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LDL aggregation susceptibility is higher in healthy South Asian compared with white Caucasian men

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ABSTRACT

Background

South Asians are more prone to develop atherosclerotic cardiovascular disease (ASCVD) compared with white Caucasians, which is not fully explained by classical risk factors. We recently reported that the presence of aggregation-prone LDL in the circulation is associated with increased ASCVD mortality.

Objective

We hypothesized that LDL of South Asians is more prone to aggregate, which may be explained by differences in their LDL lipid composition.

Methods

In this cross-sectional hypothesis-generating study, LDL was isolated from plasma of healthy South Asians (n=12) and age- and BMI-matched white Caucasians (n=12), and its aggregation susceptibility and lipid composition were analyzed.

Results

LDL from South Asians was markedly more prone to aggregate compared with white Caucasians. Among all measured lipids, sphingomyelin 24:0 and triacylglycerol 56:8 showed the highest positive correlation with LDL aggregation. In addition, LDL from South Asians was enriched in arachidonic acid containing phosphatidylcholine 38:4 and had less phosphatidylcholines and cholesteryl esters containing monounsaturated fatty acids. Interestingly, body fat percentage which was higher in South Asians (+26%), positively correlated with LDL aggregation and highly positively correlated with triacylglycerol 56:8, sphingomyelin 24:0 and total sphingomyelin.

Conclusions

LDL aggregation susceptibility is higher in healthy young South Asians compared with white Caucasians. This may be partly explained by the higher body fat percentage of South Asians, leading to sphingomyelin-enrichment of LDL. We anticipate that the presence of sphingomyelin-rich, aggregation-prone LDL particles in young South Asians may increase LDL accumulation in the arterial wall and thereby contribute to their increased risk of developing ASCVD later in life.

INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) is the primary cause of death worldwide and puts a major burden on global health care.[1] People originating from the South Asian subcontinent (India, Nepal, Bangladesh, Bhutan, Pakistan and Sri Lanka), who comprise one fourth of the world population, are particularly prone to develop ASCVD compared with other ethnic groups.[2] Moreover, South Asians suffer from higher ASCVD morbidity and mortality rates and experience their first myocardial infarction on average ten years prior to western white Caucasians.[3] Factors contributing to this ASCVD risk that are highly present in the South Asian population include smoking, a low level of physical activity and a diet enriched with carbohydrates and saturated fats. In addition to these lifestyle factors, South Asians have a high body fat percentage and are susceptible to develop obesity, insulin resistance, hypertension and dyslipidemia. However, the high ASCVD risk of South Asians cannot be solely explained by a high prevalence of classical risk factors in this population.[4-6]

Even though a high plasma LDL-cholesterol level is a risk factor for ASCVD,[7] measurement of LDL-cholesterol levels only does not capture the qualitative properties of LDL particles affecting the progression of atherosclerosis.[8] Current theories of atherogenesis emphasize retention of LDL by the subendothelial proteoglycans in the arterial intima as an initial step.[9] The retained LDL is susceptible to modification by oxidation, glycation, and proteolytic and lipolytic enzymes,[10] which can induce aggregation of the modified LDL particles.[11] An enzyme that induces formation of extremely large LDL aggregates is sphingomyelinase.[12-15] LDL aggregation enhances its binding to arterial proteoglycans[11] and thereby can increase extracellular lipid accumulation, thereby further aggravating atherosclerosis development.[16] In addition, macrophages and other inflammatory cells can take up aggregated LDL particles, leading to foam cell formation.[17] Over decades, these processes result in formation of atherosclerotic lesions containing aggregated LDL-derived particles.[13, 18-20] We have recently developed a test to measure the susceptibility of LDL particles isolated from plasma to aggregate, and observed substantial inter-individual variation in LDL aggregation.[21] Importantly, LDL aggregation susceptibility was found to predict future cardiovascular deaths independently of conventional risk factors for ASCVD, such as LDL-cholesterol levels.[21]

Differences in the aggregation susceptibility of LDL particles depend on their surface lipid composition. We observed that LDL particles having a high proportion of sphingomyelins (SMs) and ceramides are prone to aggregate, whereas LDL particles having a relatively high content of phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) are more resistant to aggregation.[21] These data are in accordance with studies showing that plasma SM levels are higher in patients having coronary artery disease than in controls[22] and that plasma ceramides predict future cardiovascular deaths.[23,48] Of

LDL core lipids, a high proportion of several triacylglycerol (TAG) species and cholesteryl esters (CEs) containing monounsaturated fatty acids was associated with decreased LDL aggregation.[21]

In this study, we hypothesized that, compared with age- and BMI-matched white Caucasians, the susceptibility of LDL to aggregate is higher in healthy South Asian individuals, which could partly contribute to their increased risk to develop ASCVD later in life. In addition, since we have previously shown that LDL aggregation is associated with specific characteristics of the LDL lipidome, we hypothesized that such a difference in LDL aggregation would be mirrored by differences in LDL lipid composition between South Asians and white Caucasians. Finally, as South Asians have relatively more body fat than BMI-matched white Caucasians, and obesity is associated with higher levels of sphingolipids, including both SM and ceramide,[24] we assessed whether body composition is related to both the aggregation susceptibility and lipidome of LDL.

MATERIAL AND METHODS

Participants

Twelve healthy Dutch South Asian and twelve Dutch white Caucasian men were matched for age (18-32 years) and BMI (18-27 kg/m^2) and were included in this study. South Asian subjects were eligible in case of being born and raised in the Netherlands and having 4 grandparents from South Asian descent. Major exclusion criteria included smoking, recent weight-loss, a significant chronic disease and/or a renal, hepatic or endocrine disease. None of the participants used any medication. The study was performed in accordance with the principles of the revised declaration of Helsinki and approved by the medical ethical committee of the Leiden University Medical Center in the Netherland.[25] All study participants provided written informed consent prior to the study.

Study design

This study was conducted as part of a clinical trial that investigated the effects of the glucagon-like peptide 1 receptor agonist exenatide on brown adipose tissue (BAT) activity and energy metabolism (Janssen & Nahon et al, in preparation, trial register number clinicaltrials.gov NCT03002675). That study was conducted between September 2016 and February 2018 at the Leiden University Medical Center, the Netherlands. In the present study, we analyzed only samples collected at the baseline. Participants had been instructed to refrain from physical exercise 48 hours prior to the study day. After a 10-hour overnight fast, body composition was determined by bio-impedance analysis (BIA; Bodystat 1500, Bodystat, Douglas, Isle of Man, UK) and blood samples were drawn.

Serum and plasma measurements

Commercially available enzymatic kits were used to measure concentrations of triglycerides, total cholesterol, HDL-cholesterol (all Roche Diagnostics, Woerden, the Netherlands) and insulin (Meso Scale Diagnostics LLC, Rockville, MD, USA) in serum, and glucose (Instruchemie, Delfzijl, the Netherlands) in plasma. LDL-cholesterol was calculated by the Friedewald equation.[26]

LDL isolation

LDL (d = 1.019 to 1.063 g/ml) was isolated from 300 µl plasma samples by D2O-based sequential ultracentrifugation,[27] and 300 µl of LDL was collected. The concentration of LDL is expressed as protein concentration, which was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA)

Production of human recombinant acid sphingomyelinase

The human recombinant acid sphingomyelinase protein was produced at the University of Helsinki, Finland. The cDNA was ordered from GenScript (Piscataway, USA) as pUC57 plasmid and subcloned to pEFIRES-P vector with an EF1a promoter[28] or to another proprietary mammalian expression vector with a CAG-promoter. Both plasmid vectors yielded essentially similar protein expression in CHO-S cells.

For production of the protein, Chinese hamster ovary (CHO) cells were transfected with the expression construct via lipofection (Fugene 6; Promega, Madison, WI) and selected with puromycin (Corning, Manassas, VA). During selection, cells were grown in F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mmol/l Ultraglutamine (Lonza, Verviers, Belgium), 100 µl/ml streptomycin, 100 IU/ml penicillin (Corning, Mediatech Inc, Manassas, VA) and 10% FBS (Gibco, LifeTechnologies, Paisley, UK). For large-scale expression, cells were adapted to CD OptiCHO medium (Gibco, LifeTechnologies, Paisley, UK) supplemented with 2 mmol/l Ultraglutamine and grown in suspension in an orbital shaker. Cell culture supernatants were clarified by filtration through 0.22 µm membranes (Steritop, Millipore, Darmstadt, Germany) and the solution was pumped through a Protino column (Macherey-Nagel, Duren, Germany). The protein was eluted with imidazole, dialyzed against 140 mM NaCl and finally concentrated with Amicon Ultra concentrator (30 kDa MWCO, Millipore Ireland Ltd, Tullagreen, Ireland).

LDL aggregation susceptibility measurement

The measurement of LDL aggregation susceptibility was performed essentially as described before.[21] Briefly, isolated LDL particles were diluted to 200 µg/ml in 20 mM MES, pH 5.5, containing 150 mM NaCl and 50 µM ZnCl2. The size of the LDL particles was measured (0 h) using dynamic light scattering (Wyatt DynaPro Plate Reader II; Wyatt Technology, CA). Sphingomyelinase was added to the wells and the wells were coated

with paraffin. Particle aggregation was followed by measuring their size approximately every 15 minutes for 6 hours. Aggregation data was collected with Dynamics V7 software (Wyatt Technology, CA).

Lipid mass spectrometry analyses

For mass spectrometry (MS), total lipids of the blood plasma LDL isolates were extracted into chloroform according to Folch et al.[29] Before MS analysis aliquots of the lipid extracts were dissolved in chloroform/methanol (1:2 v/v) and spiked with the quantitative internal standard mixture designed for human plasma lipids (SPLASH® LIPIDOMIX® Mass Spec Standard No 330707; Avanti Polar Lipids, Inc., AL). This mixture contained separate deuterium labelled standard compounds with exact concentration for each of the LDL lipid classes, which thus were quantified against their own standards having similar efficiency of detection as the natural lipid species in LDL. Just before the analysis NH_4OH was added to sample aliquots (to give 1% solution by vol) to support ionization and prevent sodium adduct formation. The sample solutions were infused via a syringe pump into the electrospray ionization (ESI) source of a triple quadrupole mass spectrometer (Agilent 6490 Triple Quad LC/MS; Agilent Technologies, Inc., Santa Clara, CA) at a flow rate of 10 µl/min. The MS+ scan was used to detect TAG species as (M+NH4)+ ions[30], whereas MS/MS precursor ion scans were used to detect PC, LPC and SM species (precursors of m/z 184) and CE species (precursors of m/z 369). The ESI-MS/MS instrument was set to a source temperature of 250°C and collision energies of 10-30 eV (optimized for each lipid class) were used. Nitrogen was used as the collision, nebulizing (20 psi) as well as drying gas (11 µl/min). Data analysis of the mass spectra were performed by using MassHunter Workstation qualitative analysis software (Agilent Technologies, Inc.) and the individual lipid species were quantified using the internal standards and Lipid Mass Spectrum Analysis software.[31] The concentrations generated by LIMSA were converted to molar percent data. In addition, the acyl chain assemblies in such TAG and PC species that clearly separated the South Asian and white Caucasian samples were studied by recording their acyl chain specific MS/MS fragments.[32] For TAG species, positive ion mode neutral loss scans of different acyl chains were detected. For PC species, their formate adducts served as mother ions for negative ion mode precursor scans of the acyl fragments.

Statistical analyses

Raw data of LDL aggregate size was analyzed with GraphPad Prism (version 8.0.1, GraphPad Software, La Jolla, CA). Due to limitations of dynamic light scattering sensitivity for large particles, the maximum aggregate size was limited to 3000 nm and the minimum size to 14 nm. LDL aggregation curves were fitted with nonlinear regression curve fit

([Agonist] vs. response – Variable slope [4 parameters]) and aggregate size at the 2-h time point was interpolated.

The results are presented as mean ± SD and the statistical significance between groups was determined by unpaired Student´s t-test or by Mann-Whitney test. These

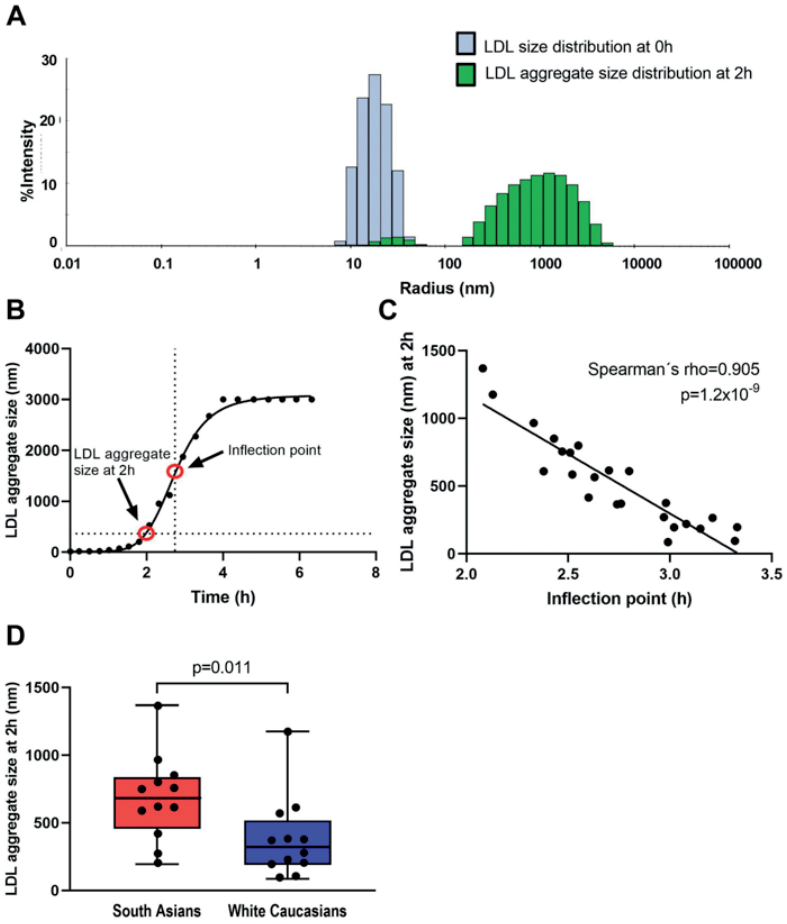

Figure 1. (A) The size distribution of both native LDL and LDL treated with human recombinant sphingomyelinase for 2 h was determined by dynamic light scattering. (B) LDL aggregation curve (LDL aggregate size (nm) vs. time (h)) after inducing aggregation with human recombinant sphingomyelinase. LDL aggregate size at time point 2 h (365 nm) is marked in the curve as well as the calculated inflection point (2.74 h). (C) Correlation of LDL aggregate size (nm) at 2 h and the calculated inflection point (h) (Spearman's rho=0.905, p=1.2x10-9). (D). LDL particles were isolated from plasma of South Asian men (n=12) and white Caucasian men (n=12), treated with sphingomyelinase, and aggregate size was measured using dynamic light scattering. Aggregate size at the 2-h time point was calculated from aggregation curves. The box plot diagram shows the median and the upper and lower quartiles of the aggregate size at 2 h in both ethnicities, the whiskers presenting the lowest and the highest values. Statistical significances of the differences between the ethnicities were studied by using Mann-Whitney U test. LDL, low-density lipoprotein. .

tests and two-tailed Spearman correlation coefficient analysis were performed using IBM SPSS Software (version 25.0, North Castle, NY). P-values < 0.05 were considered to be significant. Correlation analyses were performed for the total study population as well as per ethnicity. In addition, the PC species profiles of the LDL isolates were subjected to principal component analysis using Sirius, PRS, Bergen, Norway (version 8.5). False discovery rate (FDR) was controlled by using two-stage step-up method of Benjamini, Krieger and Yekutieli using GraphPad Prism.

RESULTS

Baseline characteristics

Twelve healthy Dutch men of South Asian descent and twelve age- and BMI-matched white Caucasian men participated in this study. **Table 1** shows the clinical characteristics of the participants. Compared with white Caucasians, South Asians had a higher body fat percentage (18.9 ± 3.2 vs. 14.5 ± 4.7%, p=0.015, unpaired Student´s t-test) at a similar BMI (24.7 ± 2.7 vs. 23.9 ± 2.4 kg/m^2, p=0.47). There were no significant differences between ethnicities in plasma glucose and serum insulin, triglycerides, HDL-cholesterol or LDL-cholesterol, while serum total cholesterol was higher in South Asians than in white Caucasians (4.8 ± 0.8 vs. 4.2 ± 0.5 mmol/l, p=0.032).

Table 1. Clinical characteristics of study participants.

Clinical characteristics	South Asians (n=12)	White Caucasians (n=12)	p-value
Age (years)	27.5 ± 3.2	25.6 ± 3.2	
Body mass index (kg/m^2)	24.7 ± 2.7	23.9 ± 2.4	
Body fat (%)	18.9 ± 3.2	14.5 ± 4.7	0.015
Systolic blood pressure (mmHg)	119 ± 6	124 ± 9	
Diastolic blood pressure (mmHg)	75 ± 9	82 ± 10	
Triglycerides (mg/dL)	73 ± 36	73 ± 25	
Total cholesterol (mg/dL)	186 ± 31	162 ± 19	0.032
HDL-cholesterol (mg/dL)	46 ± 12	44 ± 9	
LDL-cholesterol (mg/dL)	126 ± 35	104 ± 15	
Insulin (pg/ml)	137 ± 133	136 ± 89	
Glucose (mmol/l)	4.8 ± 0.3	4.6 ± 0.2	

Data are presented as mean ± SD. Statistical differences between the ethnicities were determined using the unpaired Student´s t-test, and the p-value is reported in the case of a statistically significant difference between the South Asians and white Caucasians. LDL, low-density lipoprotein; HDL, high-density lipoprotein.

LDL from South Asians is more prone to aggregate than LDL from white Caucasians

To determine if LDL of South Asians is more prone to aggregate than LDL of white Caucasians, we isolated LDL from the plasma samples and measured LDL aggregation susceptibility. Treatment of LDL with sphingomyelinase induced rapid formation of large aggregates (**Figure 1A**). In accordance with our earlier report,[21] LDL aggregate size at 2 hours correlated tightly and significantly with the calculated inflection point of the curve describing the aggregate size as a function of time (Spearman's rho = -0.905, $P=1.2 \times 10^{-9}$) (**Figures 1B** and **C**). There were no significant differences in the size of LDL particles in the beginning of the incubation or in the end of the incubation. However, LDL from South Asians aggregated more rapidly than LDL from white Caucasians, as indicated by a larger LDL aggregate size at 2 h (620 ± 320 nm vs 350 ± 290 nm, p=0.011; **Figure 1D**).

LDL lipid composition correlates with LDL aggregation and differs between the ethnic groups

LDL particles have an amphiphilic surface monolayer containing phospholipids and unesterified cholesterol and a hydrophobic core containing CEs and TAGs. A single copy of apoB-100 surrounds the particle. Since LDL lipid composition has been shown to influence the aggregation susceptibility of LDL particles,[21] we next analyzed the LDL lipidome in both ethnicities (n=24). Within this pooled data, SM 23:0, SM 24:0 and TAG 56:8 were associated with aggregation-prone LDL, while TAG 54:1, TAG 52:2 and CE 16:0 and CE18:1 were associated with aggregation-resistant LDL (**Figure 2A**). When the relative amounts of these lipids in LDL particles were compared between the 2 ethnic groups, South Asians had significantly more SM 24:0 and TAG 56:8, 2 lipids associated with aggregation-prone LDL, and less CE 18:1 and TAG 52:2, two lipids that were associated with aggregation-resistant LDL (**Figure 2B–D**). In addition, we determined associations between LDL lipid composition and LDL aggregation susceptibility in South Asians and white Caucasians separately (**Supplemental Figure 1**). Although in South Asians only LPC 20:3 correlated with LDL aggregation, in white Caucasians several lipid species correlated with LDL aggregation, including lipids that positively (TAG 56:8 and SM 24:0) and negatively (TAG 54:1) correlated with LDL aggregation when ethnicities were combined.

In addition to these lipids that significantly associated with LDL aggregation, there were also other differences between the LDL lipidomes of South Asians and white Caucasians (**Figure 3** and **Supplementary Table 1**). LDL from white Caucasians had a higher proportion of 2 highly unsaturated PC species; 36:5 and 38:5, but lower proportion of PC38:4 (**Figure 3A**). The PC species profiles, trait of the LDL surface, were also studied by multivariate principal component analysis (**Figure 3B**), and also in this analysis, the South Asians were found to have relative enrichment of PC 38:4 and 36:2, which species

contained arachidonic acid (20:4) and its precursor linoleic acid (18:2), respectively (as evidenced by acyl chain specific MS/MS precursor scans). In addition, several monounsaturated PC species were present with higher proportions in white Caucasians (**Figure 3A and 3B**). Of all core lipids, monounsaturated CEs (16:1 and 18:1) were higher in white Caucasians, while CE 18:2, the most common lipid in LDL, was higher in South Asians (Figure 3C). Of note, the elevated TAG 56:8 of South Asians was comprised of two main molecular species 16:0/18:2/22:6 and 16:0/20:4/20:4 (as evidenced by studying the acyl chain specific MS/MS neutral losses from the TAG molecule). Of these, the amount of 16:0/18.2/22:6 was similar in different samples but the amount of the arachidonic acid containing varied from sample to sample and was on average higher in the South Asian samples.

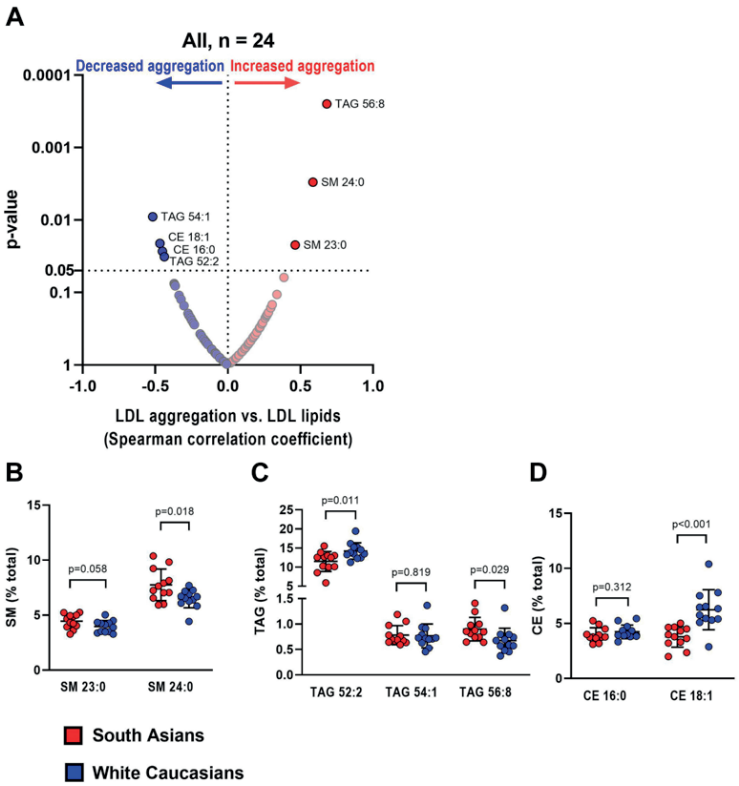

Figure 2. (A) Volcano plot showing the Spearman´s correlation coefficients of LDL aggregate size at 2 h (LDL aggregation susceptibility) vs. LDL lipids (n=24). Only those lipids with significant p-values ($p<0.05$) are annotated within the figure. Positive correlations are indicated with red circles and negative correlations with blue circles. (B-D) Scatter plot diagrams (mean ± SD) showing the proportions of the (B) surface lipid species (SM, sphingomyelin), (C) core triacyclglycerols (TAGs) and (D) core cholesteryl esters (CE), which significantly correlate with aggregate size at 2 h, in South Asians and white Caucasians. Statistical significances of the differences between the groups were determined using the unpaired Student's t-test. The differences remain significant after FDR correction for all lipids with p-value < 0.05. .

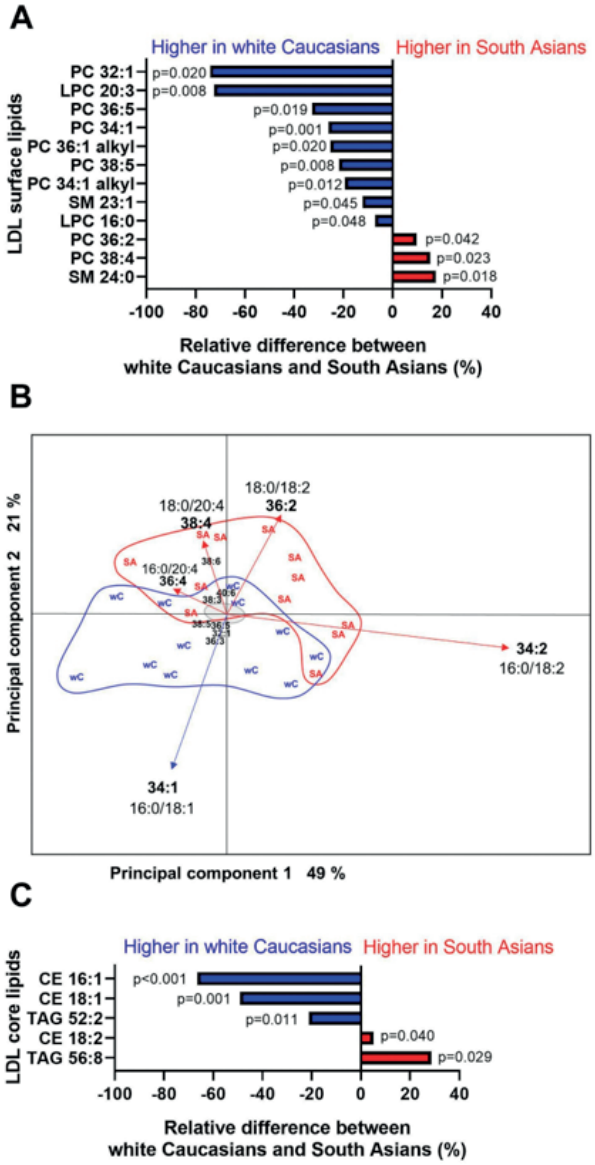

Figure 3. (A) Relative differences between South Asians and white Caucasians (((Average South Asians – Average white Caucasians) / Average all) x 100) in their LDL surface lipids. (B) Phosphatidylcholine (PC) species profile differences between the ethnicities were further demonstrated by PCA using untransformed data (the grey area at the origin of the PCA biplot represents the location of several species having little separation power in the analysis). (C) Relative differences between South Asians and white Caucasians (((Average South Asians – Average white Caucasians) / Average all) x 100) in their LDL core lipids. Statistical differences between the groups were determined using the unpaired Student's t-test. The difference in LPC 16:0, PC 36:2, SM 23:1, SM 24:0, TAG 52:2 and TAG 56:8 did not remain significant after FDR correction. CE, cholesteryl ester; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PCA, principal component analysis; SM, sphingomyelin; TAG; triacylglycerol.

Body fat percentage positively correlates with LDL aggregation susceptibility

Since obesity may modulate ASCVD risk by inducing alterations in the plasma lipidome, we next examined whether anthropometric measurements correlated with LDL lipid components and LDL aggregation. While no correlation with BMI was observed (data not shown), LDL aggregate size at 2 h significantly and positively correlated with body fat percentage (Spearman's rho=0.486, p=0.016) (**Figure 4A**). Interestingly, a higher body fat percentage was associated with a higher proportion of total SM and lower proportion of total PC in the surface of LDL particles (**Figure 4B**), which were previously shown to be characteristics of aggregation-prone LDL.[21] High body fat percentage was also associated with a high relative content of CE 18:2 and TAG 56:8 and low proportion of monounsaturated CE species 16:1 and 18:1 in the core of LDL particles. Notably, many of these LDL components were associated in a similar manner with LDL aggregation (**Figure 2**). Moreover, some of these specific lipid species that correlated positively with both LDL aggregation and body fat percentage were present to a higher extent in LDL of South Asians (SM 24:0 and TAG 58:6), and vice versa, CE 18:1 with low levels in South Asians correlated negatively with both LDL aggregation and body fat percentage (**Figure 2** vs. **Figure 4B**). Strikingly, the LDL lipid surface proportion of many PCs (PC 38:5, PC 34:1 and PC 32:1) correlated negatively with body fat percentage and were lower in South Asians than in white Caucasians. When the groups were evaluated separately, higher total SM and lower total PC content statistically significantly correlated with a higher body fat percentage only in South Asians but not in white Caucasians (**Supplemental Figure 2**).

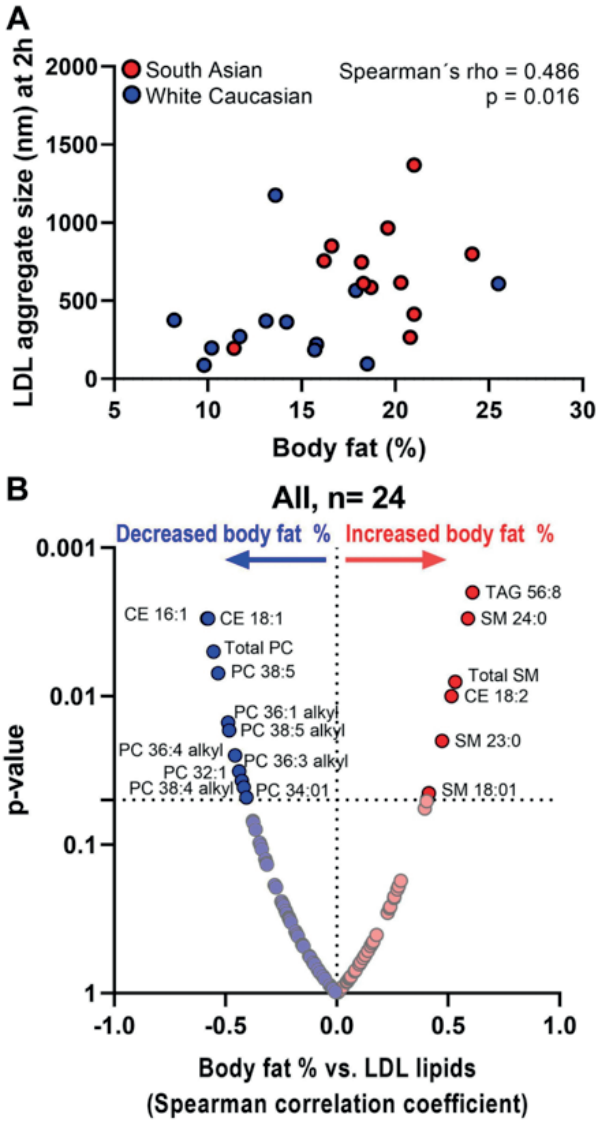

Figure 4. (A) Correlation between LDL aggregation susceptibility and body fat percentage (r=0.486, p=0.016). (B) Volcano plot showing the Spearman correlation coefficients of body fat percentage vs. LDL lipids (n=24). Only those lipids with significant p-values (p<0.05) are identified in the figure. Positive correlations are indicated with red circles and negative correlations with blue circles. CE, cholesteryl ester; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; TAG; triacylglycerol.

DISCUSSION

In this study we show that South Asians, who have higher ASCVD morbidity and mortality than white Caucasians,[4-6, 33] have LDL that is more prone to aggregate than LDL from white Caucasians. Recently, we showed that increased LDL aggregation susceptibility is a marker of increased ASCVD risk independently of conventional risk factors.[21] As aggregation of modified LDL is one of the key steps in atherogenesis by promoting LDL retention,[9, 11, 16] foam cell formation,[35-37] inflammation[20] and plaque destabilization,[21] the presence of aggregation-prone LDL, besides being a marker, can also be a maker in several crucial steps in atherogenesis. Therefore, the unbeneficial LDL quality among South Asians could partly explain their increased risk for ASCVD compared with white Caucasians.

The susceptibility of LDL to aggregation is influenced by the lipid composition of the surface monolayer of LDL, which, in turn, influences the conformation of apo-B100.[21] Such conformational changes mediate formation of LDL aggregates.[15, 21] In addition to the surface lipids, also the core lipid composition has been identified to influence the conformation of apoB-100 within LDL.[37, 38] We previously showed that a high proportion of SM within the surface of circulating LDL is associated with aggregation-prone LDL, whereas a high proportion of PC is associated with aggregation-resistant LDL. Moreover, causality of the differences in the lipid composition in LDL aggregation was previously shown by modifying the proportions of SM and PC *in vitro* or *in vivo* in mice.[21] In the present study, we observed that the proportion SM 23:0 and SM 24:0 were higher in aggregation-prone LDL, which is thus in accordance with our previous results. In addition, of the core lipids, the proportion of highly unsaturated TAG 56:8 was higher in aggregation-prone LDL, whereas the proportion of TAGs 52:2 and 54:1 and CEs 18:1 and 16:0 were higher in aggregation-resistant LDLs. In accordance, TAGs and CEs harbouring saturated and monosaturated fatty acids in LDL, were also previously shown to associate with lower aggregation susceptibility of LDL particles.[21]

Of the individual lipid species that correlated with LDL aggregation susceptibility, TAG 56:8 is comprised largely of two main molecular species (16:0/18:2/22:6 and 16:0/20:4/20:4) of which particularly the arachidonic acid (20:4)-containing TAG species was higher in South Asians. Furthermore, the LDL surface PC profile of the South Asians was characterized by high proportion of PC 38:4 and PC 36:2, which also contained arachidonic acid or its precursor linoleic acid (18:2), respectively. In line with these findings, plasma levels of arachidonic acid have been reported to be higher in South Asians than in white Caucasians.[39] Arachidonic acid can be converted intracellularly into either pro-inflammatory or pro-resolving lipid mediators, while docosahexaenoic acid (22:6) is converted into pro-resolving lipid mediators. The balance between these pro-inflammatory and pro-resolving lipid mediators controls the inflammatory state of an

atherosclerotic plaque.[40] We propose that LDL enriched in arachidonic acid-containing TAGs and PCs species contributes to increased LDL aggregation in South Asians.

We observed for the first time that body fat percentage (mean 16.7%, range 8.2%-25.5%) correlated positively with aggregation susceptibility of LDL particles. Thus, in addition to having aggregation-prone LDL particles, South Asians had a higher body fat percentage than white Caucasians (18.9 ± 3.2 vs. 14.5 ± 4.7%) at a similar BMI (24.7 ± 2.7 vs. 23.9 ± 2.4 kg/m^2). This latter finding is in accordance with previous reports.[41, 42] Of note, BMI is a relatively poor predictor of body fat,[45] and indeed, we did not observe any association between BMI and LDL aggregation in either this study or in our previous study cohorts.[21] When investigating the LDL lipidome in relation to adiposity, we observed a positive correlation between body fat percentage and total SM content of LDL particles only in South Asians (Spearman's rho=0.678, p=0.014). SMs are bioactive lipids that are modulated by adiposity, as obesity-induced inflammation has been suggested to increase SM biosynthesis, and SMs have the potential to increase metabolic dysfunction and ASCVD risk.[22, 23, 44] In line with this, loss of visceral fat was recently associated with a decrease in plasma SM levels and reduced inflammation in athletes with a healthy body weight.[45] Furthermore, similar changes in circulating SM levels and inflammation status were observed during a 7-year follow-up period of individuals who lost body weight, whereas opposite effects were observed in individuals who gained weight.[45] Indeed, in addition to a higher body fat percentage in South Asians compared with white Caucasians, the surface of LDL particles of South Asians was enriched in SM 24:0. Collectively, these data support a link between being overweight and increased ASCVD risk,[46,47] and we suggest that adiposity may modulate the lipidome of LDL particles to affect their aggregation susceptibility.

Limitations of the study

The sample size in this study is relatively small and we investigated LDL aggregation susceptibility in blood samples only from healthy subjects in a cross-sectional study. Therefore, these results should be verified in larger study cohorts, also including patients with ASCVD. In addition, it would be highly interesting to investigate in a prospective study setting whether the susceptibility of LDL particles to aggregate indeed contributes to the development of ASCVD particularly in South Asians. Dietary information of the participants in this study is limited and the relative effects of diet vs. body composition on LDL aggregation susceptibility remain to be examined in future studies. In addition, the LDL aggregation assay requires isolation of LDL particles, which may challenge the clinical use and the use in of the assay in large cohorts. In the present study we found differences in the lipid composition of LDL particles between South Asian and white Caucasian participants. However, ethnicity may also influence the apolipoproteins other

than apoB-100 carried in LDL particles. Such differences and their potential effect on LDL aggregation remain to be studied.

Conclusions

This study provides evidence that LDL aggregation susceptibility is higher in young lean South Asians compared with BMI-matched white Caucasians. Mechanistically, this may be explained by the higher body fat percentage of South Asians, leading to SM enrichment of the LDL particle surface. We anticipate that the presence of SM- and arachidonic acid-rich, aggregation-prone LDL particles in young South Asians may increase LDL accumulation in the arterial wall and thereby contribute to their increased risk of developing ASCVD later in life.

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Authors' contributions: KÖ and PCNR designed and supervised the study together with MRB. LJ recruited the study participants and collected the samples, participant data, and analyzed baseline characteristics together with KJN. MR, LÄ, and FTS performed the experiments and analyzed the data together with HR, RK, and KÖ. OR produced the human recombinant sphingomyelinase. MR prepared the figures and tables and wrote the first draft of the manuscript to which LJ, KÖ, and PCNR provided critical comments and edits. All authors commented on the manuscript and have read and approved the final manuscript.

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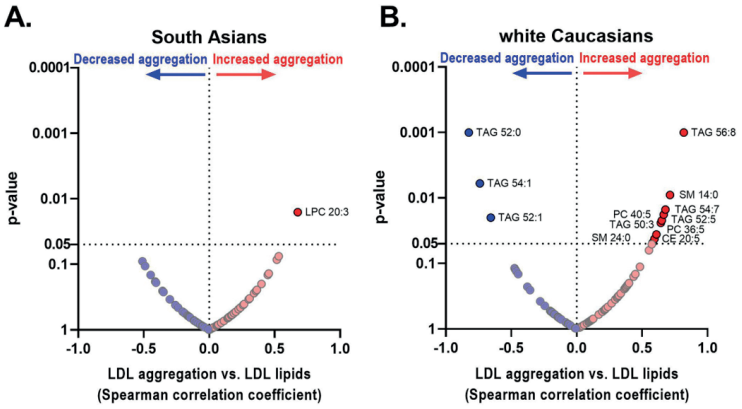
SUPPLEMENTAL APPENDIX

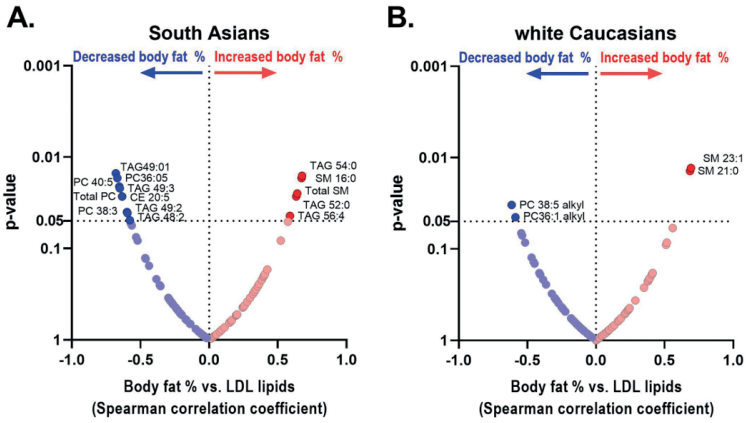
Supplementary Table 1. LDL lipids that differ significantly between white Caucasians and South Asians

LDL Surface Lipids	White Caucasians (mean % of lipid class)	South Asians (mean % of lipid class)	*P*-value
LPC 16:0	50.20 ± 3.85	46.75 ± 4.23	.048
LPC 20:3	0.80 ± 0.41	0.38 ± 0.27	.008
PC 32:1	0.61 ± 0.40	0.28 ± 0.18	.020
PC 34:1	11.47 ± 1.74	8.83 ± 1.58	.001
PC 34:1 alkyl	0.57 ± 0.10	0.47 ± 0.08	.012
PC 36:1 alkyl	0.26 ± 0.04	0.20 ± 0.07	.020
PC 36:2	14.02 ± 1.46	15.44 ± 1.75	.042
PC 36:5	1.21 ± 0.36	0.84 ± 0.29	.019
PC 38:4	5.94 ± 0.79	6.92 ± 1.14	.023
PC 38:5	2.95 ± 0.36	2.37 ± 0.57	.008
SM 23:1	3.40 ± 0.39	3.00 ± 0.51	.045
SM 24:0	6.50 ± 0.83	7.75 ± 1.44	.018
LDL core lipids			
CE 16:1	1.7 ± 0.6	0.9 ± 0.6	<.001
CE 18:1	6.2 ± 1.8	3.9 ± 1.5	.001
CE 18:2	66.5 ± 4.7	69.9 ± 3.5	.040
TAG 52:2	14.2 ± 2.1	11.5 ± 2.4	.011
TAG 56:8	0.67 ± 0.24	0.90 ± 0.26	.029

CE, cholesteryl ester; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; TAG; triacylglycerol. LDL lipids are expressed as mean percentages per lipid class (mean ± SD, n = 12 per group). Statistical differences between the groups were determined using the unpaired Student's t-test. The difference in LPC 16:0, PC 36:2, SM 23:1, and SM 24:0 did not remain significant after FDR correction.



Supplementary Figure 1. Volcano plot showing the Spearman correlation coefficients of LDL aggregate size at 2 h (LDL aggregation susceptibility) vs LDL lipids in (A) South Asians (n = 12) and (B) white Caucasians (n = 12). Only those lipids with significant P-values (P < .05) are annotated within the figure. Positive correlations are indicated with red circles, and negative correlations with blue circles. CE, cholesteryl ester; LPC lysophosphatidylcholine; PC phosphatidylcholine; SM, sphingomyelin; TAG; triacylglycerol.



Supplementary Figure 2. Volcano plot showing the Spearman correlation coefficients of body fat percentages vs LDL lipids in (A) South Asians (n = 12) and (B) white Caucasians (n = 12). Only those lipids with significant P-values (P < .05) are annotated within the figure. Positive correlations are indicated with red circles, and negative correlations with blue circles. CE, cholesteryl ester; LPC, lysophosphatidylcholine; PC phosphatidylcholine; SM, sphingomyelin; TAG; triacylglycerol.