Plant stanol esters reduce LDL aggregation by altering LDL surface lipids. The BLOOD FLOW randomized intervention study.

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Abstract

Objective

Plant stanol ester supplementation (2-3 g plant stanols/d) reduces plasma LDL cholesterol concentration by 9-12% and is therefore recommended as part of prevention and treatment of atherosclerotic cardiovascular disease. In addition to plasma LDL cholesterol concentration, also qualitative properties of LDL particles can influence atherogenesis. However, the effect of plant stanol ester consumption on the proatherogenic properties of LDL has not been studied.

Approach and Results

Study subjects (n=90) were randomized to consume either a plant stanol ester-enriched spread (3.0 g plant stanols/day) or the same spread without added plant stanol esters for 6 months. Blood samples were taken at baseline and after the intervention. The aggregation susceptibility of LDL particles was analysed by inducing aggregation of isolated LDL and following aggregate formation. LDL lipidome was determined by mass spectrometry. Binding of serum lipoproteins to proteoglycans was measured using a microtiter well-based assay.

LDL aggregation susceptibility was decreased in the plant stanol ester group, and the median aggregate size after incubation for 2h decreased from 1490nm to 620nm, $p=0.001$. Plant stanol ester-induced decrease in LDL aggregation was more extensive in participants having BMI<25 kg/m^2. Decreased LDL aggregation susceptibility was associated with decreased proportion of LDL-sphingomyelins and increased proportion of LDL-triacylglycerols. LDL binding to proteoglycans was decreased in the plant stanol ester group, the decrease depending on decreased serum LDL-cholesterol concentration.

Conclusions

Consumption of plant stanol esters decreases the aggregation susceptibility of LDL particles by modifying LDL lipidome. The resulting improvement of LDL quality may be beneficial for cardiovascular health.

This study is an ad hoc analysis of the original BLOOD FLOW Study. The Clinical Trial Registry number of the original BLOOD FLOW Study is: ClinicalTrials.gov, # NCT01315964

Abbreviations: apo, apolipoprotein; CE, cholesteryl ester; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; MS, mass spectrometry; PC, phosphatidylcholine; SM, sphingomyelin; SMase, sphingomyelinase; TAG, triacylglycerol

Introduction

Low density lipoprotein (LDL) cholesterol (LDL-C) concentration is a causal and measurable risk factor for atherosclerotic cardiovascular disease (ASCVD).[1] Genetically controlled low LDL-C levels for lifetime are associated with remarkable decrease in ASCVD, as revealed by Mendelian randomization studies.[2] Likewise, ASCVD risk reduction can be achieved by lowering LDL-C with pharmacological and nonpharmacological means. In a recent meta-analysis including over 300 000 participants from 33 trials, LDL-C reduction of 1 mmol/l was found to predict a 23% relative risk reduction in major cardiovascular events.[3] In these studies, LDL-C reduction was accomplished by upregulating LDL receptor expression using either statin or non-statin therapies, the latter including also dietary trials. Interestingly the relative risk reduction of major vascular events per change in LDL-C was similar in the statin and non-statin treatment modalities.

It is well established that lifestyle and especially dietary changes can lower the circulating LDL-C concentration by up to 20 %, such lifestyle changes being included in international guidelines as a means to reduce the cardiovascular risk.[4, 5] Foods with added plant stanol esters were developed to lower plasma LDL-C levels via inhibition of cholesterol absorption, so that less intestinal cholesterol is transported to the liver. Thus, plant stanol esters can be used as a dietary supplement to safely lower the LDL-C concentration, a daily intake of 2-3 g/d plant stanols lowering the concentration on average by 9-12%.[6-8] Indeed, the plant stanol ester -enriched spread has been estimated to be as effective for reducing cardiovascular risk as the Mediterranean diet.[9] Regarding plant stanols (and plant sterols), the European Atherosclerosis Society Consensus Panel of Phytosterols[10] considered that large randomised outcome studies in low to moderate risk subjects are not practically feasible. However, differences in the proatherogenic properties of LDL particles could add information when attempting to detect individuals at high risk for ASCVD.

The proatherogenic properties of LDL particles are associated with increased retention and accumulation of LDL in the arterial wall.[11] Thus, after the circulating LDL particles have passed the arterial endothelium and entered the tunica intima, the lipoproteins are prone to bind to intimal proteoglycans and are exposed to

107 modification by intimal extracellular enzymes and oxidants.[12-15] Modified LDL
108 particles can aggregate [14] and, indeed, LDL aggregates are found in atherosclerotic
109 lesions.[16-18] Individual differences in these processes may partly explain differences
110 in atherogenesis between individuals having similar LDL-C levels. The binding
111 propensity of LDL to proteoglycans shows inter-individual variation and is stronger in
112 individuals with ASCVD.[19] Similarly, we recently showed that LDL aggregation
113 susceptibility depends on the lipid composition of the particles, varies among
114 individuals and, importantly, an increased aggregation susceptibility can predict
115 future ASCVD death in patients with diagnosed coronary stenosis.[20]

116 Both the binding of LDL to proteoglycans and LDL aggregation susceptibility
117 can be modified by diet and by medications that lower plasma LDL-C.[21, 22] To this
118 end, the aim of the present study was to investigate whether consumption of plant
119 stanol esters has an effect on LDL lipid composition and two atherogenic properties
120 of LDL particles, *i.e.* binding of LDL to proteoglycans and LDL aggregation
121 susceptibility. This study is an *ad hoc* analysis of the original BLOOD FLOW Study, in
122 which the outcome measures were to evaluate the effects of plant stanol ester
123 consumption on serum lipids and on arterial stiffness and endothelial function.[8, 23] In
124 the present examination, LDL aggregation susceptibility and LDL binding to
125 proteoglycans were defined as exploratory end-point measures.

126

Subjects and Methods

Data Sharing

Data described in the manuscript, code book, and analytic code will be made available upon request.

Study participants

The original study called BLOOD FLOW was carried out in Helsinki, Finland in 2011 and has been described earlier in detail.[8, 23] In short, 94 Finnish, white Caucasian subjects were recruited by advertisements in four large companies having mainly office employees and in two research institutes. Ninety-two subjects completed the study. For the present study, in 2018 these 92 subjects were contacted again, and 90 of them (56 females and 34 men) with a median age of 52 years (range 24-66 years) gave their consent to use the frozen sera from the 2011 intervention in the exploratory analyses of the present study. **Supplemental Figure I** displays the flow chart of the study participants. In the original study, lipid-lowering medication or consumption of nutrient supplements interfering with cholesterol metabolism were exclusion criteria, as well as gravidity or breast feeding, unstable CAD, abnormal liver, kidney, or thyroid function, inflammatory bowel disease, and abundant alcohol consumption. No inclusion criteria were set for serum and lipoprotein lipids. The study was performed according to the principles of the Declaration of Helsinki, and written informed consent was obtained from all study participants. The Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the study protocol.

Study design, diet, and basic measurements

The original study was a randomized, double-blind, placebo-controlled, parallel clinical intervention (Clinical Trials Register #NCT01315964).[8] The participants were randomized using a computer-generated randomization list into two groups. The plant stanol ester group consumed a plant stanol ester enriched rapeseed oil-based spread (3.0 g of plant stanols/day, STAEST group, n=44) three times/day during regular meals. The control group consumed the same spread without added plant stanols (CONTROL group, n=46) and followed the same

157 instructions for frequency and timing. Both the study participants and the
158 researchers were blinded to the spreads, which were coded with computer-generated
159 different colors and provided by Raisio Group Ltd. The color codes were broken after
160 all analyses of the original study had been performed. The subjects kept their
161 habitual home diet except for replacing 20 g/day of their regular spread intake by the
162 test spreads. A dietician counselled the subjects twice, and the subjects kept a 3-day
163 food record at baseline and at the end of the study. The nutrient intakes were similar
164 between the groups throughout the intervention.[8]

165 The intervention phase of the original study lasted for 6 months, and blood samples
166 were collected after 12-hour fast at baseline and at the end of the study. Laboratory
167 measurements employed routine standard methods, and the serum and lipoprotein
168 lipids were enzymatically determined using automated analyser systems at the
169 Central Laboratory of Helsinki University Hospital. Plasma lipoprotein subclasses
170 were determined using nuclear magnetic resonance (NMR) spectroscopy at
171 LipoScience Inc. (Raleigh, NC). The rest of the samples were frozen in -80˚C.

172 **Measurement of LDL aggregation susceptibility**

173 In the present *ad hoc* study, LDL particles (d = 1.019 to 1.063 g/ml) were
174 isolated from frozen serum samples after thawing by D_2O-based sequential
175 ultracentrifugation.[24] As shown previously,[20] frozen samples are suitable for the LDL
176 aggregation assay. LDL aggregation analysis was performed blinded. LDL protein
177 concentration was determined with Pierce™ BCA Protein Assay Kit (Thermo
178 Scientific, Rockford, USA), and the amounts of LDL are expressed as their protein
179 concentration. LDL samples were diluted to 200 µg of protein/mL in 20 mM MES, pH
180 5.5, containing 150 mM NaCl and 50 µM $ZnCl_2$. LDL particle size was determined
181 using dynamic light scattering, Wyatt DynaPro Plate Reader II (Wyatt Technology,
182 California, USA). Human recombinant sphingomyelinase (SMase, produced in
183 house[25]) was used to induce LDL aggregation. Aggregate size was followed every
184 15-30 minutes for 6 hours. LDL aggregation data was collected with Dynamics V7
185 software (Wyatt Technology, California, USA). Apolipoprotein (apo) B-100, apoE, and
186 apoCIII contents of the isolated LDL particles were measured with ELISA assays
187 (Cat. 3715-1A-6 for apoB-100 and Cat. 3712-1H-6 for apoE, Mabtech, Sweden; Cat
188 KSP-123 for apoCIII, Nordic Biosite, Sweden).

Mass spectrometry analyses of LDL lipid composition

Total lipids of the isolated LDL particles were extracted for lipid mass spectrometry (MS) with the method of Folch et al.[26] 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., Alabama, USA). Just prior to MS, NH_4OH was added to aliquots of the sample extracts to give 1% solution, which supported ionization and prevented sodium adduct formation. The samples were introduced via a syringe pump into the electrospray ionization (ESI) source of a triple quadrupole MS (Agilent 6410 Triple Quad LC/MS; Agilent Technologies, Inc., Santa Clara, USA) at a flow rate of 10 µl/min. MS+ scan was used to detect TAG species as $(M+NH_4)^+$ ions[27] and MS/MS precursor ion scans of m/z 184 and m/z 369 were used to detect phosphorylcholine –containing phospholipid species (phosphatidylcholine PC, lysophosphatidylcholine LPC, and SM) and cholesteryl ester (CE) species, respectively. The ESI-MS/MS instrument was set to a source temperature of 250°C and collision energies optimized for each lipid class (10-30 eV) were used. Nitrogen was used as the collision, nebulizing (20 psi), and 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 and converted to molar percent data using the internal standards and Lipid Mass Spectrum Analysis (LIMSA) software, which has an inbuilt deisotoping routine that will automatically correct for an overlap of isotope peaks.[28] The proportions of the various lipid species are expressed as percentages of surface lipids (PC, SM, and LPC species) and percentages of core lipids (CE and TAG species).

Measurement of lipoprotein binding to proteoglycans

Proteoglycans were isolated from human aortas as described previously[29] and used to coat 96-well plates overnight at 4°C and blocked with 3% BSA, 1% fat-free milk powder, and 0.05% Tween 20 in phosphate-buffered saline for 1 h at 37°C. 1 µL of each serum sample was diluted in 100 µL 20 mM MES, 140mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ pH 5.5 and incubated in the wells for 1h at 37°C. The 96-well plate was then washed with the same buffer containing 50 mM NaCl and the amount

of bound cholesterol was measured using Amplex red cholesterol kit (Thermo Scientific). Each sample was analysed blinded and in duplicate. To make it easier to compare the proteoglycan-binding of plasma lipoproteins to data published earlier, we also performed the binding assay at neutral pH. For this purpose, only the binding and washing buffers were changed. Thus, 1 µL of each serum sample was diluted to 100 µL of 20 mM HEPES, 50 mM NaCl, 5 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.2, and incubated for 1 h at 37°C. After the incubation, the wells were washed using the same buffer and the amount of cholesterol bound to the proteoglycans was determined as described above.

Statistical analysis

Statistical differences between baseline values and after intervention were calculated using IBM SPSS Software (version 25.0, North Castle, New York, USA). Clinical characteristics are presented as median and range or mean and standard deviation (SD) or number of cases (n) and percent from total. Paired Student´s t-test was used to compare normally distributed and Wilcoxon signed rank test to compare non-normally distributed values before and after intervention. LDL aggregation data were analysed with GraphPad Prism software (version 8.0.1, La Jolla, USA). Missing values from raw LDL aggregate size-data were replaced with average of previous and following value. The aggregate size vs. time curves were fitted using nonlinear regression curve fit ([Agonist] *vs.* response – Variable slope (four parameters)) and inflection points were defined. To analyse correlations between LDL aggregation susceptibility or proteoglycan binding and LDL composition or NMR measurements or between changes in these parameters, Spearman correlation coefficient analysis was used.

Results

Clinical characteristics and outcome of the intervention

The baseline characteristics of the original study population have been reported earlier.[8] In brief, the study population was asymptomatic normo-and mildly-to-moderately hypercholesterolemic subjects with normal median body mass index (BMI) in both the CONTROL and in the STAEST group. None of the subjects had diagnosed ASCVD. The primary outcome of the original study was that 3.0 g of plant stanol consumption as esters/day reduced arterial stiffness in small arteries in both genders and in men also in large arteries. The secondary outcome revealed that when compared to controls, plant stanol ester consumption lowered LDL-C and non-HDL-C concentrations by 10.2% and 10.6%, respectively.[8] Regarding the present *ad hoc* study, one subject of the original study population declined to participate, and one subject could not be reached, both from the STAEST group, and thus these subjects were dropped out. The baseline characteristics and matching between the groups were similar (**Table 1**). Of the participants, one in the CONTROL group had type 2 diabetes and one in each group was a smoker. Blood pressure values, plasma glucose, and high-sensitive C-reactive protein (hs-CRP) concentrations were similar between the groups, and they remained within normal limits throughout the study. As shown in **Table 1**, BMI and HDL-C concentrations of the participants were slightly increased after the intervention in both groups. LDL-C, total cholesterol, and non-HDL-cholesterol concentrations were all decreased in the STAEST group.

Serum concentrations of triglyceride-rich lipoproteins (chylomicrons+VLDL and IDL), LDL, and HDL and their subclasses as well as the sizes of the lipoproteins at baseline and after the intervention were determined using NMR spectroscopy (**Table 2**). In line with the clinical parameters, the concentration of LDL particles decreased in the STAEST group by about 15%. No changes in the proportions of the various LDL subclasses (large, medium, and very small LDL) or LDL size were observed, indicating that consumption of plant stanols influenced similarly all LDL subclasses. There was a modest increase in the concentration of large VLDL particles, which was accompanied by a slight increase in VLDL size. In addition, the proportion of small HDL particles decreased, but this change did not influence the overall size of the HDL particles.

Dietary information of the participants was collected from a 3-day food diary prior to baseline measurements and prior to the end of the study period. In the STAEST group, the intake of mono- and polyunsaturated fatty acids increased (**Table 3**). In the CONTROL group, no statistically significant changes in the diet were observed. Sitosterol, a biomarker of cholesterol absorption efficiency[30] was measured in serum. As expected, in the STAEST group sitosterol-to-cholesterol – ratio was decreased by 33 % (from 1.41 ± 0.62 to 0.95 ± 0.32 µmol/mmol cholesterol, $p<0.000$), while no changes in this ratio was observed in the CONTROL group (from 1.49 ± 0.61 to 1.51 ± 0.59 µmol/mmol cholesterol).

LDL aggregation susceptibility is decreased in STAEST group

To study whether a plant stanol ester-rich diet would influence LDL aggregation susceptibility, LDL particles were first isolated from the serum samples. The LDL samples were diluted to a concentration 200 µg of protein/mL, and LDL aggregation was induced with human recombinant SMase. LDL aggregate formation and increase in the aggregate size was measured with dynamic light scattering every 15-30 minutes, and LDL aggregate size after incubation for 2 h was determined from the aggregate size vs. time –curves. Previously, aggregate size at this time point has been shown to best reflect the aggregation susceptibility of the particles.[20] **Figure 1A** shows LDL aggregation measurement of two participants from the STAEST group at baseline and after the intervention. Consistently with previously published data,[20, 25] there was a large inter-individual variation in LDL aggregation susceptibility, and these aggregation measures did not differ between the STAEST and the CONTROL groups at baseline (blue boxes in **Figure 1 B**). In the STAEST group, there was a significant decrease in LDL aggregation susceptibility, found in about 2/3 of the participants. Consequently, the median LDL aggregate size at 2 h shifted from 1490 nm at baseline to 620 nm after intervention. In the CONTROL group the aggregate size did not change significantly. LDL aggregation susceptibility did not correlate with LDL particle size, as also shown before.[20, 25]

Overweight and obesity has been shown to influence cholesterol metabolism and the efficacy of dietary fats on LDL-C lowering.[31, 32] Therefore, we compared the effects of plant stanol esters and the control diet on LDL aggregation susceptibility in normal-weight (BMI<25 kg/m^2) and overweight/obese (BMI≥25 kg/m^2) participants.

The clinical characteristic of the participants at baseline and after the 6-month intervention in each group are shown in **Table 4.** The baseline levels of the lipids did not differ significantly between the four groups. In the STAEST groups, total cholesterol, LDL-C, and non-HDL cholesterol levels decreased more in the BMI<25 kg/m^2 group than in the BMI≥25 kg/m^2. Interestingly, there was also a significant difference (P<0.001) in cholesterol absorption (measured as sitosterol-to-cholesterol –ratio) between the two BMI groups consuming plant stanol esters. Thus, in the BMI<25 kg/m^2 group, the serum sitosterol-to-cholesterol -ratio decreased on average by 0.66±0.44 µmol/mmol cholesterol (-38%), while in the BMI≥25 kg/m^2 group the decrease was 0.30±0.24 µmol/mmol cholesterol (-25%).

LDL aggregation susceptibility has been shown to be independent of plasma LDL-C concentration or BMI.[20] Also in this study, LDL aggregation susceptibility at baseline did not differ between the two BMI groups (blue boxes in **Figures 1C and D**). However, as a result of the plant stanol ester intervention, LDL aggregation decreased strongly in the normal-weight participants (from 1210 nm to 300 nm median aggregate size), while in the overweight/obese group, no significant decrease was observed (from 1600 nm to 1240 nm) (**Figure 1C**). In the CONTROL group, no significant differences in the LDL aggregate sizes between the normal weight and the overweight/obese participants was observed (**Figure 1D**).

Changes in LDL composition explain changes in LDL aggregation susceptibility

We have previously shown that LDL aggregation susceptibility is controlled by LDL lipid composition.[21] Accordingly, we analysed LDL lipidome and, as also shown previously[20, 25], high proportions of SM species of LDL surface lipids were associated with increased LDL aggregation, and high proportions of several polyunsaturated PC species were strongly associated with the aggregation resistance (**Figure 2A**). Also, in accordance with previous data[20], high proportions of TAGs of the core lipids were associated with decreased LDL aggregation susceptibility (**Figure 2B**).

We next examined whether changes in LDL lipidome were associated with changes in LDL aggregation susceptibility in the STAEST and CONTROL groups. In the STAEST group, the surface lipids that strongly associated with changes in LDL aggregation were the short SM species 15:0 and 14:0. Changes in these SM species correlated positively with changes in LDL aggregation susceptibility (**Figure 3A**). Since LDL aggregation was decreased in the STAEST group, reduction within the proportion of LDL-SMs provided an explanation for the decreased aggregation. In the CONTROL group, LDL aggregation susceptibility was not significantly changed, but the slight changes in LDL aggregation susceptibility were negatively associated with changes in several highly unsaturated LDL-PC species (**Figure 3B**). Thus, an increase in these PCs could explain the nonsignificant decrease in LDL aggregation susceptibility in the CONTROL group. Of LDL core lipids, changes in the proportion of TAGs in the core lipids correlated negatively with changes in LDL aggregation in the STAEST group (**Figure 3C**). In the CONTROL group only CEs containing highly polyunsaturated fatty acids correlated negatively with LDL aggregation susceptibility (**Figure 3D**).

The susceptibility of LDL to sphingomyelin hydrolysis by *Bacillus cereus* spingomyelinase and the resulting aggregation of the lipolyzed LDL particles has been shown to be increased if LDL particles are enriched in apoC-III.[33, 34] On the other hand, addition of small exchangeable apolipoproteins to aggregating LDL particles inhibits their aggregation.[35] Therefore, we next analysed the apoCIII and apoE contents of LDL particles from samples obtained at baseline and after the intervention. In the STAEST group the molar ratios of apoE/apoB and apoCIII/apoB were 12.2±6.0 mmol/mol and 286±150 mmol/mol, respectively, at baseline and 13.4±8.4 mmol/mol and 315±121 mmol/mol after the intervention. In the CONTROL group the values were similar (12.2±8.0 mmol/mol of apoE/apoB and 293±142 mmol/mol of apoCIII/apoB at baseline and 13.4±10.9 mmol/mol of apoE/apoB and 328±188 mmol/mol of apoCIII/apoB after the intervention). These ratios did not differ either between the groups or between the time points. Surprisingly, however, we observed that both the apoE/apoB-ratio and the apoCIII/apoB-ratio correlated inversely with LDL aggregation at baseline (**Figure 4**). In addition, the differences in apoE/apoB and apoCIII/apoB were associated significantly with differences in various LDL lipids (**Figure 4**). In particular, increased amount of apoE or apoCIII was

associated with higher proportion of TAGs in the core of LDL particles. Of note, differences in either the apoE/apoB or apoCIII/apoB or in the proportion of TAGs of the core lipids were not associated with differences in the ratio of surface lipids to core lipids (i.e. potential differences in LDL size) or LDL size measured with NMR spectroscopy.

LDL binding to proteoglycans is decreased in the STAEST group

The binding of lipoproteins to human aortic proteoglycans was determined using microtiter wells that had been coated with the proteoglycans, BSA-blocked, and incubated with serum diluted in a buffer containing 20 mM MES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 150 mM NaCl and having pH 5.5.[29, 36, 37] Unbound lipoproteins were removed by washing, and the amounts of bound lipoproteins were determined by measuring the cholesterol concentration in the proteoglycan-coated wells. The interaction of plasma lipoproteins to proteoglycans is much stronger at acidic pH than at neutral pH,[29, 37] but as shown in **Supplemental Figure II**, binding of the lipoproteins at pH 5.5 correlated significantly with binding of the same lipoproteins to proteoglycans at pH 7.2, but at lower ionic strength (50 mM NaCl).

The binding of serum lipoproteins to proteoglycans at baseline correlated significantly with the concentration of VLDL and LDL particles in the serum samples measured by NMR spectroscopy (**Figure 5A**). Of the lipoprotein subfractions, small VLDL and both large and very small LDL particles correlated positively and VLDL size correlated negatively with the proteoglycan-binding. The apoE/apoB or apoCIII/apoB-ratios did not correlate with either the proteoglycan-binding or with any of the lipoprotein subclasses. The binding of lipoproteins to proteoglycans was associated with differences in the proportion of several LPC species (**Supplemental Figure III**).

In the STAEST group, consumption of plant stanol esters led to decrease in the binding of LDL to proteoglycans (from 4.1 µmol/L to 3.7 µmol/L, p=0.032), whereas in the CONTROL group there were no changes (from 4.3 µmol/L to 4.4 µmol/L, p=0.604). Similarly to plant stanol -induced changes in LDL aggregation, also the change in the proteoglycan binding was statistically significant in the normal-

403 weight participants, but not in the overweight/obese (**Figure 5B and C).** The
404 decrease in the proteoglycan-binding of plasma lipoproteins correlated significantly
405 with a decrease in LDL particles (r=0.360, p=0.016) and with the decrease in LDL-C
406 (r=0.383, p=0.011). When the proteoglycan-binding propensity values were
407 standardized for serum LDL-C concentrations, the significance disappeared in the
408 STAEST group (p=0.407) and remained non-significant in the CONTROL group
409 (p=0.566).

410

Discussion

It is shown for the first time in this *ad hoc* study of the original BLOOD FLOW intervention that consumption of 3 g/day of plant stanols as esters for 6 months reduces LDL aggregation susceptibility and the binding of plasma lipoproteins to proteoglycans. Interestingly, the effects of plant stanols were stronger in normal weight than in overweight or obese participants.

The binding of lipoproteins to proteoglycans determines their potential to be retained in the arterial wall, where the retained particles are subjected to modifications by enzymes and oxidizing agents.[38] The modifications can induce lipoprotein aggregation[14] and, importantly, aggregated LDL particles are found in atherosclerotic lesions.[16-18] The atherogenicity of aggregated LDL has been accredited to increased lipid accumulation and induction of secretion of biologically active products from foam cells that recruit macrophages and other cells into the developing atherosclerotic plaques.[13, 39] A particularly potent enzyme in inducing LDL aggregation is secretory SMase,[34, 40] an enzyme also used in our LDL aggregation assay.

Consumption of plant stanol esters decreased LDL-C concentration and the number of LDL particles in the serum samples and led to decreased binding of LDL to isolated human aortic proteoglycans. Similarly, we previously found that consumption of α-linolenic acid, which decreased plasma LDL-C levels, also decreased LDL binding to proteoglycans LDL-C concentration-dependently.[21] On the other hand, simvastatin was recently found to decrease the binding of LDL to proteoglycans to a larger extent than explainable by the decrease in LDL-C concentration alone.[22]

LDL aggregation susceptibility decreased in 2/3 of the participants of the STAEST group with consumption of a plant stanol ester -enriched spread. In contrast, only minor changes in LDL aggregation were observed when the corresponding control spread was consumed. Similar results were observed previously, when diet rich in unsaturated fats was found to reduce LDL aggregation susceptibility in 2/3 of the participants with minimal changes observed in the control group.[20] Consistent with our previous results,[20] LDL aggregation susceptibility correlated positively with the proportions of total SM and negatively with the

443 proportions of several PCs and TAGs in the LDL particles. Interestingly, in plant
444 stanol ester consuming group, differences in the changes in the proportions of SMs
445 and TAGs in LDL explained changes in LDL aggregation susceptibility, whereas in
446 CONTROL group the changes in the different PC proportions explained the individual
447 changes in LDL aggregation.

448 We also analysed the amounts of apoE and apoCIII in the isolated LDL
449 particles. These two small exchangeable apolipoproteins have been linked with
450 increased binding of lipoproteins to a small proteoglycan, biglycan, apoE by directly
451 interacting with glycosaminoglycans and apoCIII via an unknown mechanism.[33, 41, 42]
452 ApoCIII in LDL particles has also been shown to enhance both sphingomyelinase-
453 and phospholipase A_2-mediated hydrolysis of LDL particles.[33, 43] Surprisingly, we did
454 not observe any correlation between the proteoglycan-binding and LDL-apoE or LDL-
455 apoCIII, while observing an inverse correlation between LDL aggregation
456 susceptibility and LDL-apoE and LDL-apoCIII. The differences between this study
457 and the previously published results may be related to differences in the respective
458 study populations. Thus, the effect of LDL-apoCIII has been shown to be particularly
459 prominent in subjects having type 2 diabetes, who carry more apoCIII in their LDL
460 particles than subjects without diabetes.[33, 43] In our study group only one person had
461 diabetes. Another possibility explaining the above-mentioned differences may relate
462 to isolation of LDL particles. Thus, even though we used a D_2O-based optimal LDL
463 isolation method,[44] it is still possible that some of the small apolipoproteins were
464 released from LDL during the isolation. Finally, in our assay we used proteoglycans
465 isolated from human aortas. The preparation is enriched in versican, rather than
466 biglycan used in the earlier studies.[33, 41] Although unlikely, we cannot rule out the
467 possibility that the interaction of lipoproteins with large versican proteoglycans differs
468 from their interaction with biglycan.

469 LDL particle aggregation depends on the degree of particle lipolysis,[45]
470 but regarding fully lipolyzed particles, such as in our LDL aggregation assay, both
471 apoE and apoCIII on LDL particles appear to inhibit particle aggregation, a finding in
472 accordance with previously published data showing that addition of small
473 exchangeable apolipoproteins stabilizes modified LDL particles.[35] Partice stabilization
474 has been suggested to depend on the ability of the apolipoproteins to incorporate into

the surface monolayer of the modified LDL particles.[36] Importantly, high content of both apo E and apoCIII also correlated with high proportion of TAGs in LDL core and high proportion of several PC species on LDL surface, in accordance with earlier data.[33, 43] Indeed, an increase in these lipids in LDL is causally associated with the decreased LDL aggregation susceptibility.[20]

In addition to the current dietary intervention with plant stanols, also "healthy Nordic diet" has been found to decrease the LDL aggregation among most of the study participants.[20] In the cited study, dietary vitamin E, a marker of vegetable oil consumption, best explained this decrease.[20] However, when we studied if α-linolenic acid–rich *Camelina sativa* oil, fatty fish, or lean fish we found no effect on LDL aggregation.[21] It is of interest to note that the participants in both the "healthy Nordic diet" study,[46] and the *Camelina sativa* oil study[47] were all overweight or obese. Here, we showed that consumption of plant stanols reduced intestinal cholesterol absorption, serum LDL-C and LDL particle concentrations, and LDL aggregation significantly more in participants having BMI < 25 kg/m^2 than in the overweight/obese participants. Together these findings suggest that excess adipose tissue influences the intestinal lipid absorption. This idea is in accordance with earlier studies showing that cholesterol absorption is lower in obese than in normal-weight subjects and that dietary effects of unsaturated fats differ between normal-weight and overweight people.[31, 32, 48]

Taken together, this study shows that dietary plant stanol esters decrease the binding of LDL to proteoglycans by lowering LDL levels in circulation and reduce LDL aggregation susceptibility by inducing qualitative changes in LDL lipids. The changes in the proatherogenic properties of LDL were more pronounced in lean individuals. We have previously demonstrated that the circulating LDL particles are more aggregation-prone in individuals having established ASCVD than in healthy subjects, and that the presence of aggregation-prone LDL in circulation predicts future ASCVD death independently of conventional risk factors.[25] Thus, plant stanol esters possess a dual potential to support our preventive efforts to combat ASCVD: they lower the concentration of LDL particles in serum and also render them more resistant against aggregation.

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Disclosures: MR, PTK, and KÖ have applied for a patent on the LDL aggregation assay. The other authors have no conflicts of interest.

Author´s contributions

HG, KÖ, and MR designed the research; HG, PS, and HL collected the patient samples and the clinical characteristic data, RK, FT-S, LÄ and MR were responsible for lipid mass spectrometry analyses, MR conducted research; MR and FT-S analysed data; MR wrote the first draft of the manuscript and it was critically reviewed by KÖ, HG, and PTK. All authors edited the text and approved the final manuscript.

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Highlights:

- LDL aggregation susceptibility has been associated with atherosclerotic cardiovascular deaths, but it has not been studied whether LDL-lowering plant stanol ester consumption affects LDL aggregation.
- LDL aggregation decreases with consumption of plant stanol esters by altering LDL lipid composition.
- Plant stanol esters are more effective among lean individuals than overweight/obese individuals.
- LDL binding to human arterial proteoglycans is decreased with plant stanol ester consumption.

Figure legends

Figure 1. Plant stanol ester –rich diet decreases LDL aggregation susceptibility. LDL aggregation was induced by incubating LDL isolated from serum samples collected at baseline and after the intervention and measuring the size of the aggregates by dynamic light scattering. (**A**) Aggregate size vs. time curves of two participants at baseline and after the intervention. The size of LDL aggregates at 2 h is used as a measure of LDL aggregation susceptibility (**B**) Aggregate sizes at 2h in STAEST group (n=44) and in CONTROL group (n=46). The groups were further divided according to BMI to normal-weight (BMI < 25 kg/m^2) and overweight/obese (BMI ≥ 25 kg/m^2). Aggregate sizes at 2 h (**C**) in STAEST group (BMI < 25 kg/m^2 n=20, BMI ≥ 25 kg/m^2 n=24) and (**D**) in CONTROL group (n=23 in both BMI groups). The box encompasses the middle 50% of the measured values and the horizontal line within the box shows the median. The whiskers show the most extreme data points. Statistical significance between the baseline and after intervention values was determined with Wilcoxon signed rank test.

Figure 2. Correlation of LDL aggregation susceptibility and LDL lipid species.Volcano plots show Spearman correlation coefficients between LDL aggregation susceptibility and (**A**) LDL surface lipid species and (**B**) LDL core lipids. PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin. Triacylglycerols (TAGs) are grouped according to the sum of acyl carbon double bonds: TAG-SAT, no double bonds, TAG-MONO, 1 double bond; TAG-DI, 2 double bonds, TAG-TRI, 3 double bonds; TAG-TETRA, 4 double bonds. CE-HUFA; cholesteryl esters having ≥ 3 double bonds in their acyl chain. Only lipids having statistically significant ($p<0.05$) correlation are indicated.

Figure 3. Correlation of changes in LDL aggregation susceptibility and LDL lipid species. Spearman correlation coefficients between changes in LDL aggregation susceptibility and changes in LDL surface lipids (**A**) in the STAEST group, n=37 and (**B**) in the CONTROL group, n=42. Spearman correlation coefficients between changes in LDL aggregation susceptibility and changes in LDL core lipids (**C**) in the STAEST group and (**D**) in the CONTROL group. PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin. Triacylglycerols (TAGs) are grouped according to the sum of acyl carbon double bonds: TAG-SAT, no double bonds, TAG-MONO, 1 double bond; TAG-DI, 2 double bonds, TAG-TRI, 3 double bonds; TAG-TETRA, 4 double bonds. CE-HUFA; cholesteryl esters having ≥ 3 double bonds in their acyl chain. Lipid species with significant p-values ($p<0.05$) are labelled.

Figure 4. Correlation of apo E and apo CIII and LDL aggregation and lipidome. Spearman correlation coefficients between apoE/apoB (**A**) or apoCIII/apoB (**B**) and LDL aggregation and LDL lipid composition at baseline (n=90). PC, phosphatidylcholine; Triacylglycerols

(TAGs) are grouped according to the sum of acyl carbon double bonds TAG-SAT, no double bonds, TAG-MONO, 1 double bond; TAG-DI, 2 double bonds, TAG-TRI, 3 double bonds; TAG-TETRA, 4 double bonds. Lipid species with significant p-values ($p<0.05$) are labelled

Figure 5. Binding of serum lipoproteins to human aortic proteoglycans. Serum samples were incubated for 1 h at 37 °C in microtiter wells coated with human aortic proteoglycans. The amount of cholesterol bound to the wells was determined. (**A**) Heatmap showing the Spearman correlation coefficients of the association at baseline between proteoglycan-binding, serum lipoproteins and their subclasses determined by NMR spectroscopy, and apoE/apoB and apoCIII/apoB (n=90). * $p<0.05$, **$p<0.01$, *** $p<0.001$. Proteoglycan-binding of the samples collected at baseline and after the intervention (**B**) in the STAEST group divided according to BMI to normal-weight (BMI<25kg/m^2, n=20) and overweight/obese (BMI≥ 25kg/m^2, n=24) and (**C**) in the CONTROL group (n=23 in both BMI groups). The statistical significance was determined using Wilcoxon signed rank test.

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Table 1. Clinical characteristics presented as mean ± SD of study participants at baseline and after the intervention. Statistical differences within groups were calculated between before and after values of the intervention using paired Student's t-test, *p<0.05, **p<0.01, ***p<0.001 BMI, body mass index; LDL-C, low density lipoprotein cholesterol, HDL-C, high density lipoprotein cholesterol, TG, triglycerides.

	CONTROL		STAEST	
Clinical characteristics	Baseline	After intervention	Baseline	After intervention
Subjects (n)	46		44	
male	14		20	
female	32		24	
Age (years)	52 (24-66)		52 (27-66)	
BMI (kg/m^2)	25.0±3.6	25.3±3.6**	25.2±3.8	25.5±3.6*
Cholesterol (mmol/l)	5.6±1.0	5.7±1.0	5.5±0.9	5.3±0.8**
LDL-C (mmol/l)	3.5±0.9	3.6±1.0	3.5±0.8	3.2±0.8***
HDL-C (mmol/l)	1.8±1.0	1.9±0.5**	1.8±0.5	1.9±0.5*
TG (mmol/l)	0.96±0.5	1.0±0.5	0.88±0.41	0.98±0.5*
non-HDL-C (mmol/l)	3.8±1.0	3.8±0.9	3.7±1.0	3.4±0.9***

Table 2. Lipoprotein subclasses and lipoprotein sizes of study participants at baseline and after the intervention were analysed by NMR spectroscopy. The concentrations of lipoproteins are expressed as nmol/l and the subclasses as % of each lipoprotein class. Normally distributed data are expressed as mean ± SD and other values as median (range). Statistical significances of the differences within groups were calculated between before and after values using paired Student's t-test (normally distributed values) or Wilcoxon signed rank test, *p<0.05, **p<0.01, ***p<0.001. TG, triglyceride.

	CONTROL		**STAEST**	
Lipoprotein particle concentration	Baseline	After intervention	Baseline	After intervention
Triglyceride-rich particles (nmol/l)	49.3 (4.0-333)	55.4 (9.6-304)	49.9 (13.6-311)	40.0 (11.1-221)
Large (% of TG-rich)	1.0 (0-11)	0.7 (0-11.5)	0.85 (0-21.5)	1.6 (0-14.2)*
Medium (% of TG-rich)	41 (3.2-99)	38 (2.7-99)	34 (2.7-99)	49 (5.8-89)
Small (%of TG-rich)	42±24	41±40	48±24	39±20
IDL (% of TG-rich)	44 (0-88)	0 (0-77)	0 (0-73)	0 (0-69)
LDL (nmol/l)	1314 (657-2887)	1238 (538-2981)	1355 (634-2573)	1147 (413-2330)***
Large (% of LDL)	63±26	61±26	61±29	58±30
Medium (% of LDL)	7.5±5.6	8.5±5.6	8.0±6.3	9.2±7.2
Very small (% of LDL)	29 (0-69)	33 (0-81)	34 (0-70)	28 (0-70)
HDL (nmol/l)	38.6±7.5	36.2±6.8	37.435.5± 7.0	35.5±6.9
Large (% of HDL)	35±15	35±14	34±16	35±16
Medium (% of HDL)	0 (0-30)	0 (0-46)	0 (0-18)	0.15 (0-32)
Small (% of HDL)	62±15	60±16	63±29	60±15*
Lipoprotein size	Baseline	After intervention	Baseline	After intervention
VLDL size (nm)	48.7 (9.6-152)	46.6 (36.8-73.6)	45.6 (34.4-66.7)	48.8 (37.2-67.8)**
LDL size (nm)	21.9 (20.0-23.0)	21.7 (19.2-22.9)	21.7 (19.9-23.0)	21.8 (19.9-22.8)
HDL size (nm)	9.4 (8.2-10.3)	9.4 (8.2-10.5)	9.5 (8.3-10.5)	9.4 (8.3-10.4)

Table 3. Dietary information was collected from 3-day food diaries at baseline and at the end of the intervention. The values of dietary energy % are presented as median (range). Statistical differences within groups were calculated between before and after values of the intervention using Wilcoxon signed rank test. *p<0.05. E%, energy %, SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, CARB, carbohydrates.

	CONTROL		STAEST	
Dietary E%	Baseline	End of intervention	Baseline	End of intervention
Protein E%	16 (10-28)	16 (10-26)	17 (12-25)	17 (10-24)
Fat E%	35 (16-47)	36 (25-50)	33 (23-50)	35 (19-45)
SFA E%	11 (5-20)	12 (7-21)	11 (7-21)	11 (5-16)
MUFA E%	12 (5-23)	12 (8-18)	11 (7-20)	13 (7-18)**
PUFA E%	5 (3-13)	5 (3-15)	5 (3-8)	6 (3-12)*
CARB E%	41 (24-56)	42 (26-53)	43 (22-56)	42 (27-54)
Alcohol E%	2 (0-11)	0 (0-15)	0 (0-15)	1 (0-13)

Table 4. Clinical characteristics of the study participants (presented as mean ± SD) divided to normal weight (BMI < 25) and overweight/obese (BMI ≥ 25). Statistical difference between baseline and after treatment were analysed with paired Student´s t-test. *p<0.05, **p<0.01, ***p<0.001. BMI, body mass index; LDL-C, low density lipoprotein cholesterol, HDL-C, high density lipoprotein cholesterol, TG, triglycerides.

	BMI < 25 kg/m^2				**BMI ≥ 25 kg/m^2**			
	CONTROL		STAEST		CONTROL		STAEST	
Clinical characteristics	Baseline	After intervention	Baseline	After intervention	Baseline	After intervention	Baseline	After intervention
Subjects (n)	23		20		23		24	
male	4		4		10		16	
female	19		16		13		8	
Age (years)	54 (24-66)		53 (37-64)		52 (36-62)		51 (27-66)	
BMI (kg/m^2)	22.3±1.9	22.6±1.9 **	22.2±2.1	22.6±2.2**	27.7±2.7	28.1±2.6	27.8±2.9	27.9±2.8
Cholesterol (mmol/l)	5.5±0.1	5.6±1.0	5.4±0.9	5.0±0.7**	5.6±1.0	5.8±1.0	5.6±0.8	5.5±0.8
LDL-C (mmol/l)	3.4±0.9	3.4±1.0	3.2±0.8	2.8±0.7***	3.7±0.9	3.8±1.0	3.8±0.7	3.6±0.7*
HDL-C (mmol/l)	2.0±0.4	2.1±0.4*	2.0±0.4	2.1±0.5	1.6±0.5	1.7±0.5	1.6±0.5	1.7±0.5
TG (mmol/l)	0.8±0.3	0.9±0.4	0.7±0.2	0.8±0.2	1.1±0.5	1.2±0.5	1.0±0.5	1.2±0.5
non-HDL-C (mmol/l)	3.6±1.0	3.6±1.0	3.4±0.8	3.0±0.7***	4.0±1.0	4.1±1.0	4.0±0.8	3.8±0.8*

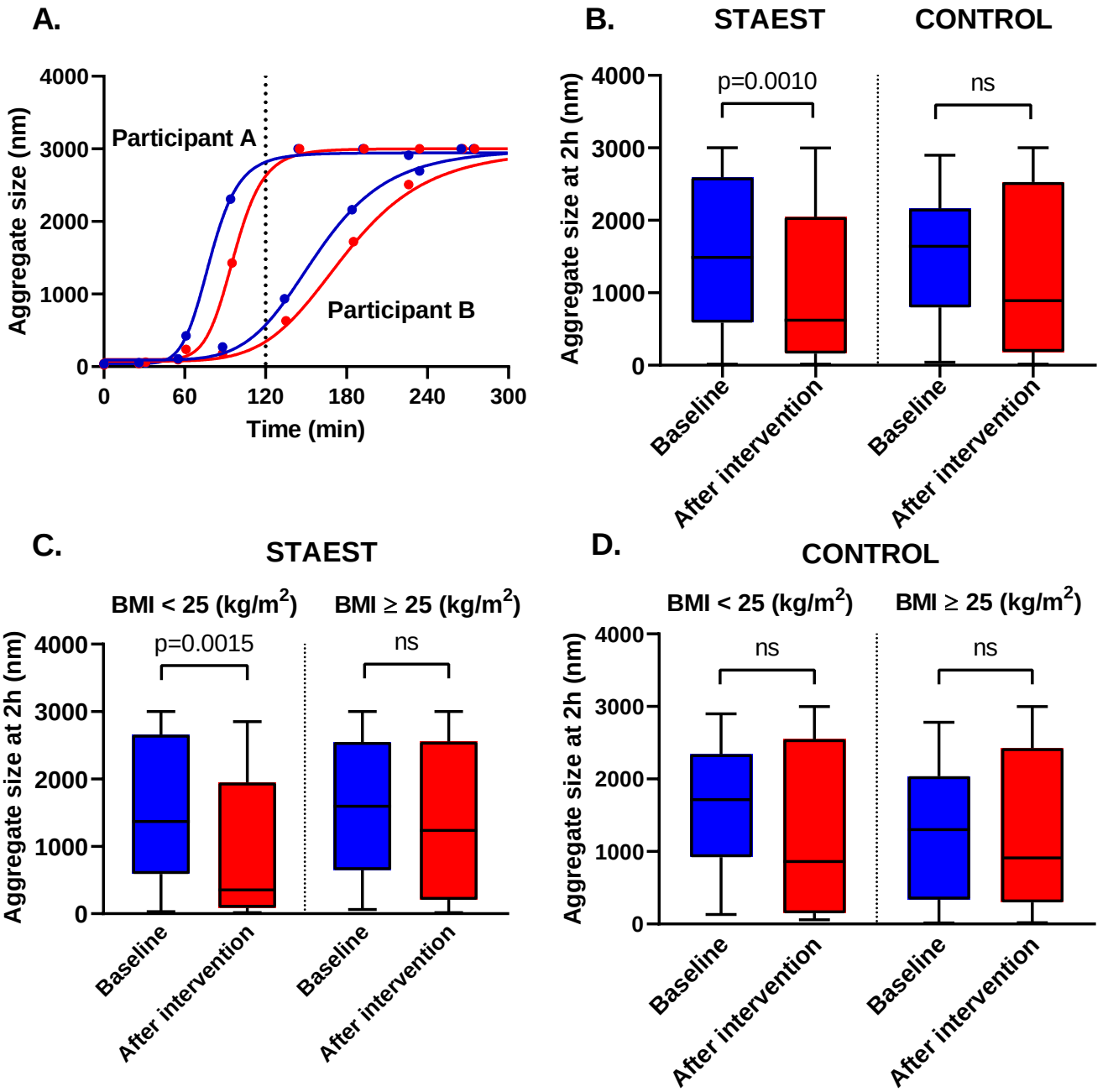
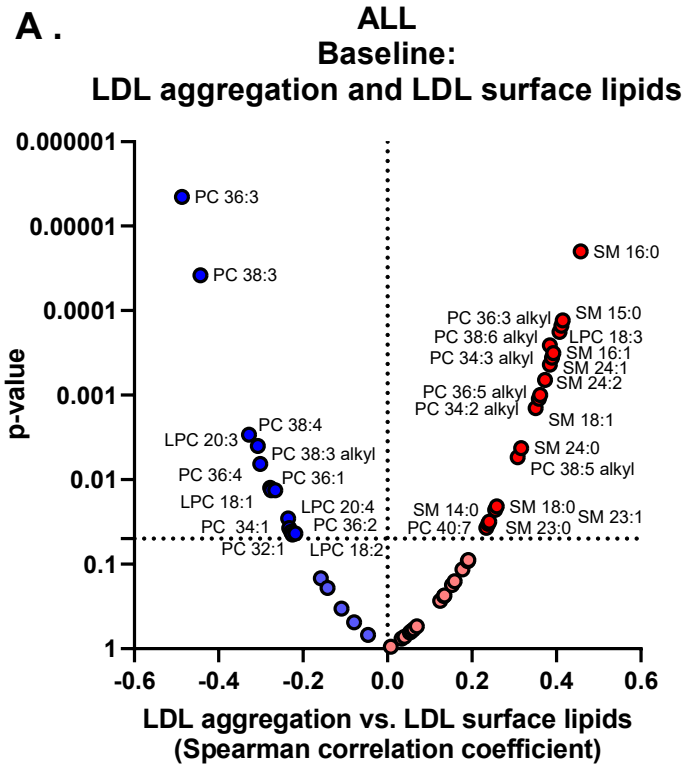

A.
Aggregate size (nm)
4000
3000
2000
1000
0
Participant A
Participant B
0
60
120
180
240
300
Time (min)
B.
STAEST
CONTROL
Aggregate size at 2h (nm)
p=0.0010
ns
Baseline
After intervention
Baseline
After intervention
C.
STAEST
BMI < 25 (kg/m2)
BMI ≥ 25 (kg/m2)
Aggregate size at 2h (nm)
p=0.0015
ns
Baseline
After intervention
Baseline
After intervention
D.
CONTROL
BMI < 25 (kg/m2)
BMI ≥ 25 (kg/m2)
Aggregate size at 2h (nm)
ns
ns
Baseline
After intervention
Baseline
After intervention

Figure 2


A .
ALL
Baseline:
LDL aggregation and LDL surface lipids
p-value
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
PC 36:3
PC 38:3
SM 16:0
PC 36:3 alkyl
SM 15:0
PC 38:6 alkyl
LPC 18:3
PC 34:3 alkyl
SM 16:1
SM 24:1
PC 36:5 alkyl
SM 24:2
PC 34:2 alkyl
SM 18:1
PC 38:4
LPC 20:3
SM 24:0
PC 38:3 alkyl
PC 38:5 alkyl
PC 36:4
PC 36:1
LPC 18:1
LPC 20:4
SM 14:0
SM 18:0
SM 23:1
PC 34:1
PC 36:2
PC 40:7
SM 23:0
PC 32:1
LPC 18:2
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
LDL aggregation vs. LDL surface lipids
(Spearman correlation coefficient)

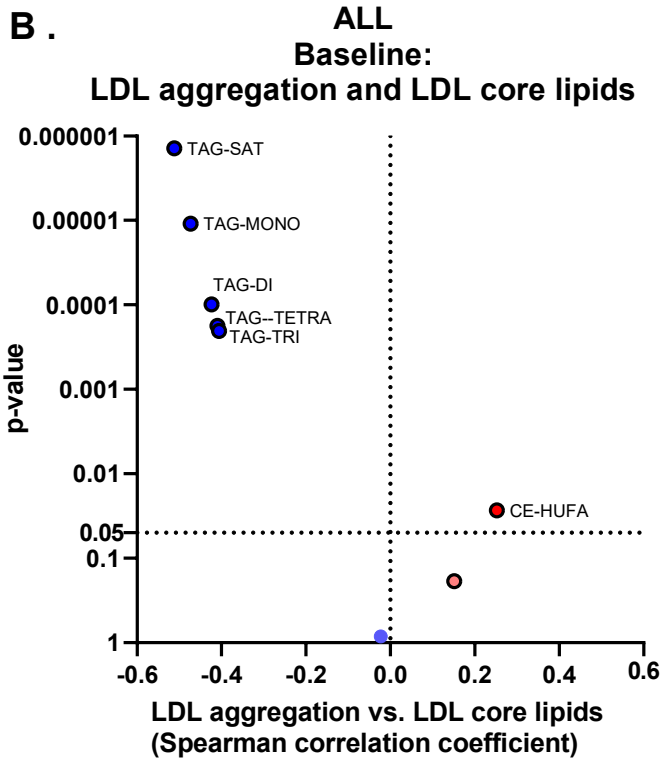

B .
ALL
Baseline:
LDL aggregation and LDL core lipids
p-value
0.000001
0.00001
0.0001
0.001
0.01
0.05
0.1
1
TAG-SAT
TAG-MONO
TAG-DI
TAG--TETRA
TAG-TRI
CE-HUFA
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
LDL aggregation vs. LDL core lipids
(Spearman correlation coefficient)

Figure 3.

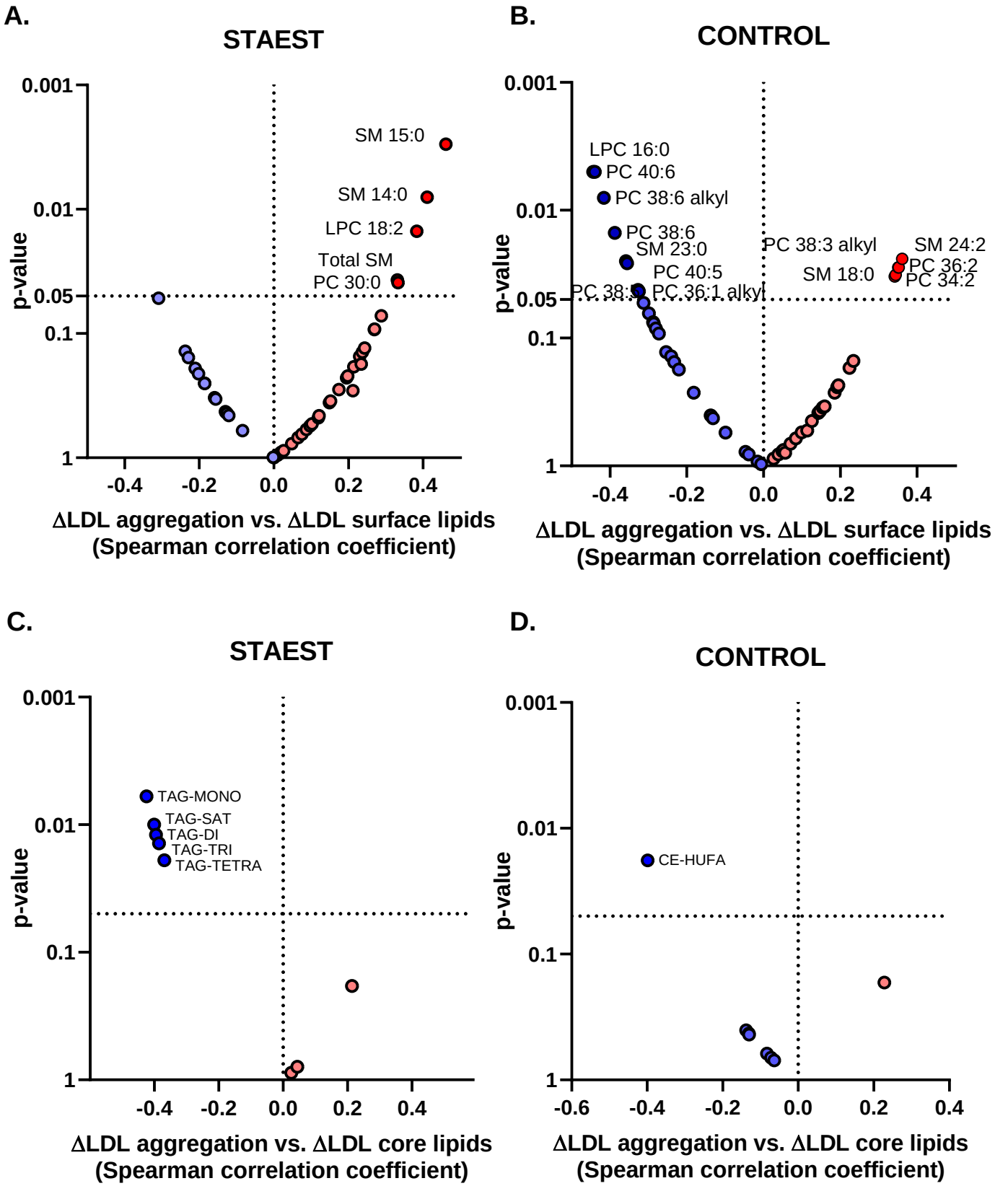

A.
STAEST
0.001
0.01
0.05
0.1
1
p-value
SM 15:0
SM 14:0
LPC 18:2
Total SM
PC 30:0
-0.4
-0.2
0.0
0.2
0.4
ΔLDL aggregation vs. ΔLDL surface lipids
(Spearman correlation coefficient)
B.
CONTROL
LPC 16:0
PC 40:6
PC 38:6 alkyl
PC 38:6
SM 23:0
PC 40:5
PC 38:3 alkyl
SM 24:2
PC 36:2
PC 34:2
SM 18:0
PC 36:1 alkyl
ΔLDL aggregation vs. ΔLDL surface lipids
(Spearman correlation coefficient)
C.
STAEST
TAG-MONO
TAG-SAT
TAG-DI
TAG-TRI
TAG-TETRA
-0.4
-0.2
0.0
0.2
0.4
ΔLDL aggregation vs. ΔLDL core lipids
(Spearman correlation coefficient)
D.
CONTROL
CE-HUFA
-0.6
-0.4
-0.2
0.0
0.2
0.4
ΔLDL aggregation vs. ΔLDL core lipids
(Spearman correlation coefficient)

Figure 4

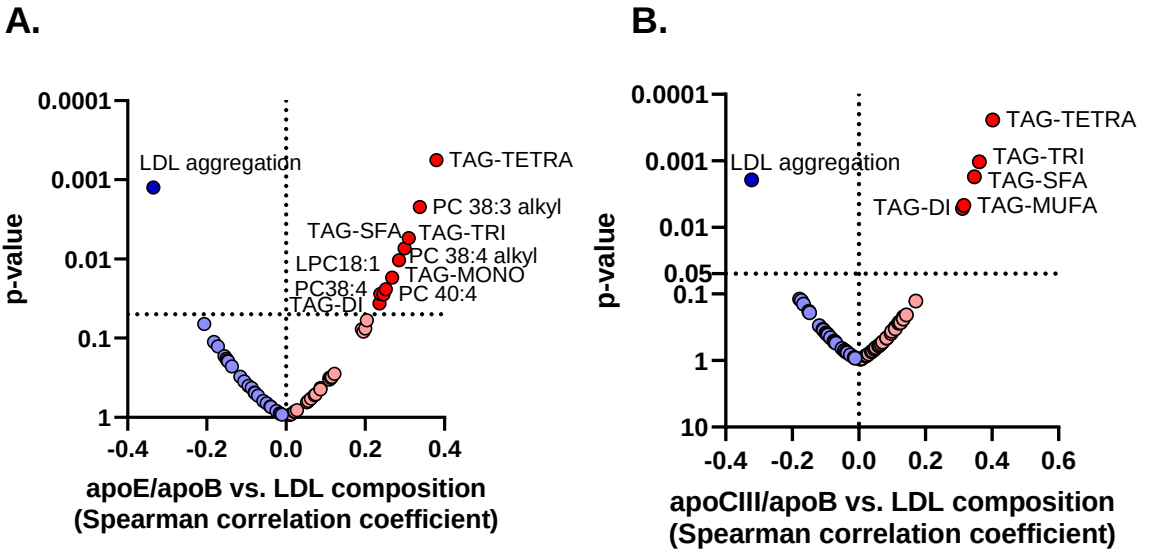
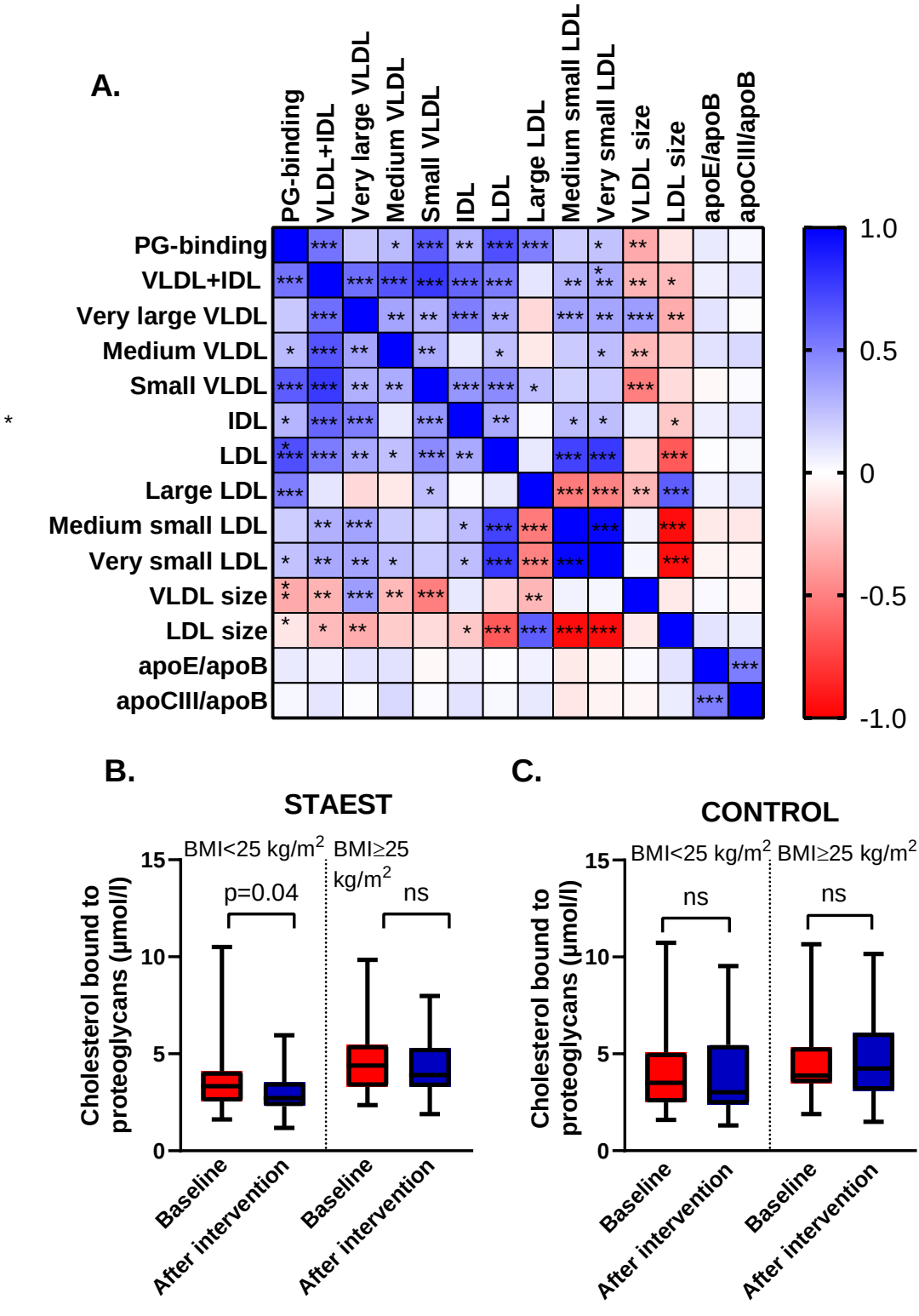

A.
0.0001
0.001
0.01
0.1
1
p-value
LDL aggregation
TAG-TETRA
PC 38:3 alkyl
TAG-SFA
TAG-TRI
LPC18:1
PC 38:4 alkyl
TAG-MONO
PC38:4
PC 40:4
TAG-DI
-0.4
-0.2
0.0
0.2
0.4
apoE/apoB vs. LDL composition
(Spearman correlation coefficient)
B.
0.0001
0.001
0.01
0.05
0.1
1
10
p-value
TAG-TETRA
LDL aggregation
TAG-TRI
TAG-SFA
TAG-DI
TAG-MUFA
-0.4
-0.2
0.0
0.2
0.4
0.6
apoCIII/apoB vs. LDL composition
(Spearman correlation coefficient)

Figure 5.


A.
PG-binding
VLDL+IDL
Very large VLDL
Medium VLDL
Small VLDL
IDL
LDL
Large LDL
Medium small LDL
Very small LDL
VLDL size
LDL size
apoE/apoB
apoCIII/apoB
1.0
0.5
0
-0.5
-1.0
B.
STAEST
BMI<25 kg/m2
BMI≥25 kg/m2
p=0.04
ns
Cholesterol bound to proteoglycans (µmol/l)
15
10
5
0
Baseline
After intervention
Baseline
After intervention
C.
CONTROL
BMI<25 kg/m2
BMI≥25 kg/m2
ns
ns
Cholesterol bound to proteoglycans (µmol/l)
15
10
5
0
Baseline
After intervention
Baseline
After intervention

Plant stanol esters reduce LDL aggregation by altering LDL surface lipids. The BLOOD FLOW randomized intervention study.

Maija Ruuth, Lauri Äikäs, Feven Tigistu-Sahle, Reijo Käkelä, Harri Lindholm, Piia Simonen, Petri T. Kovanen, Helena Gylling & Katariina Öörni

Supplemental files:

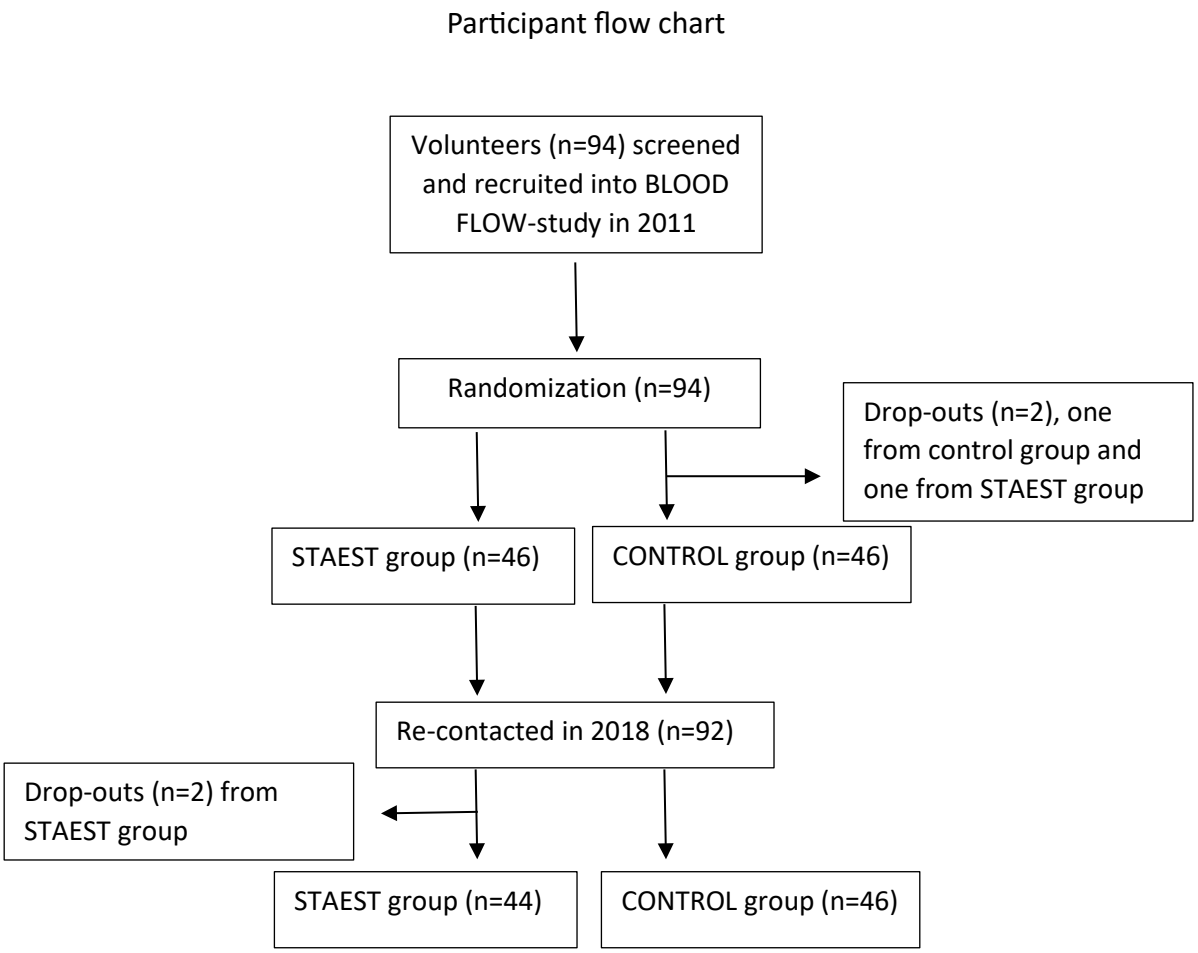
Supplemental Figure I: Participant flow chart

Supplemental Figure II: Binding of serum lipoproteins to proteoglycans at pH 5.5 and pH 7.2

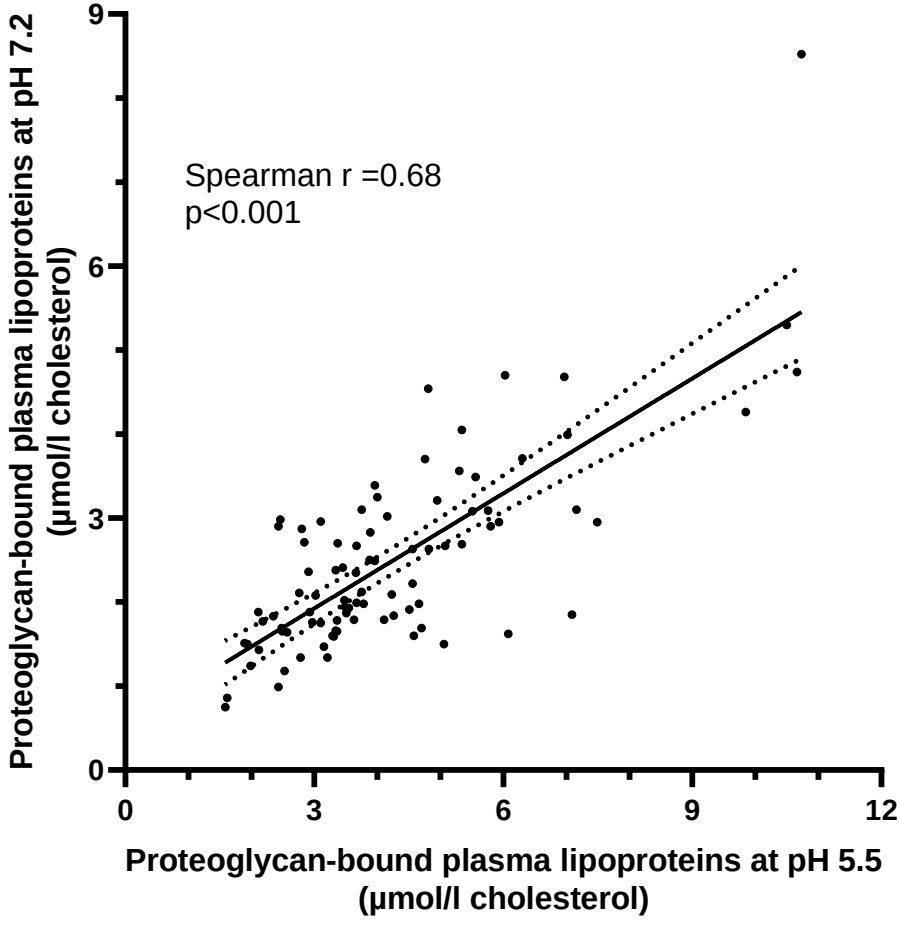
Supplemental Figure III: Correlation of proteoglycan binding and LDL lipid species

Major Resources Table

Supplemental Figure I

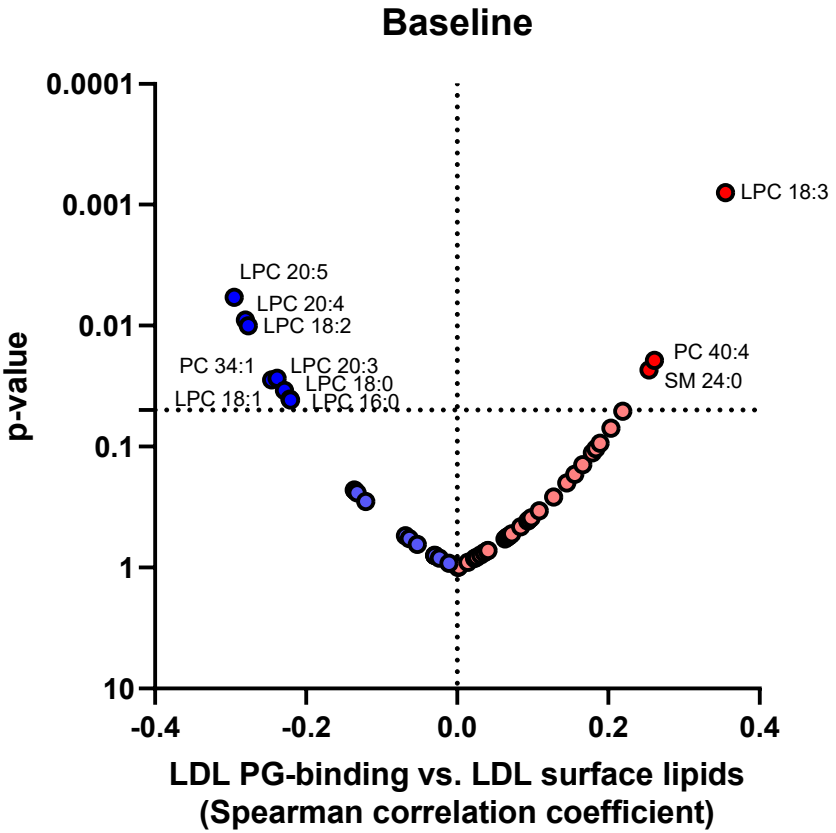


Supplemental Figure II



Binding of serum lipoproteins to human aortic proteoglycans at pH 5.5 and pH 7.2.
The binding of serum lipoproteins (n=90) to human aotic proteoglycans was determined at pH 5.5 (20 mM MES-150 mM NaCl, 2 mM $CaCl_2$-2 mM $MgCl_2$) and at pH 7.2 (20 mM HEPES-50 mM NaCl, 2 mM $CaCl_2$-2 mM $MgCl_2$) in microtiter wells. One µL of the serum samples was incubated in the proteoglycan-coated wells for 1 h, the wells were washed with the same buffer containing 50 mM NaCl in both pH 5.5 and pH 7.2, and cholesterol in each well was determined.

Supplemental Figure III



Spearman correlation coefficient of the PG-binding and LDL lipid species at baseline (n=79). PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin. Lipid species with significant p-values ($p<0.05$) are labelled.

Major Resources Table

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Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
N/A				

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	N/A				
Parent - Female					

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
N/A					

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
N/A			

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
N/A			

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
The data that support the findings of this study are available from the corresponding author upon reasonable request.		

Other

Description	Source / Repository	Persistent ID / URL
Human recombinant sphingomyelinase	University of Helsinki/ Wihuri Research Institute	Produced in house as described in doi: 10.1016/j.jacl.2019.09.011
Human aortic proteoglycans	Wihuri Research Institute	Isolated in house as described in doi: 10.1074/jbc.272.34.21303

DOI [to be added]