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## HDL enhances oxidation of LDL *in vitro* in both men and women

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### Abstract

**Background:** Oxidative modification of low-density lipoprotein (LDL) is a key event in the oxidation hypothesis of atherogenesis. Some *in vitro* experiments have previously suggested that high-density lipoprotein (HDL) co-incubated with LDL prevents Cu<sup>2+</sup>-induced oxidation of LDL, while some other studies have observed an opposite effect. To comprehensively clarify the role of HDL in this context, we isolated LDL, HDL<sub>2</sub> and HDL<sub>3</sub> from sera of 61 free-living individuals (33 women and 28 men).

**Results:** When the isolated LDL was subjected to Cu<sup>2+</sup>-induced oxidation, both HDL<sub>2</sub> and HDL<sub>3</sub> particles increased the rate of appearance and the final concentration of conjugated dienes similarly in both genders. Oxidation rate was positively associated with polyunsaturated fatty acid content of the lipoproteins in that it was positively related to the content of linoleate and negatively related to oleate. More saturated fats thus protected the lipoproteins from damage.

**Conclusion:** We conclude that *in vitro* HDL does not protect LDL from oxidation, but is in fact oxidized fastest of all lipoproteins due to its fatty acid composition, which is oxidation promoting.

### Background

Epidemiological studies show an inverse correlation between high-density lipoprotein (HDL) concentration and the risk of developing coronary artery disease [1]. According to a widely accepted hypothesis, HDL or its subfractions play an important role in recruiting and transporting cholesterol from peripheral tissues to the liver for excretion, a series of events known as reverse cholesterol transport [2]. Other properties of HDL link its antiatherogenic functions to its antioxidative effects. Some studies have shown that co incubation of LDL with

HDL in the presence of divalent copper prevents the oxidative modification of LDL [3]. In some reports this finding could not be confirmed, and in fact it has been demonstrated that *in vitro* HDL is oxidized faster than other lipoproteins [4]. When HDL is oxidatively modified, it alters to a form that causes macrophages to accumulate cholesterol. [5]. It has been suggested that systemic inflammation gives rise to prooxidant and proinflammatory HDL particles [6]. Oxidatively modified HDL is found in atheromatous plaques from human aorta [7]. Oxidatively modified HDL is no longer capable of remov-

**Table 1: Characteristics of the study subjects.**

	Women	Men	All
N	32	27	59
Age (years)	39.3 ± 10.5	39.3 ± 11.0	39.3 ± 10.6
Body mass index (kg/m <sup>2</sup> )	23.2 ± 3.1	25.8 ± 3.6**	24.4 ± 3.6
Total cholesterol (mmol/l)	5.23 ± 0.87	5.76 ± 1.02*	5.47 ± 0.97
Triacylglycerol (mmol/l)	0.96 ± 0.41	1.81 ± 1.27***	1.35 ± 0.71
HDL cholesterol (mmol/l)	1.88 ± 0.32	1.38 ± 0.23***	1.65 ± 0.38
LDL cholesterol (mmol/l)	2.91 ± 0.84	3.63 ± 0.92**	3.21 ± 0.96
ApoA-I (g/l)	1.70 ± 0.20	1.46 ± 0.13***	1.59 ± 0.21
ApoB (g/l)	0.81 ± 0.20	1.00 ± 0.22**	0.90 ± 0.23
Lp(a) (U/l)	266 ± 226	165 ± 201	220 ± 219
fB-Glucose (mmol/l) <sup>a</sup>	4.4 ± 0.4	4.6 ± 0.6	4.5 ± 0.5
LDL diameter (nm)	26.94 ± 0.45	26.40 ± 0.55***	26.70 ± 0.51

Values are mean ± SD. <sup>a</sup> fB, fasting blood, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001 compared to women by Mann-Whitney U-test.

ing cholesterol from cells, and it enhances LDL oxidation [8].

The contradictory findings on the role of HDL on LDL oxidation *in vitro* may be due to rather small study populations, and the reported heterogeneity of oxidation kinetics between lipoprotein preparations *in vitro* [9] which might be due to individual intrinsic properties of the lipoproteins. In the present paper we report the results of a study of the effect of HDL subtractions and gender on *ex vivo* oxidation of LDL from a population of 61 healthy free-living human subjects.

## Results

Background characteristics of the men and women participating in the study are shown in Table 1. Compared with women, men had higher body mass indices, serum total cholesterol as well as LDL and apoB concentrations. Men had smaller LDL size and smaller concentrations of serum HDL and apoA-I than women.

The fatty acid compositions of ultracentrifugally isolated LDL, HDL<sub>2</sub> and HDL<sub>3</sub> fractions were analyzed by gas liquid chromatography (Table 2). There were no gender differences in the total amounts of saturated, monounsaturated and polyunsaturated fatty acids of LDL, HDL<sub>2</sub> and HDL<sub>3</sub>. The calculated peroxidizability indices were also similar in men and women. In both genders this index increased significantly from LDL to HDL<sub>2</sub> to HDL<sub>3</sub> (p < 0.001, Wilcoxon's matched pairs test).

The oxidation of LDL of all subjects gave rise to typical conjugated diene vs. time -curves, where the different phases of hydro peroxide formation were clearly discernible. Co incubations of LDL with either HDL<sub>2</sub> or HDL<sub>3</sub> produced biphasic profiles with faster oxidation in the beginning, followed by a slower rate and finally a faster

propagation phase. The profiles looked similar in all participants.

Co incubation of LDL with HDL<sub>2</sub> or HDL<sub>3</sub> decreased the mean lag time of diene formation in both women and men (Table 3). Likewise, the mean propagation rate and the maximum diene concentration increased significantly in the presence of HDL. These oxidation parameters did not differ between women and men.

Multiple forward stepwise regression analysis was performed to estimate the effect of lipids and factors related to lipoprotein metabolism on oxidation parameters. Predictors for the multivariate analysis were selected on the basis of initial correlation analyses using Spearman's correlation coefficients. The resulting models formed consistent patterns of predictors. The results of the models for the mixtures of LDL + HDL<sub>2</sub> are shown in Table 4. The results were similar for mixtures of LDL and HDL<sub>3</sub>, and for LDL alone (not shown). In these incubations, an increase in lag time was related to fasting blood glucose concentration, and a decrease in lag time was related to the peroxidizability index. Oxidation rate was positively associated with PUFA content of the lipoproteins. Maximum concentration of dienes was positively related to the content of linoleate and to the ratio of LDL to apoB, and negatively related to oleate.

Although the mean lag time was shorter in the presence of HDL<sub>2</sub> or HDL<sub>3</sub>, there were nine subjects who had longer lag time when HDL<sub>2</sub> was co incubated with LDL. We analyzed, whether there were any differences between these nine subjects and the rest of the study group that could explain the increased lag time. These nine subjects had a significantly smaller peroxidizability index of HDL<sub>2</sub> (88.0 ± 12.3 vs. 95.4 ± 9.5, p < 0.05) than the rest of the group. Their LDL lag time was slightly shorter (55.7 ± 4.7 vs. 61.0

**Table 2: Percentage composition of fatty acids of LDL, HDL<sub>2</sub> and HDL<sub>3</sub> in 59 healthy subjects.**

Fatty acid	Women			Men		
	LDL	HDL <sub>2</sub>	HDL <sub>3</sub>	LDL	HDL <sub>2</sub>	HDL <sub>3</sub>
14:0	0.80 ± 0.22	0.67 ± 0.19	0.61 ± 0.17	0.97 ± 0.29*	0.94 ± 1.41***	0.76 ± 0.26*
16:0	19.50 ± 1.27	22.68 ± 1.41	22.45 ± 1.51	19.39 ± 1.23	22.79 ± 1.30	22.25 ± 1.10
16:1(n-7)	2.28 ± 0.64	1.72 ± 0.51	1.69 ± 0.51	2.22 ± 0.79	1.82 ± 0.71	1.87 ± 1.21
18:0	5.44 ± 0.58	8.18 ± 0.91	8.24 ± 0.90	5.71 ± 0.35	8.41 ± 0.67	8.71 ± 0.60
18:1T	0.35 ± 0.11	0.38 ± 0.12	0.36 ± 0.12	0.38 ± 0.15	0.45 ± 0.19	0.42 ± 0.16
18:1(n-9)	21.44 ± 1.63	17.79 ± 1.21	16.91 ± 1.02	21.12 ± 2.37	19.62 ± 3.09	17.77 ± 2.29
18:2(n-6)	34.92 ± 2.88	30.20 ± 2.93	30.71 ± 3.00	34.92 ± 4.16	28.38 ± 3.90	29.41 ± 3.72
18:3(n-3)	0.87 ± 0.22	0.67 ± 0.18	0.65 ± 0.18	0.87 ± 0.20	0.77 ± 0.23	0.69 ± 0.18
18:3(n-6)	0.43 ± 0.14	0.28 ± 0.09	0.28 ± 0.01	0.53 ± 0.26	0.34 ± 0.20	0.35 ± 0.20
20:0	0.31 ± 0.04	0.26 ± 0.03	0.22 ± 0.03	0.26 ± 0.04***	0.23 ± 0.03***	0.19 ± 0.02**
20:3(n-6)	1.22 ± 0.29	1.85 ± 0.44	1.97 ± 0.49	1.34 ± 0.26	1.86 ± 0.39	2.09 ± 0.40
20:4(n-6)	5.40 ± 0.91	7.08 ± 1.04	7.70 ± 1.20	5.53 ± 1.14	6.67 ± 1.42	7.64 ± 1.49
20:5(n-3)	1.12 ± 0.59	1.28 ± 0.67	1.38 ± 0.75	1.26 ± 0.59	1.33 ± 0.55	1.50 ± 0.59
22:0	0.91 ± 0.10	0.75 ± 0.13	0.63 ± 0.14	0.86 ± 0.11	0.60 ± 0.12	0.55 ± 0.13
22:5(n-3)	0.40 ± 0.11	0.65 ± 0.16	0.68 ± 0.16	0.47 ± 0.07**	0.74 ± 0.12*	0.82 ± 0.11**
22:6(n-3)	2.18 ± 0.50	3.46 ± 0.73	3.70 ± 0.79	1.96 ± 0.53	3.25 ± 0.86	3.37 ± 0.88
24:0	0.84 ± 0.07	0.65 ± 0.09	0.56 ± 0.08	0.83 ± 0.14	0.63 ± 0.13	0.56 ± 0.12
24:1(n-9)	1.59 ± 0.21	1.43 ± 0.23	1.23 ± 0.21	1.39 ± 0.28**	1.17 ± 0.24**	1.04 ± 0.19*
ΣSAFA	27.82 ± 1.21	33.21 ± 1.16	32.77 ± 1.23	27.88 ± 1.18	33.54 ± 1.50	32.94 ± 1.12
ΣMUFA	25.33 ± 2.03	20.98 ± 1.54	19.86 ± 1.34	24.48 ± 2.67	22.28 ± 3.16	20.51 ± 2.63
ΣPUFA	46.51 ± 2.55	45.44 ± 2.00	47.02 ± 1.82	47.26 ± 3.66	43.75 ± 4.13	46.14 ± 3.48
PI	83.2 ± 7.4	96.1 ± 8.3	101.2 ± 9.3	83.7 ± 9.2	92.2 ± 12.0	99.2 ± 10.9

Values are mean ± SD. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to women. ΣSAFA, sum of percentages of saturated fatty acids, ΣMUFA, sum of percentages of monounsaturated fatty acids, ΣPUFA, sum of percentages of polyunsaturated fatty acids, PI, peroxidizability index (see methods).

± 7.7 μmol/l/min,  $p < 0.05$ ) and their fasting blood glucose concentration was lower ( $4.1 ± 0.5$  vs.  $4.5 ± 0.5$  mmol/l,  $p < 0.05$ ).

## Discussion

We found that co incubation of HDL<sub>2</sub> or HDL<sub>3</sub> with LDL in the presence of Cu<sup>2+</sup> resulted in shortening of the mean lag time and acceleration of the oxidation rate in comparison with that of incubation of LDL alone. If lag time or propagation rate are thought of as indices of oxidation resistance, this outcome contradicts the role of HDL as an antioxidant. Our findings are in line with the studies by Bowry et al. [4], Suzukawa et al. [10], Schnitzer et al. [11], Ohmura et al. [12] and Raveh et al. [9], who have come to the conclusion that HDL is more easily oxidized than LDL. In other studies, HDL has appeared to be less prone to oxidation and even to protect LDL against copper-induced oxidation [3,13-15]. In the study of Kontush et al. [16] all subfractions of HDL exhibited limited capacities to protect LDL at early stages of oxidation. At later phases, small dense HDL particles were the most potent inhibitors of LDL oxidation under mildly oxidative conditions. If strongly oxidative conditions were used [5 μmol/l Cu<sup>2+</sup>], none of the HDL subfractions offered any protection to LDL. The results were fairly similar whether the subspecies were isolated from serum or EDTA-plasma despite their widely differing paraoxonase activities suggesting that

paraoxonase may have had a smaller role in the inhibition. In our study the HDL<sub>2</sub> subfraction of the majority of the subjects had properties that enhanced the onset of propagation phase. However, in 9 participants this phase was delayed in the presence of a moderate concentration of Cu<sup>2+</sup> emphasizing that the individual variation of intrinsic characteristics of lipoproteins can not be overlooked.

In all, kinetic analyses by different investigators of the effects of HDL on copper-induced peroxidation of LDL are difficult to compare. (a) Firstly, the concentrations of LDL and HDL have been inconsistent and their expression has been variably based on protein, total lipid, total mass, phospholipid or cholesterol concentration, molar concentrations or particle numbers. The investigations into the kinetics of lipoprotein oxidation of Raveh et al. [9] and Ziouzenkova et al. [17] showed that the lag time and the propagation rate are dependent on LDL concentration. (b) Secondly, copper concentrations have also differed between the experiments. This has profound implications, since it has been shown in kinetic experiments that the lag time and the oxidation rate are correlated with the copper concentration until a saturating concentration is reached [9,16,18]. However, until more data are available, there is reason to think that the number of copper binding sites of lipoproteins is not constant but

**Table 3: Kinetic parameters of LDL, LDL + HDL<sub>2</sub> and LDL + HDL<sub>3</sub> oxidation in 59 healthy subjects.**

	Women	Men	All
<b>LDL</b>			
Lag time (min)	60.9 ± 7.9	59.3 ± 6.9	60.2 ± 7.4
Rate (μmol/l/min) <sup>a</sup>	0.509 ± 0.067	0.502 ± 0.067	0.506 ± 0.066
Max (nmol/mg) <sup>b</sup>	541 ± 41	537 ± 51	539 ± 45
<b>LDL + HDL<sub>2</sub></b>			
Lag time (min)	56.0 ± 5.7***	56.0 ± 6.7***	56.0 ± 6.1***
Rate (μmol/l/min) <sup>a</sup>	0.687 ± 0.062***	0.654 ± 0.084***	0.671 ± 0.073***
Max (nmol/mg) <sup>b</sup>	774 ± 40***	745 ± 71***	762 ± 56***
<b>LDL + HDL<sub>3</sub></b>			
Lag time (min)	55.8 ± 5.4***	54.8 ± 5.4***	55.3 ± 5.4***
Rate (μmol/l/min) <sup>a</sup>	0.616 ± 0.064***	0.607 ± 0.070***	0.612 ± 0.066***
Max (nmol/mg) <sup>b</sup>	687 ± 40***	674 ± 60***	681 ± 50***

Values are mean ± SD. <sup>a</sup> Rate means maximal formation rate of conjugated dienes during oxidation. Calculation of the diene concentration is based on  $\epsilon = 29500$  of the conjugated dienes <sup>b</sup> Max is the maximal amount of dienes produced per mg of LDL protein. \*\*\*  $p < 0.001$  in comparison with LDL alone in Wilcoxon's matched pairs test.

varies [18]. (c) Thirdly, the ratio of copper to lipoprotein has varied between studies. It has been found that the kinetic profile of LDL oxidation changes in response to copper concentration and that the familiar monophasic, auto accelerating profile is only obtained when the copper concentration is relatively high [17]. All of the compound kinetic curves in our study had a biphasic shape. The first phase of rapid oxidation in such a profile, whether observed with HDL or LDL, has been interpreted to occur via a tocopherol-mediated mechanism [9] where vitamin E acts as a prooxidant. Because the rate of oxidation is regulated by the ratio of bound copper/lipoprotein as outlined above, the addition of HDL to LDL should, theoretically, have lengthened the lag time and oxidation rate instead of shortening it, since HDL bound part of the copper. Obviously, many factors are involved in determining the outcome of this kind of experiment. Furthermore, it is apparent that ex vivo oxidation experiments with lipoproteins require standardization.

Earlier studies have shown that there are several intrinsic properties of lipoproteins that can affect their susceptibility to oxidation. Lipoprotein antioxidant content [19,20], fatty acid composition [21,22], presence of various enzymes [13] and LDL and HDL size [16,23] are among the factors that have been shown to have an impact on oxidation parameters, the former especially in supplementation studies. Also, long-term habitual diets with different fatty acid contents have been shown to influence LDL oxidation susceptibility [24]. We analyzed the fatty acid compositions of LDL, HDL<sub>2</sub> and HDL<sub>3</sub> particles. Fatty acids are highly intercorrelated, and therefore their use in multivariate analysis as predictors is problematic. We tried to overcome this difficulty by uniting the information in the fatty acid profiles into a single term – the peroxidizability index – which describes the combined reactivity of fatty acids towards reactive oxygen species

[25]. The results (Table 4) show that this index was significantly larger in both HDL<sub>2</sub> and HDL<sub>3</sub> than in LDL in men as well as in women, suggesting that HDL particles might be more susceptible to oxidation than LDL. This opinion was further strengthened by the findings that in multiple regression analysis the peroxidizability index of LDL, HDL<sub>2</sub> or HDL<sub>3</sub> in combination with fasting blood glucose concentration were the best predictors of lag time when LDL was oxidized alone or in mixtures with HDL<sub>2</sub> or HDL<sub>3</sub>, respectively. Furthermore, our finding of a smaller peroxidizability index in those subjects whose lag time lengthened in the presence of HDL<sub>2</sub> is in line with the suggestion that the rate of oxidation is governed by the ratio of bound copper to oxidizable lipids [26]. The results of our experiments also confirm the findings of earlier studies and suggest that the proportions of polyunsaturated fatty acids as well as those of linoleic acid and oleic acid [27-29] are related to the oxidation rate and the amount of dienes formed during in vitro oxidation. We have no ready explanation as to why the glucose concentration had a positive correlation with lag time of oxidation especially since all our subjects were normoglycemic. It has been shown that LDL isolated from patients with poorly controlled type I diabetes is more susceptible to copper-induced oxidation than LDL from control subjects [30]. Consequently, it has been suggested that glycated LDL might be particularly prone to oxidation. Nonetheless, our result is more in line with the results obtained in well-controlled type I diabetics, where glycated LDL gave a longer lag time than that of nonglycated LDL [31].

## Conclusion

In conclusion, we report that the lag time, the maximum propagation rate for the formation of dienes and the amount of dienes formed by Cu<sup>2+</sup>-induced oxidation of LDL alone or in the presence of HDL<sub>2</sub> or HDL<sub>3</sub> do not differ between healthy men and women despite significant

**Table 4: Multivariate regression models of factors predicting oxidation parameters in mixtures of LDL and HDL<sub>2</sub>.**

Dependent variable	Independent variable	Standardized regression coefficient $\beta$	p-Value	Total model
LDL + HDL <sub>2</sub> Lag time (min)	fB-Glucose	0.410	0.00037	R <sup>2</sup> = 0.296 p < 0.00005
	PI of HDL <sub>2</sub>	-0.351	0.00276	
LDL + HDL <sub>2</sub> Oxidation rate ( $\mu$ mol/ml/ min)	$\Sigma$ PUFA of LDL	0.424	0.0325	R <sup>2</sup> = 0.57 p < 0.00000
	$\Sigma$ PUFA of HDL <sub>2</sub>	0.352	0.0129	
LDL + HDL <sub>2</sub> Maximum diene concentration (nmol/mg)	HDL <sub>2</sub> 18:2n-6	0.429	0.00015	R <sup>2</sup> = 0.55 p < 0.00000
	LDL 18:1n-9	-0.292	0.0071	
	LDL/apoB	0.287	0.0033	

Abbreviations: PI, peroxidizability index;  $\Sigma$ PUFA, sum of percentages of polyunsaturated fatty acids; LDL/apoB, the ratio of LDL cholesterol to apoB concentration. Stepwise forward regression analysis

differences in lipid concentrations. Our findings do not support the concept that co incubation of LDL with HDL in the presence of divalent copper prevents its oxidative modification. Rather, our findings support previous results that in vitro HDL is oxidized fastest of all lipoproteins [4] partly because of its fatty acid composition, which is oxidation promoting.

## Methods

### Subjects

61 healthy subjects from the personnel and medical students of the Department of Medical Sciences of Tampere University and Tampere University Hospital volunteered. The age range of the subjects was 20 to 58 years. 33 were women and 28 were men. All participants filled in a questionnaire, where emphasis was given to their health status (diseases and use of medication) in addition to health related behavior (smoking, use of alcohol and vitamins). Ten subjects were current smokers and two abstained from alcohol. Fasting blood glucose concentration was  $\leq 5.7$  mmol/l in all subjects. Nine women and two men reported the use of vitamins and 12 women used hormone preparations. The results of two of the participants were later removed from analysis because of reported diseases. Thus, 59 subjects remained. All participants gave their written consent to the study. The study protocol was approved by the ethics committee of the Tampere University Hospital.

### Blood Samples

Fasting (12 h) blood samples were taken into suitable tubes (Vacuette, Greiner) from the antecubital vein in a sitting position after a 15-min rest using minimal stasis. Samples for the isolation of lipoproteins and for LDL size determination were taken into pre-chilled EDTA tubes, which were immediately placed in ice. Plasma was separated after centrifugation (Heraeus, 2000 xg, +4 °C). EDTA plasmas were supplemented with sucrose (0.6 % w/v final

concentration). This procedure has been shown to preserve LDL from oxidation for at least two months and the oxidation curve does not differ from that of a fresh sample [32]. All samples were kept frozen at -70 °C until analyzed. Fasting blood glucose concentration was determined from capillary blood using Hemocue Glucose Analyzer (Hemocue, Ängelholm Sweden).

### Analysis of Lipids and Lipoproteins

Cholesterol, HDL cholesterol, triacylglycerol, apoA-I and apoB concentrations were measured with Cobas Integra 700 automatic analyzer (Roche Diagnostics, Basel, Switzerland) using reagents and calibrators as recommended by the manufacturer. LDL cholesterol was calculated according to Friedewald. Lp(a) concentrations were analyzed by radioimmunoassay (Pharmacia, Uppsala Sweden) according to the manufacturer's instructions.

### Isolation of Lipoproteins

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation using a Beckman SW40 Ti rotor in a Beckman L60 centrifuge (36000 rpm, 40 hours, 10 °C). 2.0 ml of plasma was gently mixed with 4.0 ml of d 1.35 g/l NaCl-KBr solution in a polyallomer 14 × 95 mm tube. The mixture was then successively overlaid with 4.5 ml of a d 1.006 salt solution and 1.0 ml of distilled water. The gradients were fractionated as described [33] and 0.4-ml fractions were collected. The fractions belonging to LDL, HDL<sub>2</sub> and HDL<sub>3</sub> were pooled on the basis of the absorbance curve. A part of the pooled fractions were immediately frozen to -70 °C and a part was used for the oxidation experiments.

### Oxidation of Lipoproteins

The susceptibility of LDL and mixtures of LDL and HDL to in vitro copper-catalyzed oxidation was assessed by the technique described in [34] as modified from Esterbauer et al. [35]. LDL (50  $\mu$ g protein/ml  $\approx$  0.1

$\mu\text{M}$ ) was incubated either alone or mixed with autologous HDL<sub>2</sub> (50  $\mu\text{g}$  protein/ml  $\approx$  0.35  $\mu\text{M}$ ) or HDL<sub>3</sub> (50  $\mu\text{g}$  protein/ml  $\approx$  0.53  $\mu\text{M}$ ). The protein concentrations were determined using the method of Markwell et al. [36]. Oxidation was started by adding 10  $\mu\text{l}$  of CuSO<sub>4</sub> to a final concentration of 1.65  $\mu\text{M}$  Cu<sup>2+</sup>. The spectrophotometer was computer-operated (UVWinlab 2.1). This program also collected the absorbance data at 2-min intervals during the oxidation. Several characteristic indices were obtained from the resulting absorbance versus time curves [32]. To control the in vitro oxidation procedure we prepared an LDL pool as described [37] and stored it at -70 °C in 0.15-M NaCl/1 mM EDTA solution containing 0.6 % sucrose. One control LDL was analyzed in every oxidation run. The inter-assay coefficient of variation for lag time was 3.1 %. This LDL preparation was also used as a standard in gradient gel electrophoresis.

#### Electrophoretic Analysis of Lipoprotein Size

For the estimation of lipoprotein particle size in EDTA-plasma samples we used the nondenaturing gradient gel electrophoretic method of Krauss and Burke [38]. However, the 2–16 % polyacrylamide gels were cast in-house according to the instructions given by Pharmacia (Uppsala, Sweden) as described [39]. A control plasma sample (peak particle diameter 27.00 nm) stored at -70 °C was included in every gel. The inter-assay coefficient of variation during this study was 1.1 %.

#### Fatty Acid Composition of Lipoproteins

The fatty acid compositions of the ultracentrifugally isolated LDL, HDL<sub>2</sub> and HDL<sub>3</sub> fractions were analyzed by capillary gas-liquid chromatography. Lipids were extracted with chloroform/methanol, partitioned, and the chloroform phase was dried under N<sub>2</sub> [40]. The lipids were then transesterified with H<sub>2</sub>SO<sub>4</sub> in dry methanol at 85 °C for 2 h under N<sub>2</sub>. Following the addition of water, methyl esters of the fatty acids were extracted with petroleum ether and analyzed in a Shimadzu GC-14A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector using a Supelco SP 2560 capillary column (100 m, 0.25 mm I.D., 0.20  $\mu\text{m}$  film thickness). The carrier gas was helium. The column temperature was held at 180 °C for 15 min and thereafter programmed to increase at 3 °C/min until 230 °C and held at this temperature for 40 min. The individual fatty acids were identified with the aid of a standard mixture of methyl esters (Lipid standards 189-15 and 189-17, Sigma). The areas were measured with a Shimadzu C-R4A Chromatopac Integrator and the results expressed as percentages of the sum of all fatty acids from 14:0 to 24:1. As a control sample we used a pool of isolated HDL that was kept frozen at -70 °C. The inter-assay coefficients of variation for the percentages of different fatty acids ranged from 0.3 to 4.4 %. From fatty acid compositions, the fol-

lowing indices were calculated: saturated fatty acids (SAFA) =  $\Sigma(\%)$  of saturated fatty acids; monounsaturated fatty acids (MUFA) =  $\Sigma(\%)$  of monoenoic fatty acids; polyunsaturated fatty acids (PUFA) =  $\Sigma(\%)$  of polyunsaturated fatty acids; peroxidizability index (PI) = [ $(\Sigma \text{ mol\% monoenoic FAs} \times 0.025) + (\Sigma \text{ mol\% dienoic FAs} \times 1) + (\Sigma \text{ mol\% trienoic FAs} \times 2) + (\Sigma \text{ mol\% tetraenoic FAs} \times 4) + (\Sigma \text{ mol\% pentaenoic FAs} \times 6) + (\Sigma \text{ mol\% hexaenoic FAs} \times 8)$ ] [25].

#### Statistical Analysis

Results are expressed as means  $\pm$  standard deviation. Plasma triacylglycerol and Lp(a) concentrations were used as their logarithms but reported as their original results. Comparisons were conducted by analysis of variance or covariance and Mann-Whitney U-test. For pair wise comparisons we used Wilcoxon's matched pairs test. Univariate associations between variables were analyzed using Spearman's correlation coefficients. Predictors for the multivariate analysis were selected on the basis of the correlation analyses. Multivariate analysis was done using the stepwise forward linear regression technique. The Statistica for Windows (version 5.1) software package (Statsoft Inc., Oklahoma, USA) was used for the statistical analyses.

#### List of abbreviations

HDL, high density lipoprotein; LDL, low density lipoprotein; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PI, peroxidizability index

#### Authors' contributions

TS and OJ conceived of the study, participated in its design, performed the statistical analysis and drafted the manuscript, NP and AS carried out the laboratory analyses, MS planned and analyzed the health questionnaire, TL, HJ and STN participated in the coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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#### References

1. Nikkilä EA: Letter: Serum high-density-lipoprotein and coronary heart-disease. *Lancet* 1976, 2:320.
2. Sviridov D, Nestel P: **Dynamics of reverse cholesterol transport: protection against atherosclerosis.** *Atherosclerosis* 2002, **161**:245-254.
3. Parthasarathy S, Barnett J, Fong LG: **High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein.** *Biochim Biophys Acta* 1990, **1044**:275-283.
4. Bowry VW, Stanley KK, Stocker R: **High density lipoprotein is the major carrier of lipid hydroperoxides in human blood**

- plasma from fasting donors. *Proc Natl Acad Sci USA* 1992, **89**:10316-10320.
5. La Ville AE, Sola R, Balanya J, Turner PR, Masana L: **In vitro oxidised HDL is recognised by the scavenger receptor of macrophages: implications for its protective role in vivo.** *Atherosclerosis* 1994, **105**:179-189.
  6. Navab M, Hama SY, Reddy ST, Ng CJ, Van Lenten BJ, Laks H, Fogelman AM: **Oxidized lipids as mediators of coronary heart disease.** *Curr Opin Lipidol* 2002, **13**:363-372.
  7. Nakajima T, Origuchi N, Matsunaga T, Kawai S, Hokari S, Nakamura H, Inoue I, Katayama S, Nagata A, Komoda T: **Localization of oxidized HDL in atheromatous plaques and oxidized HDL binding sites on human aortic endothelial cells.** *Ann Clin Biochem* 2000, **37**:179-186.
  8. Nagano Y, Arai H, Kita T: **High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification.** *Proc Natl Acad Sci USA* 1991, **88**:6457-6461.
  9. Raveh O, Pinchuk I, Schnitzer E, Fainaru M, Schaffer Z, Lichtenberg D: **Kinetic analysis of copper-induced peroxidation of HDL, autoaccelerated and tocopherol-mediated peroxidation.** *Free Rad Biol Med* 2000, **29**:131-146.
  10. Suzukawa M, Ishikawa T, Yoshida H, Nakamura HJ: **Effect of in-vivo supplementation with low-dose vitamin E on susceptibility of low-density lipoprotein and high-density lipoprotein to oxidative modification.** *J Am Coll Nutr* 1995, **14**:46-52.
  11. Schnitzer E, Pinchuk I, Fainaru M, Schafer Z, Lichtenberg D: **Copper-induced lipid oxidation in unfractionated plasma: the lag preceding oxidation as a measure of oxidation-resistance.** *Biochem Biophys Res Commun* 1995, **216**:854-861.
  12. Ohmura H, Watanabe Y, Hastumi C, Sato H, Daida H, Mokuno H, Yamaguchi H: **Possible role of high susceptibility of high-density lipoprotein to lipid peroxidative modification and oxidized high-density lipoprotein in genesis of coronary artery spasm.** *Atherosclerosis* 1999, **142**:179-184.
  13. Mackness MI, Durrington PN: **HDL, its enzymes and its potential to influence lipid peroxidation.** *Atherosclerosis* 1995, **115**:243-253.
  14. Tribble DL, Chu BM, Gong EL, Van-Venrooij F, Nichols AV: **HDL antioxidant effects as assessed using a nonexchangeable probe to monitor particle-specific peroxidative stress in LDL-HDL mixtures.** *J Lipid Res* 1995, **36**:2580-2589.
  15. Kunitake ST, Jarvis MR, Hamilton RL, Kane JP: **Binding of transition metals by apolipoprotein A-I-containing plasma lipoproteins: inhibition of oxidation of low density lipoproteins.** *Proc Natl Acad Sci USA* 1992, **89**:6993-6997.
  16. Kontush A, Chantepie S, Chapman MJ: **Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress.** *Arterioscler Thromb Vasc Biol* 2003, **23**:1881-1888.
  17. Ziouzenkova O, Gieseg SP, Ramos P, Esterbauer H: **Factors affecting resistance of low density lipoproteins to oxidation.** *Lipids* 1996:571-76.
  18. Gieseg SP, Esterbauer H: **Low density lipoprotein is saturable by pro-oxidant copper.** *FEBS Lett* 1994, **343**:188-194.
  19. Abbey M, Nestel PJ, Baghurst PA: **Antioxidant vitamins and low-density lipoprotein oxidation.** *Am J Clin Nutr* 1993, **58**:525-532.
  20. Goulinet S, Chapman MJ: **Plasma LDL and HDL subspecies are heterogeneous in particle content of tocopherols and oxygenated and hydrocarbon carotenoids. Relevance to oxidative resistance and atherogenesis.** *Arterioscler Thromb Vasc Biol* 1997, **17**:786-796.
  21. Reaven PD, Parthasarathy S, Grasse BJ, Miller E, Steinberg D, Witztum JL: **Effects of oleate-rich and linoleate-rich diets on the susceptibility of low-density lipoproteins to modification in mildly hypercholesterolemic volunteers.** *J Clin Invest* 1993, **91**:668-676.
  22. Schnell JW, Anderson RA, Stegner JE, Schindler SP, Weinberg RB: **Effects of high polyunsaturated fat diet and vitamin E on high-density lipoprotein oxidation in humans.** *Atherosclerosis* 2001, **159**:459-466.
  23. Chait A, Brazg RL, Tribble DL, Krauss RM: **Susceptibility of small, dense low-density lipoproteins to oxidative modification in volunteers with the atherogenic lipoprotein phenotype, Pattern B.** *Am J Med* 1993, **94**:350-356.
  24. Korpela R, Seppo L, Laakso J, Lilja J, Karjala K, Lähteenmäki T, Solantunturi E, Vapaatalo H, Tikkanen MJ: **Dietary habits affect the susceptibility of low-density lipoprotein to oxidation.** *Eur J Clin Nutr* 1999, **52**:802-807.
  25. Lambert AJ, Portero-Otin M, Pamplona R, Merry BJ: **Effect of ageing and caloric restriction on specific markers of protein oxidative damage and membrane peroxidizability in rat liver mitochondria.** *Mech Ageing Dev* 2004, **125**:529-538.
  26. Pinchuk I, Schnitzer E, Lichtenberg D: **Kinetic analysis of copper-induced peroxidation of LDL.** *Biochim Biophys Acta* 1998, **1389**:155-172.
  27. Bonanome A, Pagnan A, Biffanti S, Opportuno A, Sorgato F, Dorella M, Maiorino M, Ursini F: **Effect of monounsaturated and polyunsaturated fatty acids on the susceptibility of plasma low density lipoproteins to oxidative modification.** *Arterioscler Thromb* 1992, **12**:529-533.
  28. Kleinveld HA, Naber AH, Stalenhoef AF, Demacker PN: **Oxidation resistance, oxidation rate and extent of oxidation of human low-density lipoprotein depend on the ratio of oleic acid content to linoleic acid content: Studies in vitamin E deficient subjects.** *Free Rad Biol Med* 1993, **15**:273-280.
  29. Scheffer PG, Bakker SJ, Popp-Snijders C, Heine RJ, Schulgens RB, Teerlink T: **Composition of LDL as determinant of its susceptibility to in vitro oxidation in patients with well-controlled type 2 diabetes.** *Diabetes Metab Res Rev* 2001, **17**:459-466.
  30. Tsai EC, Hirsch IB, Brunzell JD, Chait A: **Reduced plasma peroxyl-trapping capacity and increased susceptibility of LDL to oxidation in poorly controlled IDDM.** *Diabetes* 1994, **43**:1010-1014.
  31. Jenkins AJ, Thorpe SR, Alderson NL, Hermayer KL, Lyons TJ, King LP, Chassereau CN, Klein RL: **In vivo glycated low-density lipoprotein is not more susceptible to oxidation than nonglycated low-density lipoprotein in type I diabetes.** *Metabolism* 2004, **53**:969-976.
  32. Ramos P, Gieseg SP, Schuster B, Esterbauer H: **Effect of temperature and phase transition on oxidation resistance of low density lipoprotein.** *J Lipid Res* 1995, **36**:2113-2128.
  33. Jaakkola O, Solakivi T, Tertov VV, Orekhov AN, Miettinen TA, Nikkari T: **Characteristics of low-density lipoprotein subfractions from patients with coronary artery disease.** *Coronary Artery Dis* 1993, **4**:379-385.
  34. Leinonen JS, Rantalaiho V, Solakivi T, Koivula T, Wirta O, Pasternack A, Alho H, Lehtimäki T: **Susceptibility of LDL to oxidation is not associated with the presence of coronary heart disease or renal dysfunction in NIDDM patients.** *Clin Chim Acta* 1998, **275**:163-174.
  35. Esterbauer H, Striegl G, Puhl H, Rotheneder M: **Continuous monitoring of in vitro oxidation of human low density lipoprotein.** *Free Rad Res Commun* 1989, **6**:67-75.
  36. Markwell MA, Haas SM, Tolbert NE, Bieber LL: **Protein determination in membrane and lipoprotein samples: manual and automated procedures.** *Methods Enzymol* 1981, **72**:296-303.
  37. Jaakkola O, Solakivi T, Ylä-Herttuala S, Nikkari T: **Receptor-mediated binding and degradation of subfractions of human plasma low-density lipoprotein in cultured fibroblasts.** *Biochim Biophys Acta* 1989, **1005**:118-122.
  38. Krauss RM, Burke DJ: **Identification of multiple subfractions of plasma low density lipoproteins in normal humans.** *J Lipid Res* 1982, **23**:97-104.
  39. Siirtola A, Antikainen M, Ala-Houhala M, Solakivi T, Jokela H, Lehtimäki T, Holmberg C, Salo MK: **Studies of LDL particle size and susceptibility to oxidation and association with glucose metabolism in children after heart transplantation.** *J Heart Lung Transplant* 2004, **23**:418-426.
  40. Folch J, Lees M, Stanley GHS: **A simple method for the isolation and purification of total lipids from animal tissues.** *J Biol Chem* 1957, **66**:375-400.