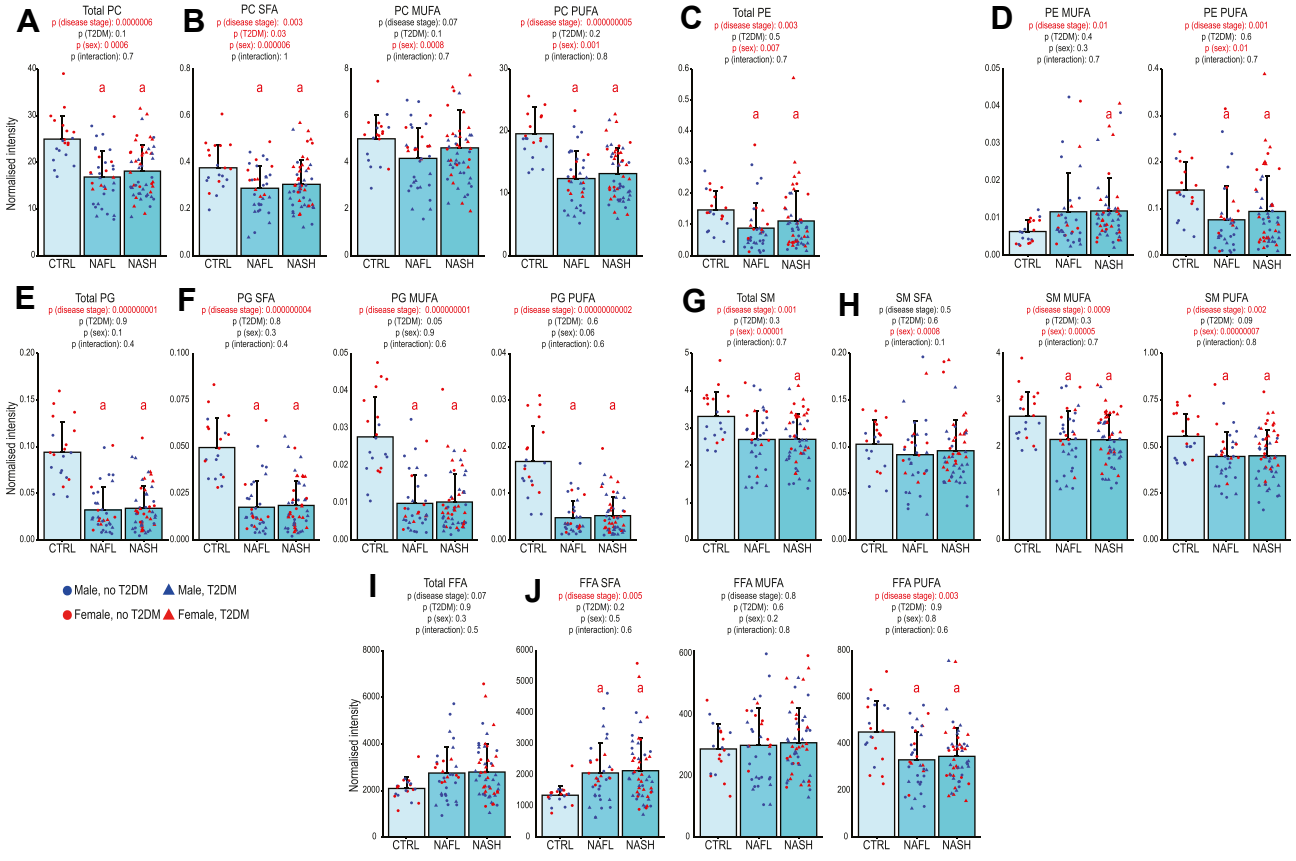

Figure 1: Whole serum levels of major lipid classes in healthy volunteers and NAFLD patients. (A,B) PC, (C,D) PE, (E,F) PG, (G,H) SM were lower across the NAFLD spectrum compared to controls. (I,J) FFA were higher in SFA and lower in PUFA in NAFLD as compared to controls. All lipid species were analysed by LC-MS. Statistical significance was assessed using three-way ANOVA controlling for sex, presence of type 2 diabetes mellitus (T2DM) and interaction between sex, T2DM and disease state, with a p-value <0.05 considered significant. Tukey HSD post hoc test was used to estimate the statistical significance among groups. Lowercase red letters indicate post hoc analysis significance: "a" means different from controls "CTRL", and "b" means different from NAFL. Data are represented as mean ± standard deviation; expression data of participants are represented as dot plots. In Supplementary Table 2 are reported all the specific lipid species analysed. Abbreviations: PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; SM, sphingomyelins; FFA, free fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

significant difference observed was in the saturated fraction, which was higher in NAFLD than controls (Supplementary Fig. 1c,d). In contrast, within the lysophosphatidylethanolamines (LPE), only the MUFA content was significantly lower in NAFL compared to controls and NASH (Supplementary Fig. 1e,f). Ceramides (Cer) did not show significant differences between NAFLD and controls (Supplementary Fig. 1g,h) but were characterised by a significant sex effect, as reported in the literature [28–30].

Lastly, within the NASH group, we clustered the patients against fibrosis (Supplementary Table 3). Apart from a mild increase of LPE MUFA, and a trend in lower SFA/MUFA-TG and FFA in NASH F3-4 *vs.* NASH F0-2, no major changes were observed in the lipid classes when clustering the patients against fibrosis (Supplementary Table 4). This trend to deflection in SFA/MUFA-TG (including TG enriched in DNL products) in end-stage NASH F3-4, was previously described by others and explained by liver dysfunction [7]; in our cohort, it also reflects similar trends in BMI, Insulin/HOMA-IR and TG (Supplementary Table 3) that could be associated to a deflection in sterol regulatory element-binding protein 1 (SREBP-1) activation, as we previously described (by next generation sequencing in a partially overlapping cohort) [26].

To better understand the extent to which whole serum lipidomic data related to metabolic impairment, we correlated the significantly different lipids from Figure 1 with critical clinical data (Figure 2). Pearson correlation analysis showed that PG (total, SFA, MUFA, PUFA) had the strongest inverse correlation with obesity and insulin resistance, whereas SFA from FFA were positively correlated with HOMA2-IR (Figure 2). Moreover, most of the lipids significantly reduced in the NAFLD groups were also positively correlated with HDL-C (Figure 2). No significant correlation between whole serum lipids and liver histology were found (data not shown), aligning with previous studies showing small/no differences across the NAFLD spectrum [17,31]. Taken together, these data show that in NAFLD, the whole serum lipidome is depleted of specific PL, and their PUFA content drove these changes, suggesting a close relationship between the circulating lipidome, IR and HDL metabolism.

3.2. HDL lipidomics of NAFLD patients suggest a reduced reverse PUFA transport from peripheral tissues to the liver

Based on the strong correlations between PL/PUFA-containing lipids and HDL-C, we posited an implication of HDL particles in the observed whole serum differences; specifically, we investigated if the lipidomic differences we observed in the whole serum were due to the sole reduction of HDL concentration or also driven by an impairment of HDL composition.

To address this question, we isolated HDL by fast protein liquid chromatography (FPLC) and studied its lipidome in a sub-cohort of 40 age-matched male subjects (9 healthy, 11 NAFL, and 20 NASH; Supplementary Table 5). We focused on male participants to reduce the variability attributable to sex differences in HDL metabolism.

Compared to controls, and in agreement with whole serum lipidomics, the HDL lipidomic profile of NAFLD patients was characterised by lower PC levels, mainly driven by their PUFA content, with NASH being most

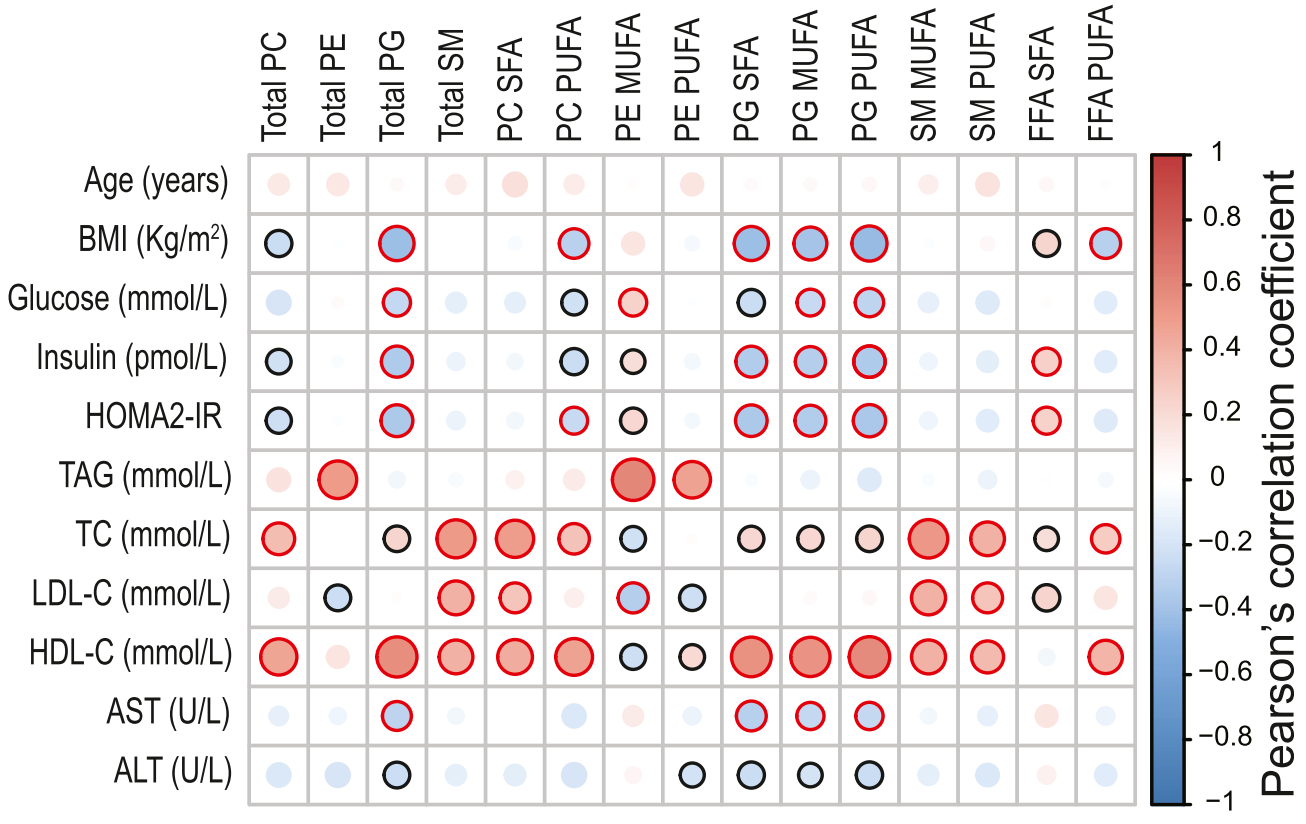

Figure 2: Correlations between significant whole-serum lipid species and clinical data. Heatmap representing a correlation matrix among significantly different whole serum lipid species and clinical data in healthy volunteers and NAFLD patients: colour represents the Pearson correlation coefficient (red: positive; blue: negative), and the size of the circle represents significance (black bold borders highlight correlations with $p < 0.05$; red bold borders highlight correlations with $p < 0.01$). Abbreviations: PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; SM, sphingomyelins; FFA, free fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; BMI, body mass index; HOMA2-IR, Homeostasis Model Assessment 2 of Insulin Resistance; TAG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase.

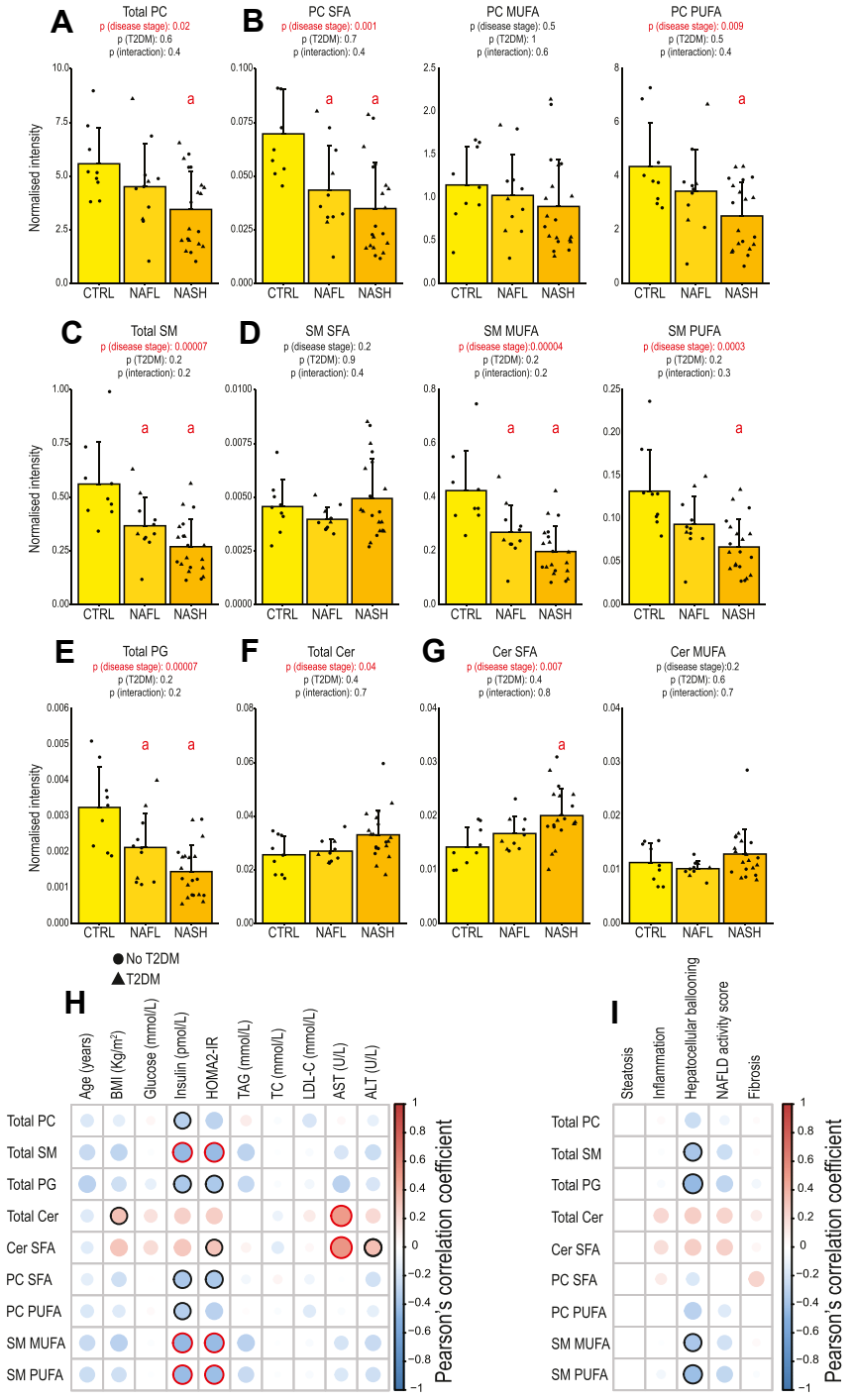

Figure 3: HDL levels of major lipid classes in healthy volunteers and NAFLD patients, and their correlation with clinical and liver histological data. (A,B) PC were lower in NAFLD as compared to controls. (C–-E) SM and PG were lower across the NAFLD spectrum as compared to controls. (F,G) Total and SFA Cer were higher in NASH compared to controls. Statistical significance was assessed using two-way ANOVA controlling for presence of type 2 diabetes mellitus (T2DM) and interaction between T2DM and disease state, with a p-value <0.05 considered significant. Tukey HSD post hoc test was used to estimate the statistical significance among groups. Lowercase red letters indicate post hoc analysis significance: "a" means different from controls "CTRL", and "b" means different from NAFL. Data are represented as mean $\pm$ standard deviation; expression data of participants are represented as dot plots. In Supplementary Table 6 are reported all the specific lipid species analysed within the HDL fraction. (H) Heatmap representing a correlation matrix among significantly different HDL lipid species and clinical data in healthy volunteers and NAFLD patients: colour represents the Pearson correlation coefficient (red: positive; blue: negative), and the size of the circle represents significance (black bold borders highlight correlations with $p < 0.05$; red bold borders highlight correlations with $p < 0.01$). (I) Heatmap representing a correlation matrix among significantly different HDL lipid species and liver histological data in NAFLD patients ($n = 31$): colour represents the Pearson correlation coefficient (red: positive; blue: negative), and the size of the circle represents significance (black bold borders highlight correlations with $p < 0.05$; red bold borders highlight correlations with $p < 0.01$). All lipid species were analysed by LC-MS and normalised to its internal standard (IS) as with whole serum (list of IS used reported in the method section), in addition to the ApoA-I concentration (Supplementary Fig. 2). Abbreviations: PC, phosphatidylcholines; SM, sphingomyelins; PG, phosphatidylglycerols; Cer, ceramides; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

significantly affected (Figure 3A,B). This observation confirmed our hypothesis that PC differences in whole serum lipidomics tightly reflect HDL abundance and composition. Indeed, reduced PC (and especially their PUFA content) have been reported in peripheral tissues (including AT and macrophages) of obese patients with IR [32—36]; these tissues are involved in HDL metabolism.

Furthermore, compared to controls, the NAFLD group showed lower absolute levels of total SM (mostly MUFA and PUFA) and total PG (Figure 3C-E). Low HDL SM concentration has been associated with a decreased HDL cholesterol efflux capacity [37]; this has also been suggested for PG, even though this lipid class represents a minor component of the HDL lipidome [38] (in our data, with few exceptions, most PG were undetectable). We also observed higher HDL total Cer in NASH patients, mainly driven by their SFA component (Figure 3F,G). No significant differences were observed in LPC, TG and PE among the groups (Supplementary Fig. 3). When clustering NASH patients against fibrosis (Supplementary Table 7), no major differences were observed in the HDL lipid classes, with the exception of a significant decline in HDL TG SFA concentration (Supplementary Table 8).

To further assess the extent to which the HDL lipidome related to metabolic parameters and liver histology, we correlated the significantly different lipids with critical clinical and histological data (Figure 3H,I). Pearson correlation analysis showed that the lipid species significantly reduced in the NAFLD group were negatively correlated with insulin resistance and, within these species, (Total/MUFA/PUFA) SM had the strongest correlation. On the other hand, we found the positive correlation of total and SFA Cer with insulin resistance and liver enzymes particularly intriguing, with AST showing the strongest correlation (Figure 3H). We also observed a significant negative correlation between hepatocyte ballooning and total PG, Total/MUFA/PUFA SM (Figure 3I). As already described for the whole serum lipidome, none of HDL lipid classes correlated with fibrosis.

We then focussed on the specific HDL PUFA-containing species where we found a generalised depletion in NASH of PUFA-PL (Figure 4A) including PC containing odd-chain fatty acids (such as PC 37:5, PC 37:6, 35:2, 35:3). Moreover, most of these PUFA-containing lipid species negatively correlated with insulinemia, HOMA2-IR (Figure 4B) and hepatocyte ballooning (Figure 4C).

In summary, these data show that, independently from the lower HDL-C concentration, HDL is characterised by PUFA PL depletion and enrichment of SFA Cer levels in NAFLD that might be intimately linked to IR, and associated to liver damage. The fact that the liver reuses HDL-derived PL, either incorporating them into membranes or converting them into different lipid classes, has been previously suggested with tracer experiments in preclinical models [39]. Our results, despite being observational, show for the first time that the HDL lipidome of NAFLD patients carries lipidomic signatures similar to those previously described in both peripheral tissues and liver of patients with MetS/NAFLD [7,32—36], therefore potentially implicating HDL as a contributor to NAFLD development and progression in IR states.

4. DISCUSSION

NAFLD is characterised by an imbalance in hepatic lipid fluxes [1], with lipoproteins (alongside FFA) being deeply involved in these processes. Apart from the characteristic elevated hepatic fat content (mainly TG), it is increasingly recognised that other lipid species, such as phospholipids and sphingolipids, are involved in the onset and progression of this condition [40]. Indeed, some lipid species are referred to as "lipotoxic species" because of their capability to induce cell toxicity.

While reports regarding characteristic hepatic lipidomic signatures of NAFLD are coherent (including higher SFA across different lipid species, oxidised lipids, ceramides or lower PUFA/PL), whole serum lipidomic studies have produced conflicting results, also sometimes discordant with hepatic findings [41]. Discordances have also been reported among major plasma lipidomic classes: for example, PL have been described higher (PC/SM [16]; PC/PE/PG [42]), and/or lower (PC/LPC/SM [13]; LPC [43]) in NAFLD compared to controls. This might be partly due to the recruiting criteria (e.g., differences in clinical parameters such as HDL-C, TG, and BMI between diseased and controls across studies) and diet, potentially affecting the circulating lipidome. Moreover, the lipidomic profiling of the whole serum/plasma provides averaged information on lipoproteins concentration and composition varying according to disease state, dietary habits, sex and many other factors [44,45]. Investigating the specific lipoprotein lipidomic profile provides a more accessible matrix to compare among studies (e.g., lipoproteins are normalised to their protein/apolipoprotein content) and give a more biologically relevant interpretation (e.g., VLDL as a more direct proxy of liver output). Understandably, mass-spectrometry-based lipoprotein lipidomics of NAFLD has mainly focused on VLDL [46—48], with limited information regarding the other lipoprotein fractions.

In this study, we first started describing NAFLD's whole serum lipidomic signature compared to healthy controls. We showed that NAFLD is characterised by a depletion in PL (PC, PE, PG, SM) specifically driven by their PUFA fraction. Moreover, NAFLD patients had higher saturated and lower polyunsaturated FFA. Circulating FFA are released by the AT, and elevated levels of FFA have been attributed to enhanced lipolysis due to AT-IR [1,9]. Elevated FFA play a major role in the onset and development of NAFLD [49,50]. The higher saturated FFA in NAFLD *vs.* controls that we reported is aligned with previous whole serum studies [51—53] as well as with the acyl chain saturation profile of the whole hepatic lipidome [17,54,55].

We also observed lower levels of PUFA in FFA and PL in whole serum of NAFLD patients (compared to controls); these results find confirmation in previous reports [40] (although polyunsaturated FFA depletion is a debated finding [51—53]). Preclinical studies have provided mechanistic insights as to how PUFA (especially essential fatty acids, EFA) deficiency promotes hepatic steatosis. EFA can negatively modulate the DNL machinery toward the negative modulation of the Liver X Receptor (LXR), of SREBP-1 and/or of the carbohydrate response element binding protein (ChREBP) [56—58]. Also, PUFA can activate the peroxisome proliferator—activated receptor-α (PPARα) promoting fatty acid oxidation [59]. Despite the solid evidence of the role of PUFA in modulating hepatic lipid metabolism, it should be said that omega-3 supplementation has yielded unsatisfactory results in NAFLD trials [60]. Our data do not clearly point to EFA deficiency but rather to a global PUFA depletion: FFA composition, a robust predictor of adipose tissue fatty acid composition (that, in turn is considered the gold standard for the representation of long term dietary fatty acids storage due to the slow turnover time [61—63]) showed a depletion in linoleic acid (FFA 18:2) that is not confirmed by other EFA and their derivatives. Reduced PUFA PL in NAFLD could therefore be also attributable to enhanced utilisation (catabolism or conversion to second messengers such as eicosanoids) [64,65].

Since 1) NAFLD liver biopsies show depletion in PC and PUFA in multiple lipid classes [40]; 2) HDL are the primary carriers of circulating PC; 3) total and PUFA PC were strongly correlated with HDL-C; we sought to better understand the lipid composition of isolated HDL to investigate whether these results were due to HDL concentration or

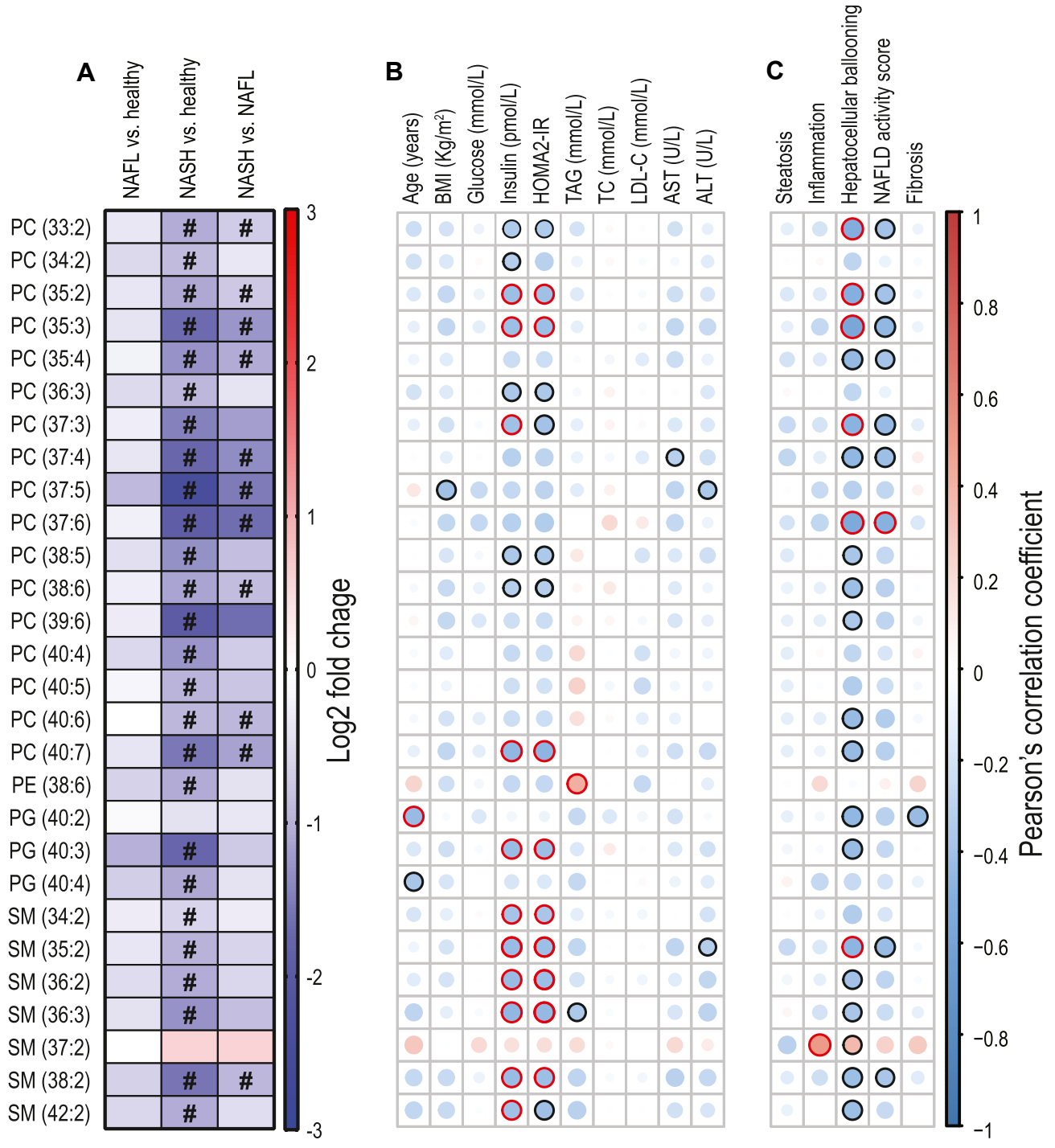

Figure 4: Significantly lower PUFA-containing phospholipids within HDL, and their correlations with clinical and liver histological data. (A) Log2 fold change among the significantly different HDL PUFA in healthy volunteers (CTRL) versus NAFLD patients, with "#" indicating a Tukey HSD post hoc significant difference $p < 0.05$ (details in Supplementary Table 6). (B) Heatmap representing a correlation matrix among significantly different HDL PUFA species and clinical data in healthy volunteers and NAFLD patients: colour represents the Pearson correlation coefficient (red: positive; blue: negative), and the size of the circle represents significance (black bold borders highlight correlations with $p < 0.05$; red bold borders highlight correlations with $p < 0.01$). (C) Heatmap representing a correlation matrix among significantly different HDL PUFA species and liver histological data in NAFLD patients ($n = 31$): colour represents the Pearson correlation coefficient (red: positive; blue: negative), and the size of the circle represents significance (black bold borders highlight correlations with $p < 0.05$; red bold borders highlight correlations with $p < 0.01$). Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SM, sphingomyelin; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; BMI, body mass index; HOMA2-IR, Homeostasis Model Assessment 2 of Insulin Resistance; TAG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase.

composition. The HDL lipidome confirmed the reduction of PC in NAFLD, being once again driven by their PUFA component and strongly negatively correlated with IR. The effects of changes in HDL PL content has been studied in the context of HDL physical properties and reverse cholesterol transport: in preclinical experiments, lower PC levels in reconstituted HDL and HDL-mimicking micelles have been associated with reduced cholesterol efflux capacity (CEC), thus rendering these particles potentially less athero-protective [66,67]. This might go along with some reports suggesting a lower CEC in NAFLD compared to healthy controls (although this matter is also debated) [68–70]: the lower PC we observed in HDL of NAFLD patients could justify, at least in part, the lower CEC described in NAFLD. Intriguingly, HDL PUFA PL were mainly depleted in NASH and negatively correlated with hepatocyte ballooning: despite this finding will require future mechanistic studies to explain the association, we are tempted to speculate that differences in HDL PL composition might contribute to hepatocyte damage. It is worth considering that HDL (together with FFA, diet, DNL [1]) might be contributing as input to the hepatic fat pool, promoting a "reverse (phospho)lipid transport" since: 1) PL constitute nearly 50% of total lipids within HDL [45]; 2) tracer studies in mice suggest that about 50% of hepatic PC is derived from the circulation (being HDL the main carriers) [39]; 3) HDL PL can be used to build up membranes and/or disassembled to use or esterify FA (into TG) [39]. The fact that our HDL lipidomics results are aligned with hepatic lipidomic signatures described in NAFLD [41] might support the hypothesis that HDL PL composition might have a direct impact on hepatocyte (dys)-function. However, the understanding of HDL fate in hepatocytes is currently at its infancy. While mechanisms are established with regards to HDL-C uptake (with the scavenger receptor class B type I (SR-BI) being a key player in RCT) [71], less studied is the uptake of HDL lipids: it has been suggested that SR-BI might also contribute to HDL-PC uptake but other mechanisms (e.g., particle endocytosis, hydrolysis of PC by phospholipases, and other unknown pathways) seem to be also at play [39,72].

Here we also report strong correlations between HDL PUFA composition and IR that merits further investigation in light of the tight association between adipose tissue dysfunction, IR, and HDL metabolism and function [20,73,74]. However, we cannot rule out the hypothesis that depletion of PUFA in the liver would influence AT via VLDL, and then fire back to the liver (via FFA and HDL-PL) in a vicious cycle [9]. Moreover, the central role of the liver in the HDL biogenesis (including its participation into HDL lipidation), renders even more difficult to disentangle in this setting the contribution of periphery *vs.* liver in the final HDL lipid composition.

We also reported a depletion of odd-chain PC-FA within the HDL: these fatty acids have been previously associated with a reduced incidence of T2DM [75,76]. Not surprisingly, the lower HDL odd-chain fatty acids observed in NAFLD finds confirmation in an independent cohort of patients with metabolic syndrome that we previously reported [20]. Whether HDL odd chain FA exert any metabolic effect is to be established and so is their derivation as dietary sources [77], gut microbiota [78], alpha-oxidation [79], and mitochondrial catabolism of BCAA [80] can potentially contribute to the pool of these lipids.

Lastly, our data show that HDL Cer were higher in NASH compared to controls and that their abundance in HDL correlated with hepatic necro-inflammatory markers. Some studies described increased hepatic Cer in NAFLD livers, and its association with lipotoxic damage [17,81]. The liver is considered a major Cer synthesis site through different pathways (*de novo*, salvage and sphingomyelin hydrolysis) and of its export to circulation [82]; however Cer can be produced in other organs and in circulation (by sphingomyelin hydrolysis) [83]. Our findings can either reflect hepatic Cer concentrations (since HDL can receive Cer from VLDL/LDL due to the activity of lipoprotein lipid transport enzymes [84]) or, vice-versa, suggest that HDL influence hepatic Cer pool. These findings, although intriguing, need further mechanistic exploration.

This study is subject to different limitations. First, the study's cross-sectional nature provides a correlation between lipids and disease states. Thus, causation cannot be drawn. Second, the contribution of HDL lipids to its functionality alongside the liver lipid pool is only speculative and requires adequate investigation. Third, dietary habits were not recorded thus the impact of diet on our findings cannot be inferred. Fourth, HDL lipidomics was performed only on males. Last, patients were not profiled for the major genetic risk alleles for NAFLD, but the marked IR state observed favours more the metabolic rather than genetic nature of NAFLD.

In conclusion, by using mass-spectrometry-based lipidomics weshow that NAFLD is characterised by substantial changes in HDL PL composition and impaired reverse PUFA transport (via FFA and HDL-PL) and that HDL PUFA-PL correlate with hepatocyte ballooning. How HDL lipidome can influence the hepatic lipid pool and function requires further investigation.

AUTHOR CONTRIBUTIONS

G.M., M.A., A.V·P., M.V. and J.L.G. conceived and designed the study. G.M., M.V. and J.L.G. wrote the manuscript. M.A., V.A., G.M., M.F., recruited the participants. G.M., B.J., Z.H., A.M., A.K., performed lipidomic analyses. G.M. and M.V. performed statistical analyses and prepared figures. G.M. performed HDL lipoprotein isolation with support from L.V·H.-M. All the authors provided useful criticism during the study, critically reviewed the manuscript and agreed to the published version of the manuscript.

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DATA AVAILABILITY

Data will be made available on request.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101728.

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