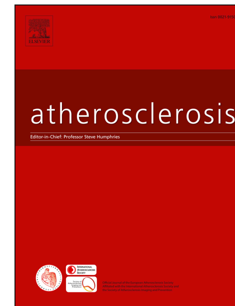
Accepted Manuscript



The effect of intakes of fish and Camelina sativa oil on atherogenic and anti-atherogenic functions of LDL and HDL particles: A randomized controlled trial

Suvi Manninen, Maria Lankinen, Arja Erkkilä, Su Duy Nguyen, Maija Ruuth, Vanessa de Mello, Katariina Öörni, Ursula Schwab

PII: S0021-9150(18)31548-X

DOI: https://doi.org/10.1016/j.atherosclerosis.2018.12.017

Reference: ATH 15818

To appear in: *Atherosclerosis*

Received Date: 25 October 2018

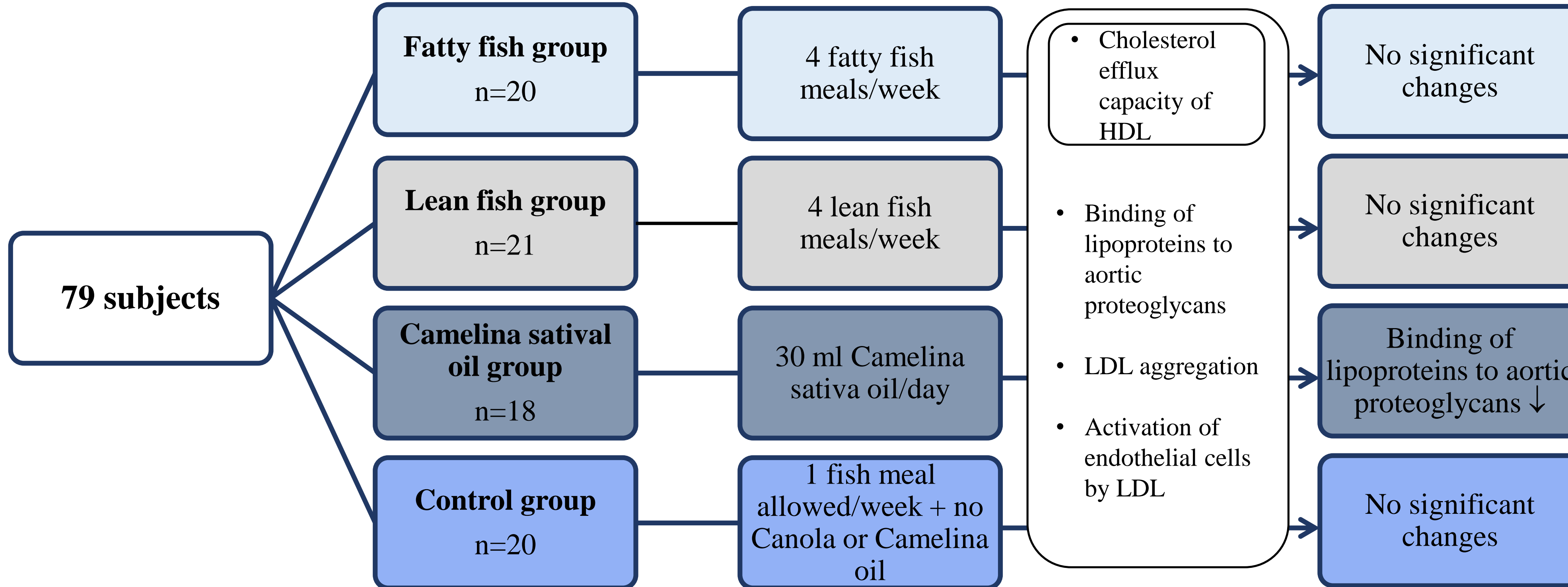
Revised Date: 29 November 2018

Accepted Date: 13 December 2018

Please cite this article as: Manninen S, Lankinen M, Erkkilä A, Nguyen SD, Ruuth M, de Mello V, Öörni K, Schwab U, The effect of intakes of fish and Camelina sativa oil on atherogenic and anti-atherogenic functions of LDL and HDL particles: A randomized controlled trial, *Atherosclerosis*, https://doi.org/10.1016/j.atherosclerosis.2018.12.017.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The effect of intakes of fish and camelina sativa oil on atherogenic and anti-atherogenic functions of LDL and HDL particles – a randomized controlled trial



ACCEPTED MANUSCRIPT

The effect of intakes of fish and Camelina sativa oil on atherogenic and anti-atherogenic functions of LDL and HDL particles: A randomized controlled trial

Suvi Manninen [a], *Maria Lankinen* [a], *Arja Erkkilä* [a], *Su Duy Nguyen* [c], *Maija Ruuth* [c,d], *Vanessa de Mello* [a], *Katariina Öörni* [c], *Ursula Schwab* [a,b]

[a] Institute of Public Health and Clinical Nutrition, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland

[b] Department of Medicine, Endocrinology and Clinical Nutrition, Kuopio University Hospital, Finland

[c] Atherosclerosis Research Laboratory, Wihuri Research Institute, Helsinki, Finland

[d] Research Programs Unit, University of Helsinki, Finland

Correspondence: Suvi M. Manninen

University of Eastern Finland, Kuopio Campus
School of Medicine
Institute of Public Health and Clinical Nutrition
P.O.Box 1627
70211 Kuopio, Finland

email: suvi.manninen@uef.fi

ACCEPTED MANUSCRIPT

1 **Abstract**

ACCEPTED MANUSCRIPT

2 *Background and aims*: Omega-3 fatty acids are known to have several cardioprotective
3 effects. Our aim was to investigate the effects of intakes of fish and Camelina sativa oil
4 (CSO), rich in alpha-linolenic acid, on the atherogenic and anti-atherogenic functions of LDL
5 and HDL particles.
6 *Methods*: Altogether, 88 volunteers with impaired glucose metabolism were randomly
7 assigned to CSO (10 g of alpha-linolenic acid/day), fatty fish (4 fish meals/week), lean fish (4
8 fish meals/week) or control group for 12 weeks. 79 subjects completed the study. The binding
9 of lipoproteins to aortic proteoglycans, LDL aggregation and activation of endothelial cells
10 by LDL and cholesterol efflux capacity of HDL were determined *in vitro*.
11 *Results*: Intake of CSO decreased the binding of lipoproteins to aortic proteoglycans in a non-
12 normalized model (p=0.006). After normalizing with serum concentrations of non-HDL
13 cholesterol, apolipoprotein B (apoB) or LDL cholesterol, which decreased in the CSO group,
14 the change was no longer statistically significant. In the fish groups, there were no changes in
15 the binding of lipoproteins to proteoglycans. Regarding other lipoprotein functions, there
16 were no changes in any of the groups.
17 *Conclusions*: Intake of CSO decreases the binding of lipoproteins to aortic proteoglycans by
18 decreasing serum LDL cholesterol concentration, which suggests that the level of apoB-
19 containing lipoproteins in the circulation is the main driver of lipoprotein retention within the
20 arterial wall. Intake of fish or CSO has no effects on other lipoprotein functions.
21
22 **Keywords**: cholesterol efflux, diet, human, interleukin-8, LDL aggregation, omega-3 fatty
23 acid, proteoglycan
24

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

25 **1. Introduction**

26

27 Progression of atherosclerosis consists of several molecular and inflammatory processes,
28 taking place in the arterial wall [1-3]. The initiating process in atherogenesis is retention of
29 lipoproteins, mainly LDL particles, in the arterial intima. Lipoproteins containing
30 apolipoprotein B (apoB) and apoE bind to proteoglycans of the intimal layer and can be
31 modified, e.g. by oxidation and lipolysis. Modified lipoproteins may further aggregate or fuse
32 [4], which increases their binding strength to proteoglycans [5, 6] and prevents their exit back
33 to the bloodstream [7]. The aggregation susceptibility of LDL varies among individuals and
34 was recently shown to associate with cardiovascular deaths [8]. LDL, particularly when
35 modified, also stimulate several atherogenic processes, such as interleukin-8 (IL-8) secretion
36 of endothelial cells, which induce the chemotactic recruitment of monocytes to the arterial
37 intima [1, 9, 10]. Modified lipoproteins are taken up by macrophages, which leads to foam-
38 cell formation. Retained and modified lipoproteins and foam cell formation leads to the
39 release of proinflammatory cytokines and bridging molecules (e.g. lipoprotein lipase),
40 smooth muscle cell proliferation, induced synthesis of proteoglycans and eventually further
41 entrapment of lipoproteins [4, 11]. These reactions trigger a self-accelerating process that
42 eventually leads to progression of atherosclerosis.

43

44 Cholesterol efflux from macrophage foam cells has a central role in the reverse cholesterol
45 transport, which is considered the primary anti-atherogenic mechanism of HDL [12]. HDL
46 particles, however, possess also several other atheroprotective activities, such as anti-
47 inflammatory and antioxidative functions [12, 13]. The profile and structure of HDL particles
48 are key mediators of their atheroprotective functions. Examples of structural components,
49 which can mediate HDL function, are apoE and serum amyloid A (SAA) [14, 15]. ApoE is

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

50 known to have several anti-atherogenic functions such as mediating plasma lipoprotein
51 clearance by the liver [14]. Furthermore, apoE has been shown to contribute to lipid retention
52 via HDL [16]. HDL particles containing apoE can bind to proteoglycans and, therefore,
53 interfere with the formation of LDL-proteoglycan complex. This competition of binding sites
54 could potentially prevent LDL accumulation in the arterial wall. Another component which
55 could impair the atheroprotective functions of HDL is SAA, which is carried primarily in
56 HDL particles in plasma [15]. SAA is considered a potential mediator of atherosclerosis by
57 acting as a bridging protein between lipoproteins and proteoglycans and impairing cholesterol
58 efflux capacity.
59
60 Diet is an important modifiable lifestyle factor in the prevention of CVD [17, 18]. Among the
61 beneficial dietary factors, fish has been considered as an important component in a
62 cardioprotective diet. Fish, especially fatty fish, is a rich source of *n*-3 polyunsaturated fatty
63 acids (*n*-3 PUFAs), which have been shown to affect several molecular mechanisms and
64 pathways related to CVD, such as physical and chemical properties of cellular membranes,
65 inflammation and regulation of gene expression [19]. Previous studies have shown that intake
66 of *n*-3 PUFAs from fish increases HDL cholesterol level but has no effect on LDL cholesterol
67 [20] whereas evidence on the effects of plant-derived *n*-3 fatty acid, alpha-linolenic acid
68 (ALA), on serum lipids is inconsistent [21]. In addition to fatty fish, also lean fish intake has
69 been found to have cardioprotective effects [22-24]. This suggests that the beneficial effects
70 of fish consumption on CVD risk factors may, in addition to *n*-3 PUFAs, be mediated
71 through other components in the fish, such as protein and other bioactive compounds or their
72 interactions.
73

ACCEPTED MANUSCRIPT

74 Although the effects of *n*-3 PUFAs on CVD have been extensively studied, little is known
75 about the effects of dietary intake of these fatty acids on lipoprotein functions related to
76 atherosclerosis. To this end, the aim of our study was to investigate the effect of Camelina
77 sativa oil (CSO), high in ALA, and fish intake on the pro- and anti-atherogenic functions of
78 LDL and HDL particles, such as binding of lipoproteins to aortic proteoglycans, LDL
79 aggregation, cholesterol efflux capacity and activation of endothelial cells in subjects with
80 impaired glucose metabolism.
81

ACCEPTED MANUSCRIPT

82 **2. Materials and methods**

83

84 **2.1 Subjects**

85 Details of the subjects, study design and recruitment were described previously [25]. Briefly,
86 volunteers aged 40 to 75 years and with impaired glucose metabolism were recruited from
87 Kuopio area. Glucose metabolism was one of the primary endpoints in this study and,
88 therefore, the main inclusion criterion was fasting plasma glucose concentration 5.6–6.9
89 mmol/l. The 2-h glucose concentration in the oral glucose tolerance test had to be ≤ 11.0
90 mmol/l. A total of 79 subjects completed the study. Subjects were randomly assigned into
91 four parallel groups for 12 weeks: CSO, lean fish, fatty fish or control group. Randomization
92 was stratified by sex and use of statins. The subjects received both oral and written
93 information and informed consent was obtained from all of the subjects. The study was
94 approved by the Ethical committee of the Hospital District of Northern Savo (55/2012) and
95 ethical guidelines were followed throughout the study.

96

97 **2.2 Study design**

98 The study diets were isocaloric and current nutrient recommendations [26] were followed
99 except for fish and ALA intakes. Fish diets included four fish meals per week, e.g. salmon,
100 Baltic herring and vendace in the fatty fish group providing around 1 g of EPA+DHA per day
101 and e.g. pike, perch, pike-perch and saithe in the lean fish group. The CSO group ingested 30
102 ml of CSO (27 g) in order to get 10 g of ALA per day. Intake of ALA > 4.5 g/day has been
103 shown to result in increased levels of EPA in the plasma phospholipids [27]. The control and
104 CSO groups were allowed to eat one fish meal per week and were instructed to consume lean
105 meat and chicken. Subjects kept daily consumption records regarding the intake of fish
106 (number of meals and type of fish) and subjects in the CSO group recorded also the intake of

ACCEPTED MANUSCRIPT

107 CSO. Food records from four consecutive days, including one weekend day, were collected
108 and checked at return by clinical nutritionists at baseline and during the study in weeks 3, 7
109 and 11. In the CSO group, intakes of total fat, PUFA and ALA increased as compared with
110 the control group. In the fatty fish group, intakes of EPA, DHA and vitamin D increased and
111 intake of carbohydrates decreased as compared with the control group. In the lean fish group
112 intake of vitamin D increased as compared with the control group. Detailed dietary intakes
113 have been reported previously [25].
114
115 The blood samples were drawn after 10-hour overnight fasting at baseline (0 wk) and at the
116 end of the study (12 wk). Physical activity, alcohol intake, smoking, body weight and use of
117 medications known to affect the measures of lipid metabolism were to be kept constant
118 during the study. As an objective measure of compliance, the proportions of plasma fatty
119 acids in phospholipids were measured using gas chromatography as previously described [28]
120 with an exception of using C19:0 as an internal standard instead of C17:0.
121
122 **2.3 Analytical methods**
123 *Binding of lipoproteins to proteoglycans*
124 Proteoglycan binding was assessed in 96-well plates coated with proteoglycans isolated from
125 human aortas as previously described [29]. 1 µl plasma was added to the wells and incubated
126 for 1 h. The amount of cholesterol bound to the wells was measured with Amplex Red
127 cholesterol assay kit.
128
129
130
131

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

132 *Isolation of LDL and HDL*

133 LDL (d = 1.019–1.063 g/ml) and HDL (d = 1.063–1.210 g/ml) were prepared from isolated
134 plasma by sequential flotation ultracentrifugation using D_2O for density adjustment as
135 described previously [30].

136

137 *LDL aggregation*

138 LDL was isolated from the plasma and LDL stability was determined by inducing LDL
139 aggregation by human recombinant sphingomyelinase as previously described [8]. LDL
140 aggregation was followed by measuring the particle size using dynamic light scattering.

141

142 *Culture of human coronary artery endothelial cells (HCAECs) and analysis of LDL effect on*
143 *endothelial cell activation*

144 HCAECs (PromoCell) were cultured in Endothelial Cell Growth Medium MV supplemented
145 with 5% fetal calf serum, 0.4% endothelial cell growth supplement, 10 ng/ml epidermal
146 growth factor, 90 µg/ml heparin, 1 µg/ml hydrocortisone (Supplement pack, Catalog Number
147 C-39220, PromoCell), 100 U/mL penicillin streptomycin solution, and 50 ng/ml amphotericin
148 B to yield Complete Medium in T-75 flask according to the manufacturer's instructions.
149 Confluent HCAECs were washed with 15 ml of PBS, trypsinized, and replated in complete
150 medium, as described below. Endothelial cell activation was measured in HCAECs between
151 passages 4 and 7. HCAECs were seeded at a density of 1-2 x 104 cells/well in 96- well plate
152 and cultured for 2-3 days until the cells reached 90-95% confluency. To determine the effect
153 of LDL on IL-8 secretion, HCAECs grown in Complete Medium were washed, placed in
154 serum-free medium and incubated with LDL (50 µg protein/ml) for 6 h. The amount of IL-8
155 released into the media was measured by an IL-8 ELISA Kit (R & D Systems, Minneapolis,
156 Minnesota, USA).

157

158 *Culture of primary human monocyte-derived macrophages*

159 Human monocytes were isolated from buffy coats (Finnish Red Cross Blood Transfusion
160 Center, Helsinki, Finland) by centrifugation in Ficoll-Paque gradient as described [31].
161 Washed cells were suspended in DMEM supplemented with 100 U/ml penicillin and 100
162 µg/ml streptomycin, counted, and seeded on 24 well-plates (1.5 million cells per well). After
163 1 h incubation, non-adherent cells were removed, and the medium was replaced with
164 macrophage-SFM medium (Gibco) supplemented with 1% penicillin-streptomycin and 10
165 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) (Biosite, San Diego,
166 USA). The cells were cultured for 7 days in the presence of GM-CSF to allow them to
167 differentiate into GM-CSF macrophages. The medium was then changed every 2 to 3 days
168 throughout the culture period.

169

170 *Cholesterol efflux from macrophage foam cells*

171 The monocyte-derived macrophages were incubated in DMEM (pH 7.4) containing 25 µg/ml
172 of [3H]CE-acetyl-LDL for 24 h to induce the formation of foam cells. To measure cholesterol
173 efflux, macrophages were washed with PBS, and fresh media containing the various HDL (25
174 µg protein/ml) were added. After incubation for 16 h, the media were collected, centrifuged
175 at 300 g for 10 min to remove cellular debris, and the radioactivity in the supernatants was
176 determined by liquid scintillation counting. Cells were solubilized with 0.2 M NaOH and
177 analyzed for radioactivity. Cholesterol efflux was expressed as the percentage radioactivity in
178 the medium relative to the sum of total radioactivity present in the medium and the cells.
179 Cholesterol efflux to the incubation medium in the absence of HDL was considered as basal
180 efflux and was subtracted from the efflux values obtained in the presence of HDL. In an
181 initial experiment using THP-1 cells, a small increase in cholesterol efflux was observed in

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT
182 the fatty fish group but not in other groups (data not shown). Therefore, the fatty fish group
183 was selected to determine cholesterol efflux capacity using human primary macrophages, a
184 relevantly physiological cell type.
185
186 *Analyses of SAA, apoE, serum lipids and lipoprotein subclasses*
187 Plasma apoE was assessed using ELISA Development Kit (catalog number 3712-1A-6;
188 Mabtech, Nacka Strand, Sweden). SAA in serum was measured using Invitrogen SAA
189 ELISA Kit (Cat nr. KHA0011, KHA0012 or KHA0011C; Thermo Fisher Scientific).
190 Concentrations of serum total, LDL and HDL cholesterol were analyzed as previously
191 described [25]. Serum concentrations of lipoprotein subclasses were measured using high-
192 throughput proton NMR spectroscopy, method has been described in detail previously [32].
193
194 **2.4 Statistical analyses**
195 IBM SPSS statistical software (v. 24, IBM Corp., Armonk, NY) was used for statistical
196 analyses. The Kolmogorov-Smirnov test was used to test the normality of the variables and
197 logarithmic transformation was performed for skewed variables. Non-parametric test was
198 used if normality was not achieved with logarithmic transformation. For the comparison of
199 baseline and endpoint values within the groups, paired samples t-test or Wilcoxon signed
200 ranks test were used. The amount of cholesterol bound to aortic proteoglycans were
201 normalized with LDL-, non-HDL cholesterol or apoB concentrations. Changes among the
202 groups were tested with the analysis of variance (ANOVA) or Kruskall-Wallis -test. Analyses
203 were performed using fold changes. Fold changes were calculated by dividing the endpoint
204 values of the variable by their baseline values. Differences between genders in LDL
205 aggregation were tested with Mann-Whitney's U -test. Change in the cholesterol efflux
206 capacity was tested with independent samples t-test. Associations between lipoprotein

ACCEPTED MANUSCRIPT

207 subclasses and concentrations of apoE and SAA were tested using Spearman rank correlation.

208 $p < 0.05$ was considered statistically significant.

209

ACCEPTED MANUSCRIPT

210 **3. Results**

211

212 **3.1. Characteristics of the participants and compliance**

213 Mean (± SD) age of the study subjects was 58.9 ± 6.5 years. Baseline characteristics of the
214 study subjects are shown in Supplementary Table 1. Compliance with the study diets was
215 good according to food records and consumption records regarding intakes of fish and CSO
216 [25]. The proportion of ALA in plasma phospholipids increased in the CSO group ($p < 0.001$)
217 and differed significantly from the other groups. Furthermore, the proportion of EPA
218 increased ($p < 0.001$) in the fatty fish group as compared with the lean fish and control
219 groups. The proportion of DHA increased in the fatty fish group ($p < 0.001$) as compared
220 with the CSO and control groups. Similar changes were also observed in the proportions of
221 these fatty acids in plasma cholesteryl esters, triglycerides and erythrocyte membranes [33].

222

223 **3.2 Binding of lipoproteins to proteoglycans**

224 Binding of lipoproteins to proteoglycans decreased in the CSO group compared with the fatty
225 fish group in a non-normalized model (overall difference among the groups p=0.006, Fig.
226 1A). After normalizing to serum LDL cholesterol, non-HDL cholesterol or apoB
227 concentrations, the change was no longer statistically significant (p=0.771, p=0.625 and
228 p=0.267, respectively), indicating that the level of apoB-containing lipoproteins in the
229 circulation is the main driver of LDL retention to proteoglycans.

230

231 **3.3 LDL aggregation**

232 The susceptibility of LDL to aggregation has recently been shown to differ significantly
233 among human donors and to be associated with cardiovascular deaths [8]. Here, we again
234 found significant individual differences in LDL aggregation, but found no statistically

ACCEPTED MANUSCRIPT

235 significant change in LDL aggregation among the groups (overall difference among the
236 groups p=0.152, Fig. 1B) or between genders (p=0.505).
237
238 **3.4 Activation of endothelial cells**
239 A previous study has shown that modulation of LDL lipid composition influences the ability
240 of LDL to induce secretion of IL-8 from endothelial cells [10]. We determined the IL-8 level
241 upon treatment of human coronary artery endothelial cells with LDL (50 µl/ml) isolated from
242 0-week and 12-week samples. We found that dietary intervention did not influence the ability
243 of LDL to stimulate IL-8 release (Fig. 1C), indicating that diet intervention does not affect
244 the pro-inflammatory properties of LDL particles as determined by IL-8 secretion.
245
246 **3.5 Cholesterol efflux capacity**
247 We examined whether diet intervention would modulate the ability of HDL particles to
248 promote cholesterol efflux. To this end, we isolated HDL particles from plasma in the same
249 subjects before and after 12 weeks of fatty fish intervention and determined their cholesterol
250 efflux from human primary foam cells. As shown in Fig. 1D, fatty fish treatment after 12
251 weeks did not significantly affect the cholesterol efflux capacity of HDL.
252
253 **3.6 Concentrations of apoE and SAA**
254 There were no statistically significant changes in plasma concentration of apoE among
255 groups (fold changes for CSO group 1.05, fatty fish group 0.92, lean fish group 1.05 and
256 control group 0.97, overall difference among the groups p=0.322). Serum concentration of
257 SAA decreased in the CSO and lean fish groups compared with the control group (overall
258 difference among the groups p=0.011, Fig. 2).
259

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

260 **3.7 Correlations between lipoprotein subclasses, SAA and apoE**

261 Correlations between lipoprotein subclasses, SAA and apoE are shown in Supplementary
262 Tables 2–4. We found weak correlations between SAA and HDL subclasses (r=0.245–0.416,
263 p<0.05) and apoE and HDL subclasses (r=0.223–0.306, p<0.05). HDL_3 also correlated with
264 LDL subclasses (r=0.383–0.629, p<0.01).

265
266
267 **4. Discussion**
268

269 In this study, we investigated the effect of fish and CSO intakes on binding of lipoproteins to
270 proteoglycans, LDL aggregation, cholesterol efflux capacity and activation of endothelial
271 cells. This is the first dietary intervention study to investigate all these lipoprotein functions
272 in humans. We showed that intake of 30 ml (27 g) of CSO rich in ALA decreases the binding
273 of lipoproteins to aortic proteoglycans compared with the fatty fish group. Previously, Jones
274 et al. [34] found that corn/safflower oil and high-oleic canola oil decreased slightly the
275 binding of LDL to biglycan. In the present study, we used whole plasma samples instead of
276 isolated LDL particles. In this way, we were able to assess the effect of all plasma
277 lipoproteins on cholesterol accumulation to proteoglycans. We have previously shown that
278 intake of CSO decreases serum LDL cholesterol compared with the fatty fish and lean fish
279 groups and IDL particle concentration compared with the lean fish group [25, 32]. Taken
280 together, our findings show a clear relation between plasma apoB containing lipoprotein
281 levels and the binding of lipoprotein-derived cholesterol to human aortic proteoglycans, a
282 finding highlighting the importance of plasma lipoprotein levels in the accumulation of
283 cholesterol within the arterial proteoglycans.

284

285 The effect of a healthy Nordic diet has recently been shown to decrease LDL aggregation in
286 most donors, the decrease associating with an increased consumption of poly- and

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

287 monounsaturated fatty acids [8]. Here, we found no change in the LDL aggregation among
288 the groups, but it should be noted that the individual differences in LDL aggregation were
289 large, as also reported earlier [8]. In that study, the diet-induced differences in LDL
290 aggregation were shown to be due to differences in the surface lipids of LDL, so that a
291 decrease in LDL-sphingomyelins was associated with decreased LDL aggregation. Neither
292 CSO, fatty fish nor lean fish induced changes in the proportion of LDL-sphingomyelins in
293 this study (unpublished data by Manninen et al.). Furthermore, it has been previously shown
294 that plasma sphingomyelins are affected by genetic factors, which may partly explain the
295 inter-individual variance found in LDL aggregation [35]. These differences in LDL
296 aggregation related to individual differences and the potential underlying mechanisms require
297 further investigation.
298
299 Modulation of LDL lipid composition has previously been shown to induce the release of IL-
300 8 from endothelial cells [10, 36]. In these studies, changes in the LDL lipidome were induced
301 enzymatically and increased content of free fatty acids and lysophosphatidylcholine was
302 found to enhance IL-8 release. Instead, studies investigating dietary effects on LDL lipidome
303 are scarce [8, 37], and to our knowledge, there are no previous studies investigating the
304 effects of dietary intervention on inflammatory properties of LDL particles. Padro et al. [37]
305 found that milk enriched with long-chain *n*-3 PUFA (0.375 g/250 ml milk) did not induce
306 changes among lipid species of LDL. *N*-3 enriched milk, however, increased long-chain *n*-3
307 PUFA content in cholesteryl esters, phosphatidylcholine and PC36:5/lysoPC16:0 ratio.
308 Authors concluded that these changes may be associated with reduced inflammatory activity
309 of LDL particles. Here, we found no change in the ability of LDL to release IL-8.
310

ACCEPTED MANUSCRIPT

311 Previous research on the effects of *n*-3 fatty acids on cholesterol efflux has been controversial
312 [38-41]. In experimental models, where macrophages have been directly exposed to EPA,
313 cholesterol efflux capacity has been reduced [38, 39], whereas EPA-supplementation with a
314 high dose (1.8 g/day) has been shown to enhance cholesterol efflux capacity of HDL from
315 THP-1 macrophages in patients with coronary risk factors [40]. In the present study, the
316 dietary intake of EPA in the fatty fish group was considerably lower: approximately 0.5 g/day
317 [25]. Here, we found that intake of fatty fish had no effect on the cholesterol efflux capacity
318 of HDL particles isolated from plasma. Furthermore, ALA-treatment has been found to
319 increase cholesterol efflux capacity in foam cells by decreasing stearoyl CoA desaturase 1
320 expression [41]. Thus, it appears that ALA-treatment may be able to influence the properties
321 of macrophages rather than that of HDL particles. Overall, further studies are needed to
322 elucidate the role of dietary *n*-3 PUFAs on cholesterol efflux capacity.
323
324 SAA and apoE are known to contribute to atherosclerosis [14, 15]. SAA has been shown to
325 have several atherogenic functions whereas apoE is best recognized for its anti-atherogenic
326 properties. Both SAA and apoE are also potential mediators in the binding of HDL to
327 proteoglycans [16, 42]. HDL_2 has been found to inhibit the formation of the LDL-
328 proteoglycan complex more efficiently than HDL_3. This is explained by the higher apoE
329 content in large HDL particles compared with small HDL particles [43]. Here, we found no
330 changes in plasma concentration of apoE in any of the groups, but there was a decrease in the
331 serum concentration of SAA in the CSO and lean fish groups as compared with the control
332 group. After excluding individuals with hs-CRP>10 mg/l at baseline or at the end of the study
333 (n=3), there were no differences between the groups in the pairwise comparison (data not
334 shown). Moreover, we found weak correlations between SAA and HDL subclasses and apoE

ACCEPTED MANUSCRIPT

335 and HDL subclasses. HDL_3 also correlated with LDL subclasses, which may be an indication
336 of dyslipidemic profile including small HDL particles [44].
337
338 The strengths of the current study include a randomized controlled study design and good
339 compliance with study diets, as previously reported [25]. However, there are also some
340 limitations to consider. Power calculations were based on differences in DHA in plasma
341 phospholipids, and it is likely that there was not enough power to see all changes in these
342 secondary outcome variables. Baseline omega-3 index of subjects was relatively high (> 8 %
343 for all groups) [33], which may have attenuated the effects. Furthermore, subjects in this
344 study were overweight and had impaired glucose metabolism. Therefore, the results of this
345 study may not apply to individuals with normal body weight and glucose metabolism.
346
347 In conclusion, intake of CSO, fatty fish or lean fish has no effect on LDL aggregation or
348 activation of endothelial cells, and fatty fish intake has no effect on cholesterol efflux
349 capacity in subjects with impaired glucose metabolism. Intake of CSO, however, decreases
350 the binding of lipoproteins to aortic proteoglycans by decreasing serum apoB-containing
351 lipoprotein concentrations. This finding highlights the importance of plasma lipoprotein
352 levels in the accumulation of cholesterol within the arterial wall.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Clinical Trial Registry

The study is registered in Clinicaltrials.gov (NCT01768429).

353

354 **Conflict of interest**

355 K.Ö. and M.R. have applied a patent for the measurement of LDL aggregation. All other
356 authors declare no conflict of interest.
357

358 **Financial support**

359 This study was supported by Yrjö Jahnsson Foundation, Diabetes Research Foundation, Juho
360 Vainio Foundation, Finnish Cultural Foundation, North Savo Regional fund, State Research
361 Funding (VTR) of Kuopio University Hospital and Finnish Cultural Foundation. Wihuri
362 Research Institute is maintained by the Jenny and Antti Wihuri Foundation. This study was
363 also supported by a grant from the Academy of Finland to K.Ö. (265940 and 315568).
364 Suomen Kasviöljyt Ltd, Kesko Ltd and Bunge Finland Ltd provided oil and fat spreads for
365 the study participants.
366

367 **Author contributions**

368 The authors' responsibilities were as follows: U.S. and A.E. are the principal investigators in
369 the AlfaFish –study and they planned and conducted the study together with M.L. and V.M.
370 S.M. analyzed the data, wrote the article and had primary responsibility for final content.
371 S.D.N. and M.R. isolated plasma LDL and HDL particles. S.D.N. performed cholesterol
372 efflux and endothelial cell activation assays, analyzed the data and interpreted the results.
373 M.R. performed LDL aggregation analyses, analyzed the data and interpreted the results. K.Ö
374 performed plasma proteoglycan binding assays, analyzed the data, and interpreted the results.
375 U.S., A.E., M.L. K.Ö. and S.D.N participated in writing and editing the manuscript. All
376 authors have read and approved the final manuscript.
377

378 **Acknowledgements**

379 We are thankful for the excellent technical assistance of Maija Atuegwu and Erja Kinnunen.

ACCEPTED MANUSCRIPT

References

[1] J. Boren, K.J. Williams, The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: a triumph of simplicity, *Curr Opin Lipidol* **27**(2016) pp. 473-83.

[2] M. Lu, O. Gursky, Aggregation and fusion of low-density lipoproteins in vivo and in vitro, *Biomol Concepts* **4**(2013) pp. 501-18.

[3] K.J. Williams, I. Tabas, The response-to-retention hypothesis of early atherogenesis, *Arterioscler Thromb Vasc Biol* **15**(1995) pp. 551-61.

[4] K. Öörni, M.O. Pentikainen, M. Ala-Korpela, P.T. Kovanen, Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions, *J Lipid Res* **41**(2000) pp. 1703-14.

[5] K. Oorni, J.K. Hakala, A. Annila, M. Ala-Korpela, P.T. Kovanen, Sphingomyelinase induces aggregation and fusion, but phospholipase A2 only aggregation, of low density lipoprotein (LDL) particles. Two distinct mechanisms leading to increased binding strength of LDL to human aortic proteoglycans, *J Biol Chem* **273**(1998) pp. 29127-34.

[6] K. Oorni, P. Posio, M. Ala-Korpela, M. Jauhiainen, P.T. Kovanen, Sphingomyelinase induces aggregation and fusion of small very low-density lipoprotein and intermediate-density lipoprotein particles and increases their retention to human arterial proteoglycans, *Arterioscler Thromb Vasc Biol* **25**(2005) pp. 1678-83.

[7] C. Bancells, S. Villegas, F.J. Blanco, S. Benitez, I. Gallego, L. Beloki*, et al.*, Aggregated electronegative low density lipoprotein in human plasma shows a high tendency toward phospholipolysis and particle fusion, *J Biol Chem* **285**(2010) pp. 32425-35.

[8] M. Ruuth, S.D. Nguyen, T. Vihervaara, M. Hilvo, T.D. Laajala, P.K. Kondadi*, et al.*, Susceptibility of low-density lipoprotein particles to aggregate depends on particle lipidome, is modifiable, and associates with future cardiovascular deaths, *Eur Heart J* **39**(2018) pp. 2562-73.

[9] A.G. Suits, A. Chait, M. Aviram, J.W. Heinecke, Phagocytosis of aggregated lipoprotein by macrophages: low density lipoprotein receptor-dependent foam-cell formation, *Proc Natl Acad Sci U S A* **86**(1989) pp. 2713-7.

[10] P. Suriyaphol, D. Fenske, U. Zahringer, S.R. Han, S. Bhakdi, M. Husmann, Enzymatically modified nonoxidized low-density lipoprotein induces interleukin-8 in human endothelial cells: role of free fatty acids, *Circulation* **106**(2002) pp. 2581-7.

[11] S.O. Olofsson, J. Boren, Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis, *J Intern Med* **258**(2005) pp. 395-410.

[12] R.S. Rosenson, H.B. Brewer Jr, B. Ansell, P. Barter, M.J. Chapman, J.W. Heinecke*, et al.*, Translation of high-density lipoprotein function into clinical practice: current prospects and future challenges, *Circulation* **128**(2013) pp. 1256-67.

[13] C.G. Santos-Gallego, J.J. Badimon, R.S. Rosenson, Beginning to understand high-density lipoproteins, *Endocrinol Metab Clin North Am***43**(2014) pp. 913-47.

[14] L. Bouchareychas, R.L. Raffai, Apolipoprotein E and Atherosclerosis: From Lipoprotein Metabolism to MicroRNA Control of Inflammation, *J Cardiovasc Dev Dis***5**(2018) pp. 10.3390/jcdd5020030.

[15] K.D. O'Brien, A. Chait, Serum amyloid A: the "other" inflammatory protein, *Curr Atheroscler Rep***8**(2006) pp. 62-8.

[16] M. Umaerus, B. Rosengren, B. Fagerberg, E. Hurt-Camejo, G. Camejo, HDL2 interferes with LDL association with arterial proteoglycans: a possible athero-protective effect, *Atherosclerosis***225**(2012) pp. 115-20.

[17] J. Gambardella, G. Santulli, Integrating diet and inflammation to calculate cardiovascular risk, *Atherosclerosis***253**(2016) pp. 258-61.

[18] D. Mozaffarian, L.J. Appel, L. Van Horn, Components of a cardioprotective diet: new insights, *Circulation***123**(2011) pp. 2870-91.

[19] D. Mozaffarian, J.H. Wu, Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events, *J Am Coll Cardiol***58**(2011) pp. 2047-67.

[20] A. Alhassan, J. Young, M.E.J. Lean, J. Lara, Consumption of fish and vascular risk factors: A systematic review and meta-analysis of intervention studies, *Atherosclerosis***266**(2017) pp. 87-94.

[21] J.M. Geleijnse, J. de Goede, I.A. Brouwer, Alpha-linolenic acid: is it essential to cardiovascular health? *Curr Atheroscler Rep***12**(2010) pp. 359-67.

[22] A.T. Erkkila, U.S. Schwab, V.D. de Mello, T. Lappalainen, H. Mussalo, S. Lehto*, et al.*, Effects of fatty and lean fish intake on blood pressure in subjects with coronary heart disease using multiple medications, *Eur J Nutr***47**(2008) pp. 319-28.

[23] S.H. Johnsen, B.K. Jacobsen, S.K. Braekkan, J.B. Hansen, E.B. Mathiesen, Fish consumption, fish oil supplements and risk of atherosclerosis in the Tromso study, *Nutr J***17**(2018) pp. 56,018-0364-8.

[24] C. Torris, M.C. Smastuen, M. Molin, Nutrients in Fish and Possible Associations with Cardiovascular Disease Risk Factors in Metabolic Syndrome, *Nutrients***10**(2018) pp. 10.3390/nu10070952.

[25] U.S. Schwab, M.A. Lankinen, V.D. de Mello, S.M. Manninen, S. Kurl, K.J. Pulkki*, et al.*, Camelina Sativa Oil, but not Fatty Fish or Lean Fish, Improves Serum Lipid Profile in Subjects with Impaired Glucose Metabolism-A Randomized Controlled Trial, *Mol Nutr Food Res***62**(2018) pp. 10.1002/mnfr.201700503. Epub 2018 Jan 31.

[26] Nordic Nutrition Recommendations 2012. Integrating nutrition and physical activity, Copenhagen: Nordic Council of Ministers (2014).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[27] G.C. Burdge, P.C. Calder, Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults, *Reprod Nutr Dev***45**(2005) pp. 581-97.

[28] J.J. Agren, A. Julkunen, I. Penttila, Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column, *J Lipid Res***33**(1992) pp. 1871-6.

[29] M. Sneck, P.T. Kovanen, K. Oorni, Decrease in pH strongly enhances binding of native, proteolyzed, lipolyzed, and oxidized low density lipoprotein particles to human aortic proteoglycans, *J Biol Chem***280**(2005) pp. 37449-54.

[30] M. Stahlman, P. Davidsson, I. Kanmert, B. Rosengren, J. Boren, B. Fagerberg*, et al.*, Proteomics and lipids of lipoproteins isolated at low salt concentrations in D2O/sucrose or in KBr, *J Lipid Res***49**(2008) pp. 481-90.

[31] P. Saren, H.G. Welgus, P.T. Kovanen, TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages, *J Immunol***157**(1996) pp. 4159-65.

[32] S.M. Manninen, M.A. Lankinen, V.D. de Mello, D.E. Laaksonen, U.S. Schwab, A.T. Erkkila, Intake of Fatty Fish Alters the Size and the Concentration of Lipid Components of HDL Particles and Camelina Sativa Oil Decreases IDL Particle Concentration in Subjects with Impaired Glucose Metabolism, *Mol Nutr Food Res***62**(2018) pp. e1701042.

[33] S. Manninen, M. Lankinen, V. de Mello, J. Agren, D. Laaksonen, U. Schwab*, et al.*, The effect of camelina sativa oil and fish intakes on fatty acid compositions of blood lipid fractions, *Nutr Metab Cardiovasc Dis*(2018).

[34] P.J. Jones, D.S. MacKay, V.K. Senanayake, S. Pu, D.J. Jenkins, P.W. Connelly*, et al.*, High-oleic canola oil consumption enriches LDL particle cholesteryl oleate content and reduces LDL proteoglycan binding in humans, *Atherosclerosis***238**(2015) pp. 231-8.

[35] T. Frahnow, M.A. Osterhoff, S. Hornemann, M. Kruse, M.A. Surma, C. Klose*, et al.*, Heritability and responses to high fat diet of plasma lipidomics in a twin study, *Sci Rep***7**(2017) pp. 3750,017-03965-6.

[36] S. Benitez, M. Camacho, R. Arcelus, L. Vila, C. Bancells, J. Ordonez-Llanos*, et al.*, Increased lysophosphatidylcholine and non-esterified fatty acid content in LDL induces chemokine release in endothelial cells. Relationship with electronegative LDL, *Atherosclerosis***177**(2004) pp. 299-305.

[37] T. Padro, G. Vilahur, J. Sanchez-Hernandez, M. Hernandez, R.M. Antonijoan, A. Perez*, et al.*, Lipidomic changes of LDL in overweight and moderately hypercholesterolemic subjects taking phytosterol- and omega-3-supplemented milk, *J Lipid Res***56**(2015) pp. 1043-56.

[38] N. Fournier, S. Tardivel, J.F. Benoist, B. Vedie, D. Rousseau-Ralliard, M. Nowak*, et al.*, Eicosapentaenoic acid membrane incorporation impairs ABCA1-dependent cholesterol efflux via a protein kinase A signaling pathway in primary human macrophages, *Biochim Biophys Acta***1861**(2016) pp. 331-41.

ACCEPTED MANUSCRIPT

[39] Y.W. Hu, X. Ma, X.X. Li, X.H. Liu, J. Xiao, Z.C. Mo, *et al.*, Eicosapentaenoic acid reduces ABCA1 serine phosphorylation and impairs ABCA1-dependent cholesterol efflux through cyclic AMP/protein kinase A signaling pathway in THP-1 macrophage-derived foam cells, *Atherosclerosis* **204**(2009) pp. e35-43.

[40] N. Tanaka, T. Ishida, M. Nagao, T. Mori, T. Monguchi, M. Sasaki, *et al.*, Administration of high dose eicosapentaenoic acid enhances anti-inflammatory properties of high-density lipoprotein in Japanese patients with dyslipidemia, *Atherosclerosis* **237**(2014) pp. 577-83.

[41] J. Zhang, P.M. Kris-Etherton, J.T. Thompson, D.B. Hannon, P.J. Gillies, J.P. Heuvel, Alpha-linolenic acid increases cholesterol efflux in macrophage-derived foam cells by decreasing stearoyl CoA desaturase 1 expression: evidence for a farnesoid-X-receptor mechanism of action, *J Nutr Biochem* **23**(2012) pp. 400-9.

[42] K.D. O'Brien, T.O. McDonald, V. Kunjathoor, K. Eng, E.A. Knopp, K. Lewis, *et al.*, Serum amyloid A and lipoprotein retention in murine models of atherosclerosis, *Arterioscler Thromb Vasc Biol* **25**(2005) pp. 785-90.

[43] W.S. Davidson, R.A. Silva, S. Chantepie, W.R. Lagor, M.J. Chapman, A. Kontush, Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function, *Arterioscler Thromb Vasc Biol* **29**(2009) pp. 870-6.

[44] A. Kontush, M.J. Chapman, Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis, *Pharmacol Rev* **58**(2006) pp. 342-74.

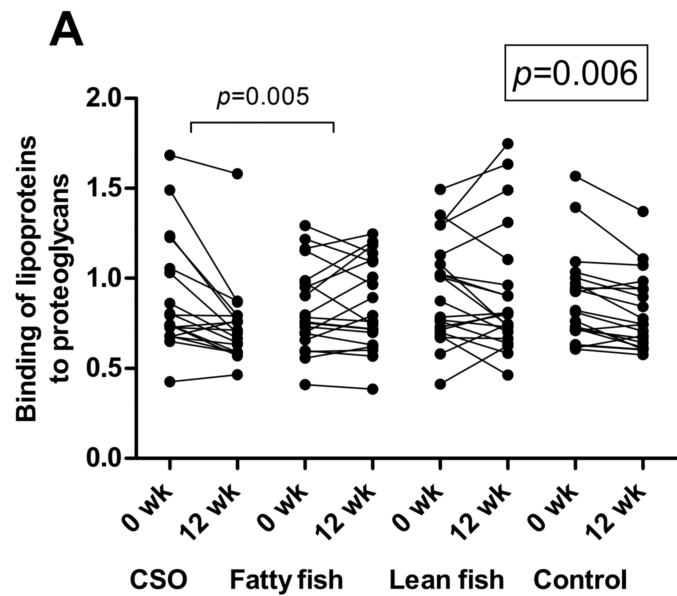
ACCEPTED MANUSCRIPT

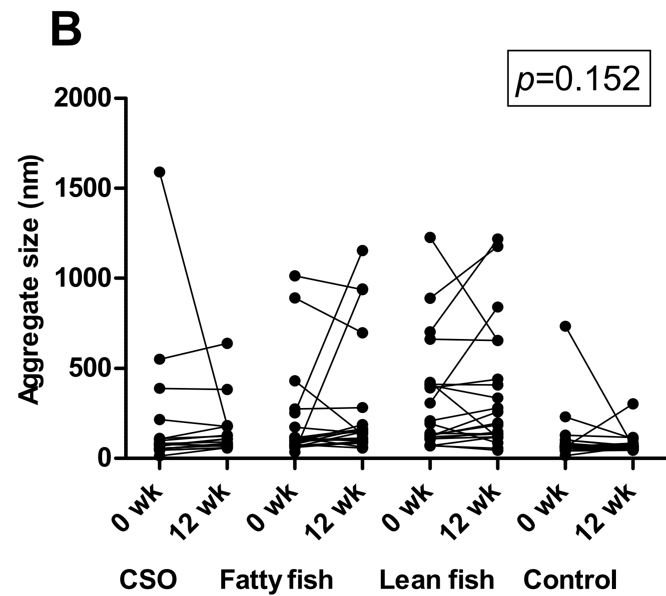
ACCEPTED MANUSCRIPT

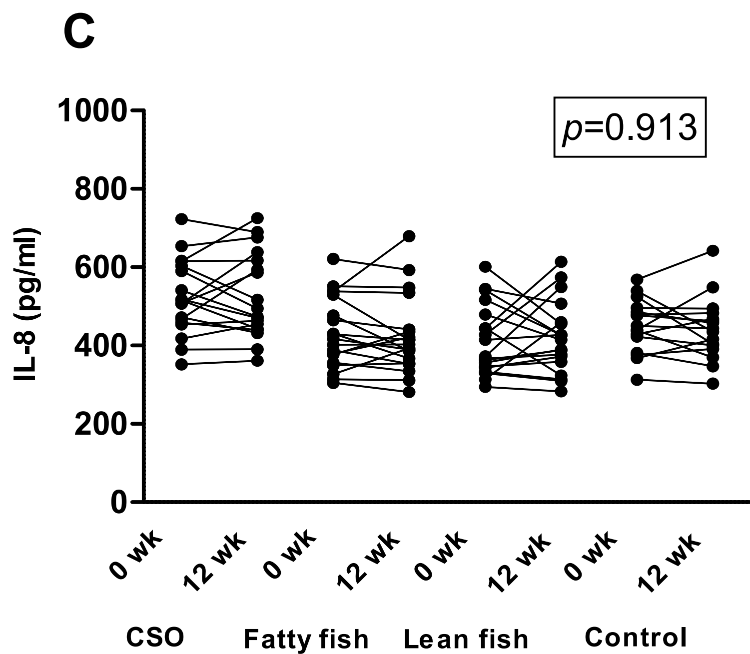
Figure 1. (A) Changes in the binding of lipoproteins to proteoglycans (n=79), (B) LDL aggregation (n=79), (C) activation of endothelial cells (n=16–19/group) and (D) cholesterol efflux capacity in the fatty fish group (n=16). Changes among groups in the binding of lipoproteins to proteoglycans, LDL aggregation and IL-8 were tested using Kruskall-Wallis test or analysis of variance followed by Bonferroni's *post hoc* tests. The *p*-value in the box represents the overall difference among groups. Change in cholesterol efflux capacity in the fatty fish group was tested with independent samples t-test. CSO, Camelina sativa oil.

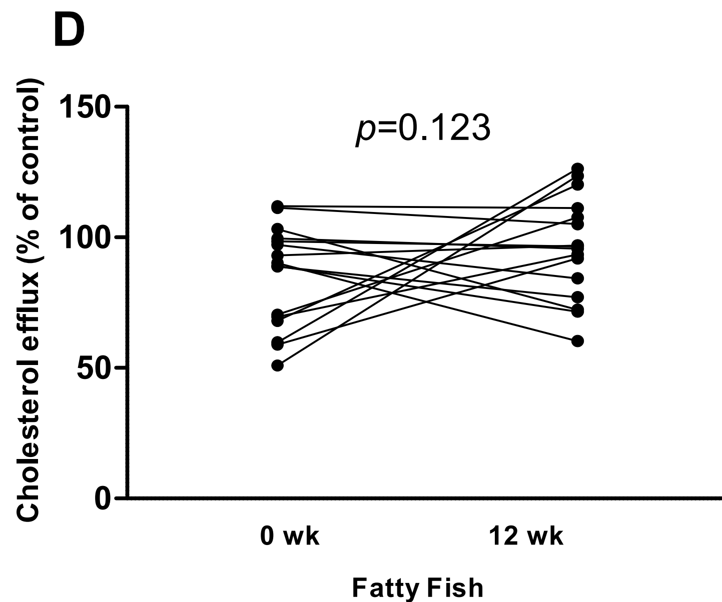
Figure 2. Changes in the concentration of SAA.
Changes within groups were tested with Mann-Whitney's U-test and changes among groups were tested using Kruskall-Wallis test. The *p*-value in the box represents the overall difference among the groups. CSO, Camelina sativa oil; SAA, serum amyloid A.

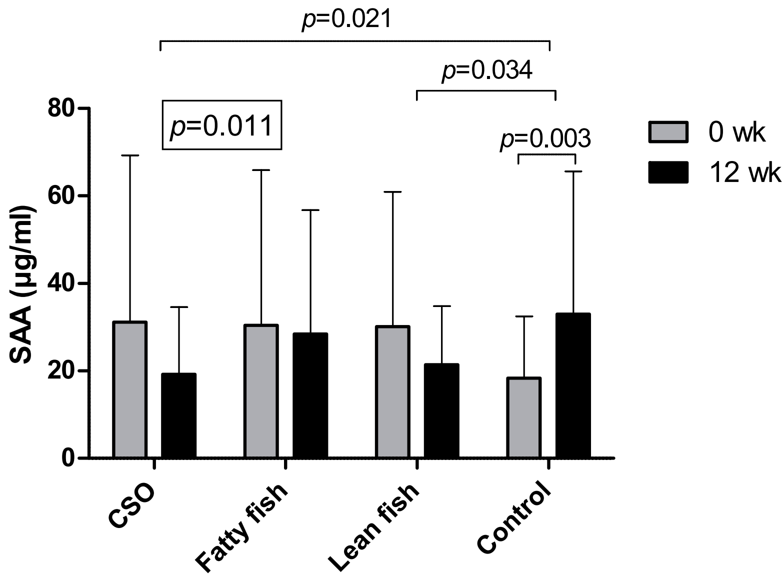
ACCEPTED MANUSCRIPT


A
p=0.006
p=0.005
Binding of lipoproteins to proteoglycans
2.0
1.5
1.0
0.5
0.0
0 wk
12 wk
0 wk
12 wk
0 wk
12 wk
0 wk
12 wk
CSO
Fatty fish
Lean fish
Control


B
p=0.152
Aggregate size (nm)
2000
1500
1000
500
0
0 wk
12 wk
0 wk
12 wk
0 wk
12 wk
0 wk
12 wk
CSO
Fatty fish
Lean fish
Control


C
p=0.913
IL-8 (pg/ml)
1000
800
600
400
200
0
0 wk
12 wk
0 wk
12 wk
0 wk
12 wk
0 wk
12 wk
CSO
Fatty fish
Lean fish
Control


D
p=0.123
Cholesterol efflux (% of control)
150
100
50
0
0 wk
12 wk
Fatty Fish


p=0.021
p=0.034
p=0.011
p=0.003
0 wk
12 wk
SAA (µg/ml)
80
60
40
20
0
CSO
Fatty fish
Lean fish
Control

ACCEPTED MANUSCRIPT

Highlights

- Camelina oil intake decreases the binding of lipoproteins to aortic proteoglycans
- Intakes of camelina oil or fish have no effect on LDL aggregation
- Intake of fatty fish has no effect on cholesterol efflux capacity of HDL

ACCEPTED MANUSCRIPT