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Plant Stanol Esters Reduce LDL (Low-Density Lipoprotein) Aggregation by Altering LDL Surface Lipids The BLOOD FLOW Randomized Intervention Study

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2020-09

Ruuth , M , Äikäs , L , Tigistu-Sahle , F , Käkelä , R , Lindholm , H , Simonen , P , Kovanen , P T , Gylling , H & Öörni , K 2020 , ' Plant Stanol Esters Reduce LDL (Low-Density Lipoprotein) Aggregation by Altering LDL Surface Lipids The BLOOD FLOW Randomized Intervention Study ' , Arteriosclerosis, Thrombosis, and Vascular Biology , vol. 40 , no. 9 , pp. 2310-2321 . https://doi.org/10.1161/ATVBAHA.120.314329

http://hdl.handle.net/10138/326484
https://doi.org/10.1161/ATVBAHA.120.314329

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

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18 **Short title**: Plant stanol esters decrease LDL aggregation

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28 **Keywords: Atherosclerosis, LDL, lipidomics, LDL aggregation, sphingomyelin**

29

30 **Word count: 7154**

31 **The number of figures: 5**

32 **The number of tables: 4**

33 **Supplemental data: 3**

34 **TOC category:** Clinical

35

36 **TOC subcategory**: Arteriosclerosis

37

38

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.

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

70

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

75 **Introduction**

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

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

103 The proatherogenic properties of LDL particles are associated with increased
104 retention and accumulation of LDL in the arterial wall.[11] Thus, after the circulating
105 LDL particles have passed the arterial endothelium and entered the tunica intima, the
106 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

127 ## Subjects and Methods

128 ### Data Sharing

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

131 ### Study participants

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

149 ### Study design, diet, and basic measurements

150 The original study was a randomized, double-blind, placebo-controlled,
151 parallel clinical intervention (Clinical Trials Register #NCT01315964).[8] The
152 participants were randomized using a computer-generated randomization list into two
153 groups. The plant stanol ester group consumed a plant stanol ester enriched
154 rapeseed oil-based spread (3.0 g of plant stanols/day, STAEST group, n=44) three
155 times/day during regular meals. The control group consumed the same spread
156 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).

189 **Mass spectrometry analyses of LDL lipid composition**

190 Total lipids of the isolated LDL particles were extracted for lipid mass spectrometry
191 (MS) with the method of Folch et al.[26] Aliquots of the lipid extracts were dissolved in
192 chloroform/methanol (1:2 v/v) and spiked with the quantitative internal standard
193 mixture designed for human plasma lipids (SPLASH® LIPIDOMIX® Mass Spec
194 Standard No 330707; Avanti Polar Lipids, Inc., Alabama, USA). Just prior to MS,
195 NH_4OH was added to aliquots of the sample extracts to give 1% solution, which
196 supported ionization and prevented sodium adduct formation. The samples were
197 introduced via a syringe pump into the electrospray ionization (ESI) source of a triple
198 quadrupole MS (Agilent 6410 Triple Quad LC/MS; Agilent Technologies, Inc., Santa
199 Clara, USA) at a flow rate of 10 µl/min. MS+ scan was used to detect TAG species as
200 $(M+NH_4)^+$ ions[27] and MS/MS precursor ion scans of m/z 184 and m/z 369 were
201 used to detect phosphorylcholine –containing phospholipid species
202 (phosphatidylcholine PC, lysophosphatidylcholine LPC, and SM) and cholesteryl
203 ester (CE) species, respectively. The ESI-MS/MS instrument was set to a source
204 temperature of 250°C and collision energies optimized for each lipid class (10-30 eV)
205 were used. Nitrogen was used as the collision, nebulizing (20 psi), and drying gas
206 (11 µl/min). Data analysis of the mass spectra were performed by using MassHunter
207 Workstation qualitative analysis software (Agilent Technologies, Inc.) and the
208 individual lipid species were quantified and converted to molar percent data using the
209 internal standards and Lipid Mass Spectrum Analysis (LIMSA) software, which has
210 an inbuilt deisotoping routine that will automatically correct for an overlap of isotope
211 peaks.[28] The proportions of the various lipid species are expressed as percentages
212 of surface lipids (PC, SM, and LPC species) and percentages of core lipids (CE and
213 TAG species).

214 **Measurement of lipoprotein binding to proteoglycans**

215 Proteoglycans were isolated from human aortas as described previously[29]
216 and used to coat 96-well plates overnight at 4°C and blocked with 3% BSA, 1% fat-
217 free milk powder, and 0.05% Tween 20 in phosphate-buffered saline for 1 h at 37°C.
218 1 µL of each serum sample was diluted in 100 µL 20 mM MES, 140mM NaCl, 2 mM
219 $CaCl_2$, and 2 mM $MgCl_2$ pH 5.5 and incubated in the wells for 1h at 37°C. The 96-well
220 plate was then washed with the same buffer containing 50 mM NaCl and the amount

221 of bound cholesterol was measured using Amplex red cholesterol kit (Thermo
222 Scientific). Each sample was analysed blinded and in duplicate. To make it easier to
223 compare the proteoglycan-binding of plasma lipoproteins to data published earlier,
224 we also performed the binding assay at neutral pH. For this purpose, only the binding
225 and washing buffers were changed. Thus, 1 µL of each serum sample was diluted to
226 100 µL of 20 mM HEPES, 50 mM NaCl, 5 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.2, and
227 incubated for 1 h at 37°C. After the incubation, the wells were washed using the
228 same buffer and the amount of cholesterol bound to the proteoglycans was
229 determined as described above.

230 **Statistical analysis**

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

245 **Results**

246 **Clinical characteristics and outcome of the intervention**

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

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

277 Dietary information of the participants was collected from a 3-day food diary
278 prior to baseline measurements and prior to the end of the study period. In the
279 STAEST group, the intake of mono- and polyunsaturated fatty acids increased
280 (**Table 3**). In the CONTROL group, no statistically significant changes in the diet
281 were observed. Sitosterol, a biomarker of cholesterol absorption efficiency[30] was
282 measured in serum. As expected, in the STAEST group sitosterol-to-cholesterol –
283 ratio was decreased by 33 % (from 1.41 ± 0.62 to 0.95 ± 0.32 µmol/mmol cholesterol,
284 p<0.000), while no changes in this ratio was observed in the CONTROL group (from
285 1.49 ± 0.61 to 1.51 ± 0.59 µmol/mmol cholesterol).

286 **LDL aggregation susceptibility is decreased in STAEST group**

287 To study whether a plant stanol ester-rich diet would influence LDL
288 aggregation susceptibility, LDL particles were first isolated from the serum samples.
289 The LDL samples were diluted to a concentration 200 µg of protein/mL, and LDL
290 aggregation was induced with human recombinant SMase. LDL aggregate formation
291 and increase in the aggregate size was measured with dynamic light scattering every
292 15-30 minutes, and LDL aggregate size after incubation for 2 h was determined from
293 the aggregate size vs. time –curves. Previously, aggregate size at this time point has
294 been shown to best reflect the aggregation susceptibility of the particles.[20] **Figure 1A**
295 shows LDL aggregation measurement of two participants from the STAEST group at
296 baseline and after the intervention. Consistently with previously published data,[20, 25]
297 there was a large inter-individual variation in LDL aggregation susceptibility, and
298 these aggregation measures did not differ between the STAEST and the CONTROL
299 groups at baseline (blue boxes in **Figure 1 B**). In the STAEST group, there was a
300 significant decrease in LDL aggregation susceptibility, found in about 2/3 of the
301 participants. Consequently, the median LDL aggregate size at 2 h shifted from 1490
302 nm at baseline to 620 nm after intervention. In the CONTROL group the aggregate
303 size did not change significantly. LDL aggregation susceptibility did not correlate with
304 LDL particle size, as also shown before.[20, 25]

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

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

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

328 **Changes in LDL composition explain changes in LDL aggregation**
329 **susceptibility**

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

338

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

355 The susceptibility of LDL to sphingomyelin hydrolysis by *Bacillus cereus*
356 spingomyelinase and the resulting aggregation of the lipolyzed LDL particles has
357 been shown to be increased if LDL particles are enriched in apoC-III.[33, 34] On the
358 other hand, addition of small exchangeable apolipoproteins to aggregating LDL
359 particles inhibits their aggregation.[35] Therefore, we next analysed the apoCIII and
360 apoE contents of LDL particles from samples obtained at baseline and after the
361 intervention. In the STAEST group the molar ratios of apoE/apoB and apoCIII/apoB
362 were 12.2±6.0 mmol/mol and 286±150 mmol/mol, respectively, at baseline and
363 13.4±8.4 mmol/mol and 315±121 mmol/mol after the intervention. In the CONTROL
364 group the values were similar (12.2±8.0 mmol/mol of apoE/apoB and 293±142
365 mmol/mol of apoCIII/apoB at baseline and 13.4±10.9 mmol/mol of apoE/apoB and
366 328±188 mmol/mol of apoCIII/apoB after the intervention). These ratios did not differ
367 either between the groups or between the time points. Surprisingly, however, we
368 observed that both the apoE/apoB-ratio and the apoCIII/apoB-ratio correlated
369 inversely with LDL aggregation at baseline (**Figure 4**). In addition, the differences in
370 apoE/apoB and apoCIII/apoB were associated significantly with differences in various
371 LDL lipids (**Figure 4**). In particular, increased amount of apoE or apoCIII was

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

377

378 **LDL binding to proteoglycans is decreased in the STAEST group**

379 The binding of lipoproteins to human aortic proteoglycans was determined
380 using microtiter wells that had been coated with the proteoglycans, BSA-blocked, and
381 incubated with serum diluted in a buffer containing 20 mM MES, 2 mM $CaCl_2$, 2 mM
382 $MgCl_2$, 150 mM NaCl and having pH 5.5.[29, 36, 37] Unbound lipoproteins were removed
383 by washing, and the amounts of bound lipoproteins were determined by measuring
384 the cholesterol concentration in the proteoglycan-coated wells. The interaction of
385 plasma lipoproteins to proteoglycans is much stronger at acidic pH than at neutral
386 pH,[29, 37] but as shown in **Supplemental Figure II**, binding of the lipoproteins at pH
387 5.5 correlated significantly with binding of the same lipoproteins to proteoglycans at
388 pH 7.2, but at lower ionic strength (50 mM NaCl).

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

398 In the STAEST group, consumption of plant stanol esters led to decrease in
399 the binding of LDL to proteoglycans (from 4.1 µmol/L to 3.7 µmol/L, p=0.032),
400 whereas in the CONTROL group there were no changes (from 4.3 µmol/L to 4.4
401 µmol/L, p=0.604). Similarly to plant stanol -induced changes in LDL aggregation, also
402 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

411 **Discussion**

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

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

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

435 LDL aggregation susceptibility decreased in 2/3 of the participants of the
436 STAEST group with consumption of a plant stanol ester -enriched spread. In
437 contrast, only minor changes in LDL aggregation were observed when the
438 corresponding control spread was consumed. Similar results were observed
439 previously, when diet rich in unsaturated fats was found to reduce LDL aggregation
440 susceptibility in 2/3 of the participants with minimal changes observed in the control
441 group.[20] Consistent with our previous results,[20] LDL aggregation susceptibility
442 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

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

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

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

506

Acknowledgement

We would like to thank Maija Atuegwu and Leena Kaipiainen for excellent technical assistance and Dr. Martin Hermansson for his help in lipidomic analysis.

Sources of Funding: Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. This study was also supported by grants from the Academy of Finland (#315568), the Finnish Foundation for Cardiovascular Research to KÖ, and MR and Raisio Nutrition Ltd.

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.

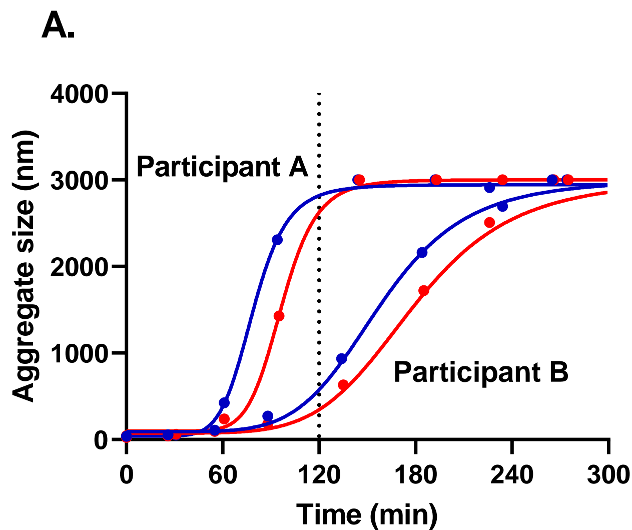
	CONTROL		**STAEST**	
Lipoprotein particle concentration	Baseline	After intervention	Baseline	After intervention
Triglyceride-rich particles (nmol/l)	49.3 (4.0-333)	55.4 (9.6-304)	49.9 (13.6-311)	40.0 (11.1-221)
Large (% of TG-rich)	1.0 (0-11)	0.7 (0-11.5)	0.85 (0-21.5)	1.6 (0-14.2)*
Medium (% of TG-rich)	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)

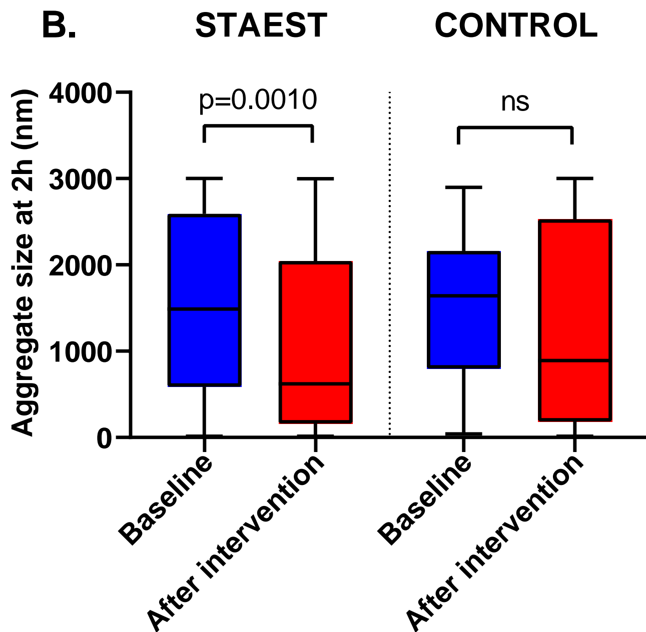
Table 3. Dietary information was collected from 3-day food diaries at baseline and at the end of the intervention. The values of dietary energy % are presented as median (range). Statistical differences within groups were calculated between before and after values of the intervention using Wilcoxon signed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

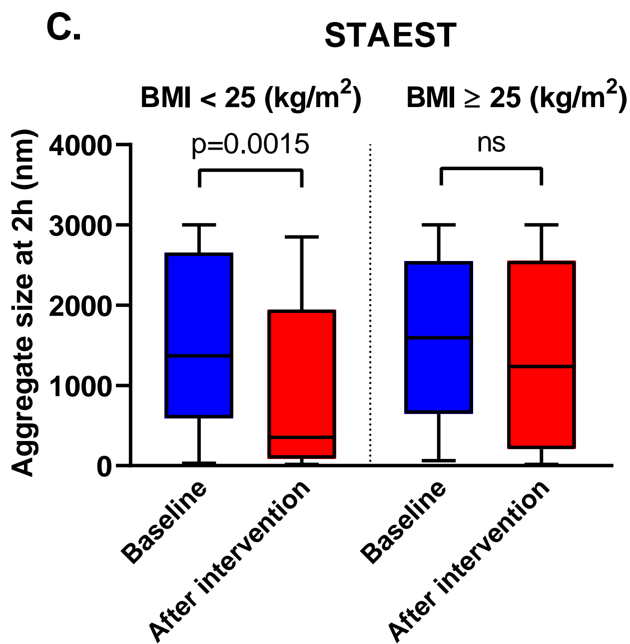
	CONTROL		STAEST	
Dietary E%	Baseline	End of intervention	Baseline	End of intervention
Protein E%	16 (10-28)	16 (10-26)	17 (12-25)	17 (10-24)
Fat E%	35 (16-47)	36 (25-50)	33 (23-50)	35 (19-45)
SFA E%	11 (5-20)	12 (7-21)	11 (7-21)	11 (5-16)
MUFA E%	12 (5-23)	12 (8-18)	11 (7-20)	13 (7-18)**
PUFA E%	5 (3-13)	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/m²				**BMI ≥ 25 kg/m²**			
	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/m²)	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.
Participant A
Participant B
Aggregate size (nm)
Time (min)
0
1000
2000
3000
4000
0
60
120
180
240
300


B.
STAEST
CONTROL
p=0.0010
ns
Aggregate size at 2h (nm)
0
1000
2000
3000
4000
Baseline
After intervention
Baseline
After intervention


C.
STAEST
BMI < 25 (kg/m2)
BMI ≥ 25 (kg/m2)
p=0.0015
ns
Aggregate size at 2h (nm)
0
1000
2000
3000
4000
Baseline
After intervention
Baseline
After intervention

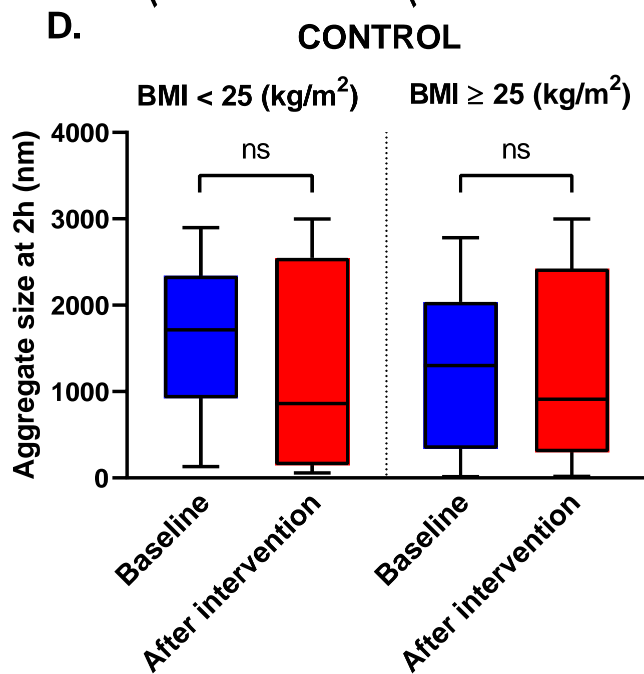
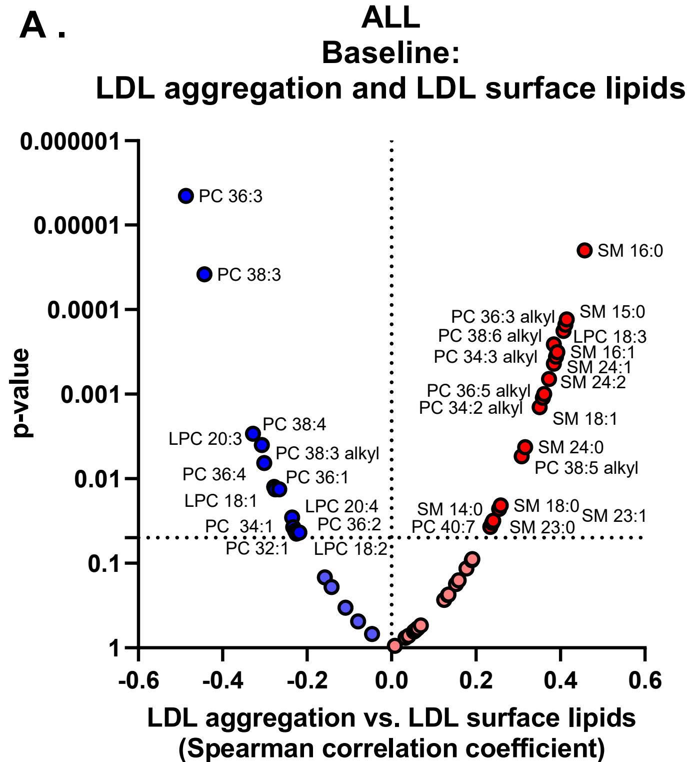

D.
CONTROL
BMI < 25 (kg/m2)
BMI ≥ 25 (kg/m2)
ns
ns
Aggregate size at 2h (nm)
0
1000
2000
3000
4000
Baseline
After intervention
Baseline
After intervention

Figure 2



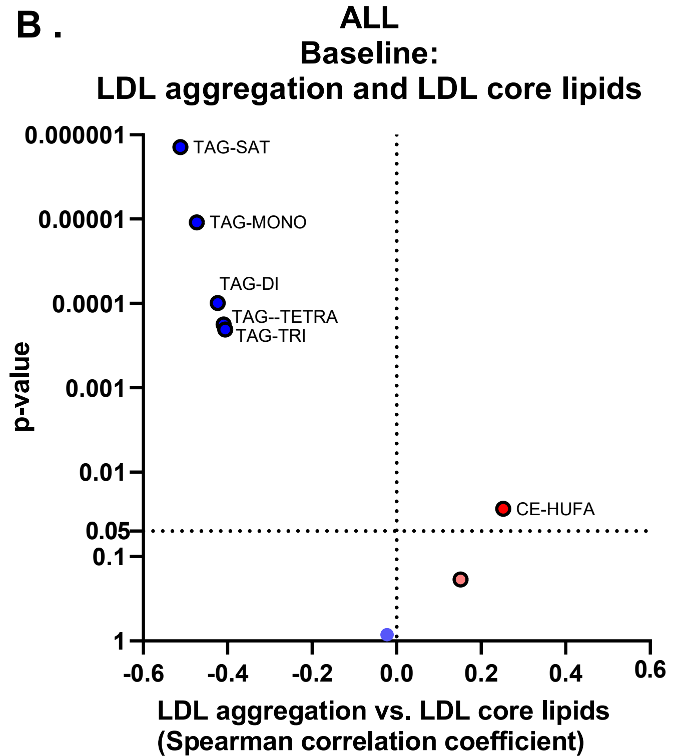


Figure 3.

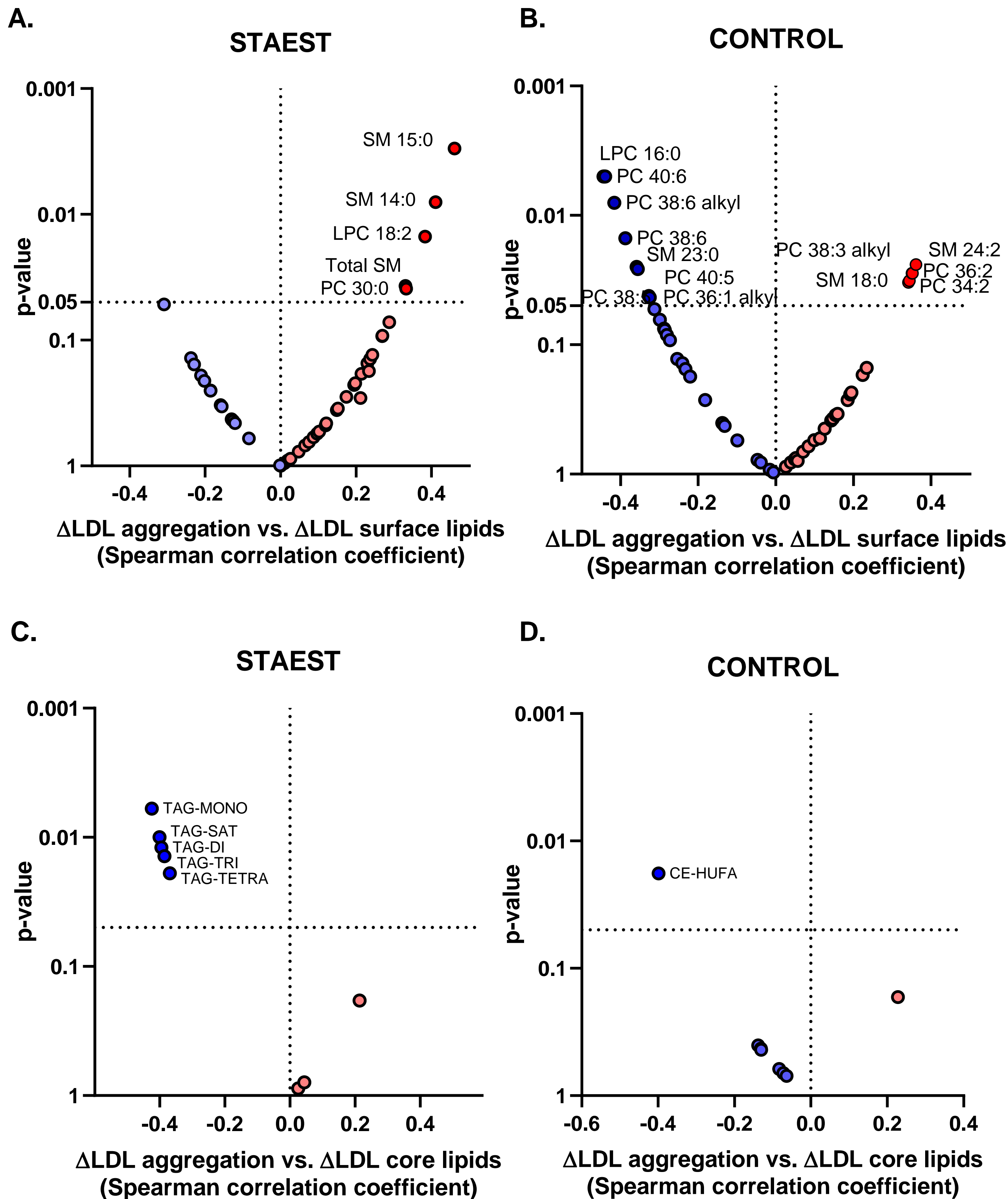
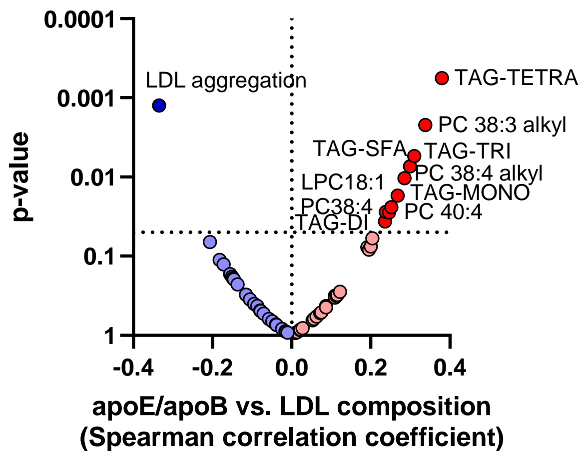


Figure 4



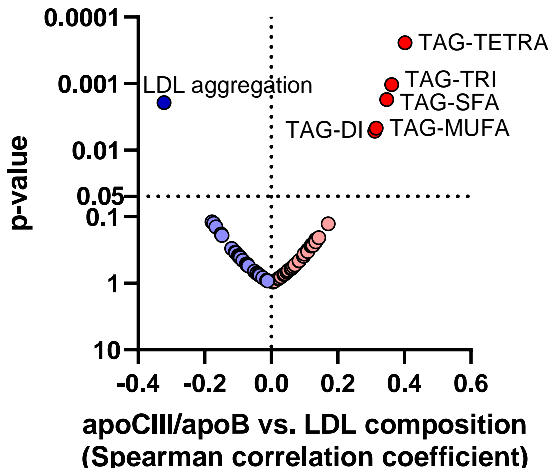


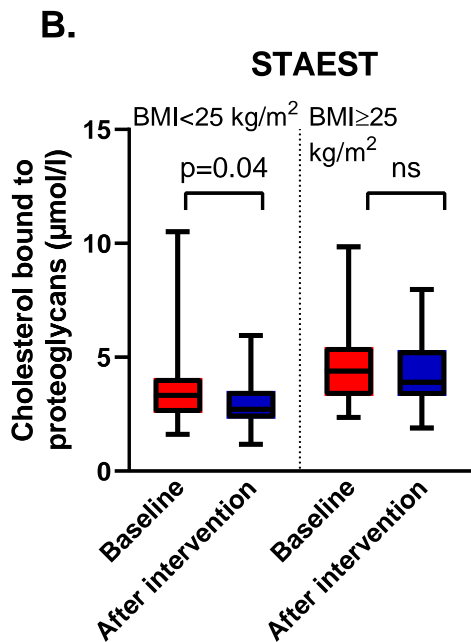
Figure 5.

A.

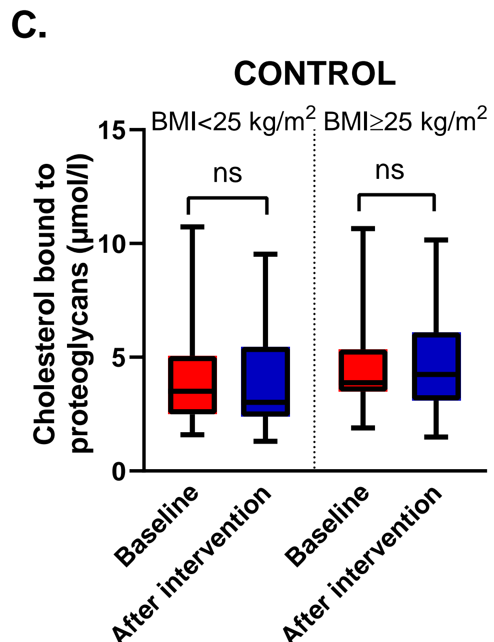
PG-binding
VLDL+IDL
Very large VLDL
Medium VLDL
Small VLDL
IDL
LDL
Large LDL
Medium small LDL
Very small LDL
VLDL size
LDL size
apoE/apoB
apoCIII/apoB

1.0
0.5
0
-0.5
-1.0

B.



C.



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

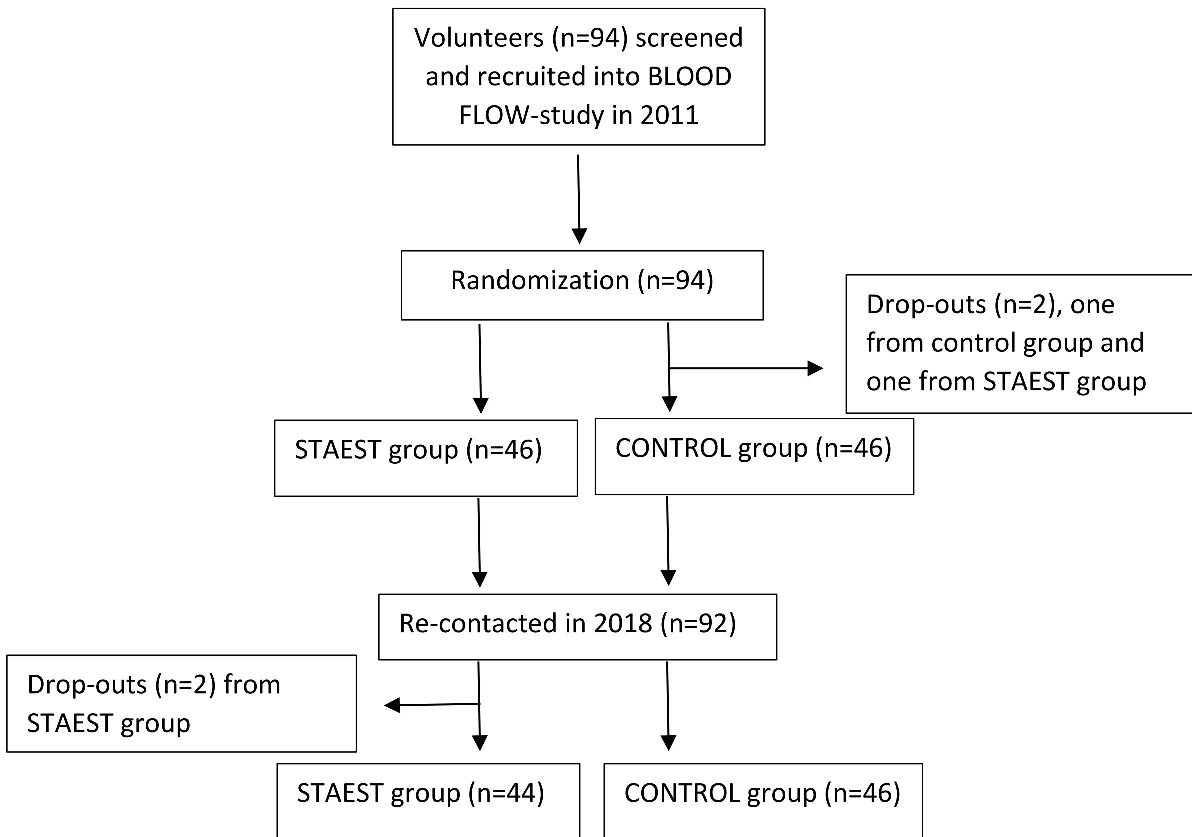
Supplemental Figure II: Binding of serum lipoproteins to proteoglycans at pH 5.5 and pH 7.2

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

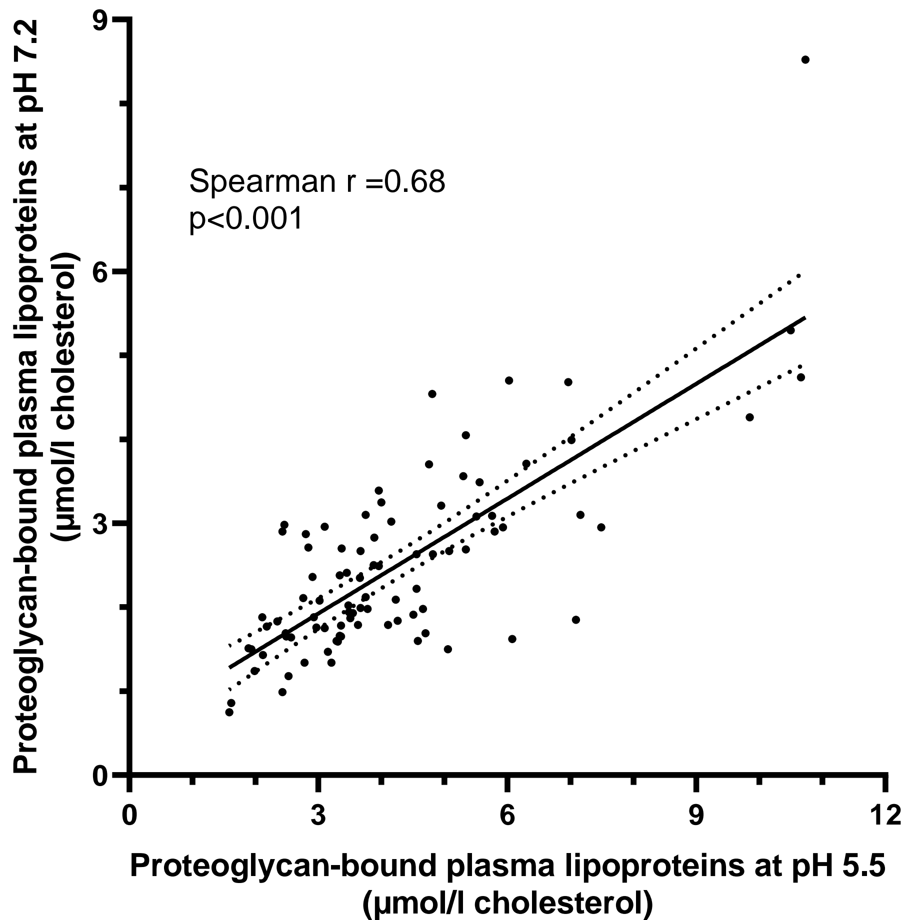
Major Resources Table

Supplemental Figure I

Participant flow chart

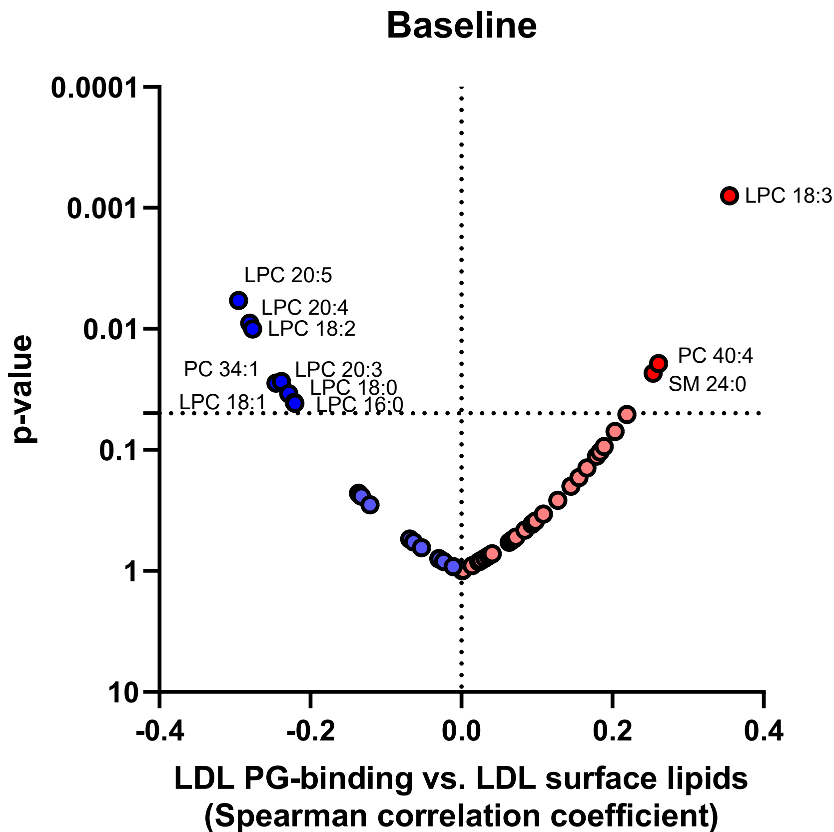


Supplemental Figure II



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]