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Seasonal Variation in Low Density Lipoprotein Oxidation and Antioxidant Status

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Accumulating evidence indicates that oxidative modification of low-density lipoproteins is atherogenic and that antioxidants may play a role in protection of LDL against oxidation. Several studies have reported a seasonal fluctuation in antioxidant levels, but to date nothing is known about seasonal fluctuations in parameters of oxidizability. We collected blood from 10 volunteers at four different periods over one year (February, May, September and December), and measured the amount of plasma lipids, plasma antioxidants, lipid and fatty acid composition of the LDL particle, LDL antioxidant content, LDL particle size and oxidation parameters (lag time and propagation rate). No seasonal fluctuation for lag time and propagation rate of copper ion-induced LDL oxidation was found. Small seasonal fluctuations were observed for some determinants of LDL oxidation, e.g. plasma and LDL vitamin E and LDL particle size, and for plasma lipids, plasma and LDL lutein and LDL β-carotene. Fatty acid composition of LDL did not change during the year. The main determinant of oxidation susceptibility was the fatty acid composition of LDL.
We conclude that LDL oxidation parameters do not change over the year.

Keywords: Within-subject seasonal variation, LDL oxidation, particle size, antioxidants

1. INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) by free radicals has been implicated as an important determinant in the atherogenic process. By oxidative modification the uptake of LDL by macrophages is accelerated which is the beginning of the fatty streak.[1] LDL is protected against the free radical attack by antioxidants in plasma and in the particle itself. High plasma levels of vitamin E,[2–4] vitamin C[2,3,5] and β-carotene[5,6] have been suggested to be associated with a decreased risk of cardiovascular diseases.

In the literature seasonal variation in plasma lipid and vitamin levels has been reported. Seasonal variation in β-carotene,[7–9] ascorbic acid[10,11] and α-tocopherol[7,8] may result either directly or indirectly in seasonal variation in LDL oxidizability through variation in plasma or LDL antioxidant activity. Other well-known parameters influencing the oxidizability of LDL

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are the size and composition of the LDL particle[12,13] and its fatty acid composition.[14,15] To our knowledge, no data are available on seasonal variation of these parameters. We therefore studied the influence of the time of the year on the antioxidant status and parameters of oxidizability of LDL cholesterol in healthy subjects.

2. METHODS

2.1 Study Design

We collected blood of 10 volunteers at four different periods over one year (February, May, September and December). The six men and four women were apparently healthy and had a mean age 39.6 ± 7.4 years. Two of them were current smokers. Two persons had only three blood collections. All participants in this study gave their informed consent.

Blood was collected in EDTA-containing Vacutainer tubes (1 mg/ml) between 8.30 and 9.00 am at all four time points. The subjects had consumed a light breakfast. Blood was immediately placed on ice and cooled to 4°C. Plasma was prepared, frozen in liquid nitrogen in small portions, leaving as little empty space as possible in the tubes, and stored at –80°C. This procedure was completed within 1 hour from venapuncture. Laboratory analyses were performed at the end of the year of study.

2.2 Preparation and Oxidation of LDL

The procedure for preparation and lipid peroxidation of LDL was adapted from the method described by Esterbauer and colleagues[16] with some major modifications as described previously in detail.[17,18] Briefly, from each subject 2 ml of frozen plasma, stored at –80°C was rapidly thawed and used for isolation of LDL by ultracentrifugation at 4°C in the presence of 10 μM EDTA. To minimize the time between isolation and oxidation and to prevent loss of lipophilic antioxidants,[19] the LDL was not dialysed.[16] By omitting dialysis a more stable LDL preparation is obtained, which can be stored in the dark at 4°C under nitrogen for several days without affecting resistance time and propagation rate.[17,18,20] This improves the precision of the method, since each LDL preparation can be oxidized consecutively in triplicate. In a representative experiment, lag time was 90 ± 2 min one hour after LDL isolation in a LDL preparation which had not been dialyzed; 24 hours after LDL isolation lag time was 91 ± 3 min (n = 3). Dialysis under nitrogen for 4 hours (2 changes) at 4°C against 1000 volumes of an oxygen-free buffer containing 150 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4 resulted in lag times of 52 ± 5 min, directly after dialysis and 23 ± 4 min after storage of this LDL under nitrogen for 24 hours (n = 3). In agreement with these observations, recently, a loss of lipophilic antioxidants during dialysis was reported.[19]

LDL oxidation is performed under hypersaline conditions (1.18 mol/L NaCl) and in the presence of 10 μmol/L EDTA. Oxidation of LDL under hypersaline conditions results in a higher lag time compared with oxidation in physiological saline (0.15 mol/L).[17] In a test we found a lag time of 90 ± 2 min in 1.18 mol/L NaCl and 58 ± 5 min in 0.15 mol/L NaCl both with 10 μmol/L EDTA and 40 μmol/L $CuSO_4$ (n = 3 independent oxidations with a reference LDL on different days). Ten μmol/L EDTA is added during ultracentrifugation to protect the LDL against oxidation and in the oxidation assay to have equal EDTA concentrations in all assays. To overcome the 10 μmol/L and because of the hypersaline conditions, 40 μmol/L $CuSO_4$ is added to initiate lipid peroxidation.[17,18] Under these conditions (high salt and 40 μmol/L $CuSO_4$) the presence of EDTA does not affect kinetics of LDL oxidation.

The kinetics of the LDL oxidation were followed by continuously monitoring the change of absorbance at 234 nm.[16–18] All samples from one person were analysed in parallel in the same oxidation run. Each LDL preparation was oxidized in three consecutive oxidation runs on the same

day. Means were calculated based upon these three observations. The intra-assay coefficients of variation for lag time and propagation rate calculated from measurements obtained at one day were 2.6% and 3.1%, respectively. The inter-assay coefficients of measurements performed on different days were 4.9% and 7.4%, respectively.[18] In every oxidation run one reference LDL, prepared from a reference plasma stored at –80°C, was used as a control. Oxidation runs with a higher than 10% deviation from the mean values of former reference measurements were omitted.[17,18] By using this highly standardized method, lag time and propagation rate do not differ between LDL prepared from plasma frozen in liquid nitrogen and that from freshly collected plasma from the same subject. In addition, no differences in these parameters were found upon storage of plasma at –80°C up to 18 months.[17,18]

2.3 Analytical Measurements

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP kit nr. 236.691 and Triglyceride kit nr. 701.904, Boehringer-Mannheim, Mannheim, Germany). Phospholipid concentrations were determined using a commercially available colour reagent (Wako Chemicals GmbH, Neuss, Germany). 100 µl of LDL (0.25 mg protein/ml) sample and 750 µl colour reagent were mixed for 10 minutes at 37°C and the concentration was measured at a wavelength of 500 nm.

High density lipoprotein (HDL)-cholesterol was measured after precipitation of very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) using the precipitation method with sodium phosphotungstate-Mg^{2+}.[21] LDL-cholesterol concentrations were calculated by the Friedewald formula.[22] LDL size was determined by analysis of 7.5–10 µl plasma using 2–16% nondenaturing polyacrylamide gradient gel electrophoresis (Pharmacia LKB, Uppsala, Sweden).[23] High molecular weight standards (Pharmacia, Piscataway, N.J., U.S.A.) were used, together with a reference serum obtained from a pool of normolipidemic sera. In our institution the reference value for LDL particle size is 25.8 nm. After staining with Sudan Black B gels were scanned with an LKB 2202 Ultrascan laser densitometer (LKB, Paramus, N.J., U.S.A.).

Fatty acid composition of LDL was determined by gas-liquid chromatography using a Chrom-pack gas chromatograph (model 438S) equipped with a CP-Sil88 column (50 m × 0.25 mm i.d.) and a flame ionisation detector, as described previously.[17] We calculated the amount of poly-unsaturated fatty acids (PUFAs = C18:2 + C20:3 + C20:4 + C22:6), mono-unsaturated fatty acids (MUFAs = C16:1 + C18:1), and saturated fatty acids (SFAs = C14:0 + C16:0 + C18:0).

Alpha-tocopherol and carotenoids in plasma and LDL (stored at –80°C) were quantified by reverse-phase HPLC with spectrophotometric detection.[24]

2.4 Statistical Analysis

Data analysis was conducted using the statistical package BMDP.[25] First, means and standard deviations for the four time periods were calculated for plasma and LDL lipid levels, antioxidant levels in plasma and in LDL, LDL particle size and fatty acid composition and oxidation parameters. Within-person seasonal fluctuation for these parameters was assessed by analysis of variance (5V module). For those parameters which showed a significant seasonal fluctuation paired t-tests were performed to detect which periods differed significantly from each other ($p < 0.05$). Further, partial correlations were calculated between the oxidation parameters and the determinants of oxidation, e.g. antioxidant vitamins, fatty acid composition and particle size. Adjustment was made for person number, to adjust for dependency of the measures of one subject over the four moments.

3. RESULTS

Table I shows the mean and standard deviation for the plasma lipids, the plasma antioxidant vitamins, and the vitamin status of the LDL particle for the four time periods. Seasonal fluctuation was found for plasma lipids, plasma vitamin E and plasma lutein. Plasma total-cholesterol and LDL-cholesterol levels were significantly lower in May compared to September and triglyceride levels were lower in May compared to both September and February. Plasma lutein was significantly lower in September compared to February and May. Further seasonal fluctuation was found for the vitamin E, β-carotene and lutein content of the LDL particle. Both LDL vitamin E and lutein had higher values in May compared to September and December, whereas for vitamin E the values in December were also lower than those in February.

Seasonal variation in oxidation parameters, LDL particle size and LDL fatty acid composition are presented in Table II. No significant seasonal variation for lag time and propagation rate was found. LDL particle size did show seasonal fluctuation, with the lowest particle size in December. There were no differences in fatty acid composition of the LDL over the four periods.

The oxidation parameters were not significantly correlated to LDL particle size as assessed by partial correlation ($r = -0.08$ and $r = 0.30$ for propagation rate and lag time, respectively). A positive correlation was found between the amount of poly-unsaturated fatty acids in LDL and propagation rate (Pearson's $r = 0.65$, $p < 0.01$) (Fig. 1). The partial correlation for the association between poly-unsaturated fatty acids and propagation rate was slightly higher ($r = 0.73$, $p < 0.01$). Further, propagation rate was negatively correlated with LDL mono-unsaturated fatty acids ($r = -0.56$, $p < 0.05$) and saturated fatty acids ($r = -0.51$, $p < 0.05$). No significant correlation was found between fatty acid composition and lag time.

4. DISCUSSION

The purpose of this study was to assess the influence of the time of the year on the antioxidant status and parameters of LDL oxidizability.

TABLE I Seasonal variation in lipids and vitamins (mean ± SD)

	February (n = 10)	May (n = 9)	September (n = 10)	December (n = 9)
Plasma:				
Cholesterol, mmol/L*	5.65 ± 0.95	5.36 ± 0.94[3]	5.63 ± 1.04[2]	5.38 ± 0.94
Triglycerides, mmol/L*	1.49 ± 0.63[2]	1.12 ± 0.45[1,3]	1.44 ± 0.64[2]	1.31 ± 0.55
HDL, mmol/L*	1.19 ± 0.22	1.32 ± 0.16	1.16 ± 0.27	1.32 ± 0.45
LDL, mmol/L*	3.78 ± 0.78	3.53 ± 0.83[3]	3.82 ± 0.78[2]	3.46 ± 0.90
Vitamin E, μmol/L*	33.08 ± 6.54	33.53 ± 6.80	33.62 ± 5.39	29.55 ± 5.60
Lycopene, μmol/L	0.31 ± 0.20	0.30 ± 0.16	0.41 ± 0.30	0.25 ± 0.11
β-Carotene, μmol/L	0.38 ± 0.16	0.34 ± 0.11	0.38 ± 0.15	0.32 ± 0.08
Lutein, μmol/L*	0.25 ± 0.07[3]	0.28 ± 0.08[3]	0.20 ± 0.05[1,2]	0.23 ± 0.09
α-Carotene, μmol/L	0.07 ± 0.03	0.06 ± 0.03	0.07 ± 0.03	0.05 ± 0.03
LDL:				
Vitamin E, nmol/mg protein*	22.18 ± 2.37[4]	24.10 ± 2.66[3,4]	19.73 ± 3.64[2]	18.91 ± 1.83[1,2]
Lycopene, nmol/mg protein	0.25 ± 0.14	0.25 ± 0.16	0.23 ± 0.16	0.21 ± 0.08
β-Carotene, nmol/mg protein*	0.22 ± 0.12	0.23 ± 0.12	0.29 ± 0.16	0.23 ± 0.11
Lutein, nmol/mg protein*	0.06 ± 0.02	0.08 ± 0.03[3,4]	0.05 ± 0.02[2]	0.05 ± 0.02[2]

*significant overall seasonal fluctuation ($p < 0.05$). [1]significantly different from levels in February ($p < 0.05$). [2]significantly different from levels in May ($p < 0.05$). [3]significantly different from levels in September ($p < 0.05$). [4]significantly different from levels in December ($p < 0.05$).

TABLE II Seasonal variation in parameters of LDL oxidation, LDL size and LDL fatty acid composition

	February (n = 10)	May (n = 9)	September (n = 10)	December (n = 9)
lag time, min	100 ± 6	99 ± 5	101 ± 8	97 ± 7
propagation rate, nmol dienes/min/mg protein	9.0 ± 0.8	9.0 ± 0.7	9.1 ± 0.6	9.2 ± 0.9
LDL particle size, nm*	26.6 ± 0.5[3,4]	27.1 ± 0.4[4]	7.0 ± 0.5[1,4]	26.0 ± 0.3[1,2,3]
LDL fatty acid composition:				
Total fatty acids, μmol/mg protein	4.2 ± 0.4	4.7 ± 0.5	4.5 ± 0.5	4.5 ± 0.4
SFA, %**	28.2 ± 3.2	28.3 ± 4.0	28.0 ± 2.8	29.5 ± 3.2
MUFA, %**	20.0 ± 2.6	20.9 ± 3.7	21.4 ± 3.1	20.6 ± 2.5
PUFA, %**	51.8 ± 4.1	50.8 ± 3.6	50.6 ± 4.5	50.0 ± 3.0

*significant overall seasonal fluctuation ($p < 0.05$). **SFA = saturated fatty acids, MUFA = mono-unsaturated fatty acids, PUFA = poly-unsaturated fatty acids. [1]significantly different from levels in February ($p < 0.05$). [2]significantly different from levels in May ($p < 0.05$). [3]significantly different from levels in September ($p < 0.05$). [4]significantly different from levels in December ($p < 0.05$).

Para-meters of oxidation remained stable throughout the year, however, small fluctuations in plasma lipid levels, plasma and LDL vitamin E and lutein levels, LDL β-carotene levels and particle size over the seasons were found.

Our study sample comprised only a small number of subjects. Of the 10 participants two subjects had no blood sample taking at one moment. To optimize the use of all available data we used the repeated measures module,

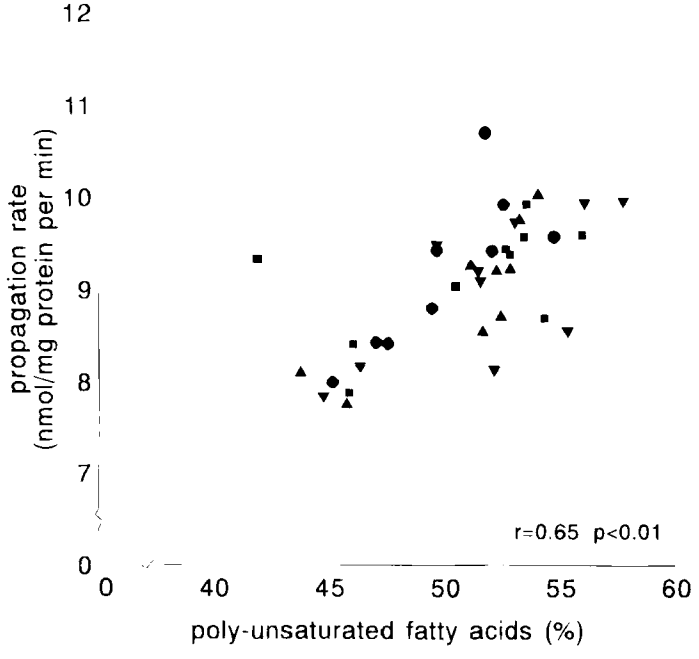

FIGURE 1 Plot showing the correlation between propagation rate of LDL oxidation and the percentage LDL poly-unsaturated fatty acids per moment; February (▼), May (▲), September (■) and December (●). The Pearson correlation is calculated over the total of 38 measures.

which adequately handles missing data. To ensure that the difference in means in the periods could not be ascribed to the different number of subjects, we calculated the means in the subset of subjects with complete data. Only marginal differences were detected and resulted in similar patterns as those presented in the Tables (results not shown).

We are aware that the differences over the seasons found in some parameters are only small. Though statistically significant, this does not imply that these differences are also of physiological relevance.

In our study significant differences in plasma lipids over the seasons were found. The fluctuation pattern for total cholesterol, LDL and triglycerides were comparable, high in February and September, low in May and December. The HDL levels showed the opposite pattern. Several groups described seasonal fluctuation in cholesterol levels,[26–29] but from these and our studies it is still inconclusive in which months the peaks and troughs occur.

For vitamins we found a seasonal fluctuation in plasma vitamin E and lutein levels and LDL vitamin E, β-carotene and lutein levels. A seasonal variation in serum vitamin E levels, however, was not shown in other studies.[7,8] Several studies considering carotenoids found seasonal variations for β-carotene,[7–9] α-carotene, β-cryptoxanthin and lutein.[8] However, Cantilena and colleagues did not find a seasonal within-person fluctuation for α- and β-carotene and cryptoxanthin[30] and no seasonal fluctuations have been found for lycopene and zeaxanthin.[8,30] To our knowledge no data are available on fluctuations of vitamin concentrations in LDL.

Seasonal fluctuations in vitamin levels in plasma and LDL may be ascribed to seasonal fluctuations in intake of these vitamins. Dietary variation of antioxidant intake over the year has been described by several groups.[9,31] As a result of better preservation techniques and marketing facilities seasonal variation in food intake gets less pronounced, but may still be important. In the present study no dietary data were assessed, so we can only speculate on influences of intake on plasma and LDL levels measured over different seasons. There is no variation in LDL fatty acid composition over the year, which suggests that qualitative intake of oils and fats (with respect to composition) remained stable over the year.

To our knowledge this is the first time that seasonal influence on oxidation parameters has been assessed. We did not find a difference in parameters of oxidation, e.g. lag time and propagation rate, over the four time periods. Oxidizability of LDL is influenced by fatty acid composition,[14,15,20,32] LDL triglycerides,[32] LDL particle size,[12,13] LDL vitamin E[17,18,33] and plasma vitamin E levels,[17,18] but not by carotenoids.[18,34] From the above mentioned parameters a significant seasonal fluctuation was found for LDL particle size and LDL vitamin E. For vitamin E correlations with parameters of oxidation have been reported only after vitamin E suppletion.[17,18,33] The same holds for fatty acids in most studies.[14,15,20,32] Therefore an intervention seems necessary to find a sufficiently large contrast. Nevertheless, we found a positive correlation between the total amount of poly-unsaturated fatty acids in LDL and propagation rate in this study. Similar associations have been reported in other studies in unsupplemented normal and hypertriglyceridemic subjects,[20,35] indicating that fatty acid composition of the LDL particle is an important parameter determining LDL oxidizability in an unsupplemented (group of the) population. The LDL particle size in our study was not correlated to lag time or propagation rate of LDL oxidation. This could be due to the small sample size of this study, or to the small diversity in particle sizes. Following the classification of LDL particles by Musliner and Krauss,[36] the predominant LDL type measured in our study is the large LDL type I. Other studies in which a relationship between particle size and oxidation parameters is reported, showed a greater diversity in particle sizes with separation of the various density

classes by ultracentrifugation.[12,13] In our study one LDL preparation has been isolated and particle size of the most abundant LDL particles has been determined.

In spite of small fluctuations in determinants of LDL oxidtion, no seasonal fluctuation in LDL oxidation was found. Therefore it appears unnecessary to consider seasonality in a study on LDL oxidation.

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