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Serum non-transferrin-bound iron and low-density lipoprotein oxidation in heterozygous hemochromatosis

Lambertus J.H. van Tits [a,*], Esther M.G. Jacobs [a,b], Dorine W. Swinkels [b], Heidi L.M. Lemmers [a], Gerly M. van der Vleuten [a], Jacqueline de Graaf [a], Anton F.H. Stalenhoef [a]

[a] *Department of General Internal Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*
[b] *Department of Clinical Chemistry, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*

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Abstract

Non-transferrin-bound iron (NTBI) is implicated in lipid peroxidation but the relation with oxidative modification of low-density lipoprotein (LDL) is not known. We assessed variables reflecting in vitro and in vivo LDL oxidation in two age- and sex-matched groups ($n = 23$) of hereditary hemochromatosis heterozygotes (C282Y), characterized by a clear difference in mean serum NTBI (1.55 ± 0.57 μmol/L vs 3.70 ± 0.96 μmol/L). Plasma level of oxidized LDL (absolute and relative to plasma apolipoprotein B), and IgG and IgM antibodies to oxidized LDL, markers of in vivo LDL oxidation, did not differ between the groups with low and high serum NTBI. Mean lag-phase of in vitro LDL oxidation was also not significantly different between both study groups. Conclusion: these findings do not support the hypothesis that NTBI promotes oxidative modification of plasma LDL.

Keywords: Low-density lipoprotein oxidation; Heterozygous hemochromatosis; Non-transferrin-bound iron; Lipid peroxidation

Although not confirmed by a meta-analysis of prospective studies [1], epidemiological and experimental data suggest evidence for a potential role of iron in atherosclerosis [2]. It is hypothesized that free iron-catalyzed oxidation of low-density lipoprotein (LDL) is causally involved [3,4]. In healthy adult men, intracellularly most iron is contained in hemoglobin, myoglobin, and ferritin, and in plasma the glycoprotein transferrin efficiently binds iron. Thus, levels of free iron are reduced to nearly zero. When body iron rises to levels above normal, however, part of it may not be safely stored. Iron then binds to ligands with less affinity than transferrin, such as citrate, phosphates, and albumin, and may be available for redox reactions [5]. Moreover, also in the presence of unsaturated transferrin, non-transferrin-bound iron (NTBI) can be detected in serum [6]. Subjects heterozygous for the C282Y mutation in the hemochromatosis gene (hereditary hemochromatosis heterozygotes) were shown to have higher serum NTBI levels than normal controls [7], despite only moderately increased serum transferrin saturation. In addition, these subjects seem to have an increased cardiovascular disease risk [8,9]. The relationship of NTBI with LDL oxidation, however, has barely been studied before. In healthy subjects who participated in feeding studies in which the type and amount of dietary fat were controlled, NTBI was not associated with measures of in vitro LDL oxidation (lag-time, rate of oxidation, and total dienes formed) [10]. No data are available on NTBI in relation to variables of in vivo LDL oxidation. Therefore, in this study we assessed variables reflecting in vitro and in vivo LDL oxidation in hereditary hemochromatosis heterozygotes. Homozygotes were excluded because it was recently reported that patients with hereditary hemochromatosis (C282Y homozygotes) have disturbed assembly, maturation, and surface

* Corresponding author. Fax: +31 24 3541346.
E-mail address: B.vantits@aig.umcn.nl (L.J.H. van Tits).

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expression of MHC class I molecules [11]. These abnormalities may lead to altered inflammatory responses and immunologic defects and might confound the relation between NTBI and LDL oxidation. As measurements of in vitro *LDL* oxidizability are labor-intensive, we performed all measurements in two age- and sex-matched groups of heterozygous subjects characterized by an obvious difference in mean level of NTBI.

Materials and methods

Subjects. Participants were selected from subjects who had visited the outpatients' clinic for hemochromatosis family screening at the Radboud University Nijmegen Medical Centre between February 2002 and September 2004. Of all these subjects, iron status (including NTBI) and plasma lipids had been determined in fresh samples, and aliquots of EDTA-plasma and serum were frozen at −80 °C with and without saccharose (0.6% (w/v) final concentration) as cryopreservant. NTBI levels of the total group ranged from 0.44 to 6.21 μmol/L and differed significantly between males (median 2.75 μmol/L) and females (median 2.30 μmol/L). For the present study we selected 23 subjects with serum NTBI levels above the sex-specific 50th percentile and 23 age- and sex-matched subjects with low serum NTBI (below sex-specific 50th percentile). Due to the matching procedure, NTBI of one of the males from the high serum NTBI group was slightly below the 50th percentile (43th percentile) and NTBI of one of the females from the low serum NTBI group was minimally above the 50th percentile (56th percentile). Only subjects heterozygous for the C282Y mutation and wild-type for the H63D mutation in the hemochromatosis gene were included. Subjects were excluded when cardiovascular disease, diabetes mellitus or infectious diseases (C-reactive protein >20 mg/L) were present. Also subjects receiving statin therapy or iron chelation medication and subjects using antioxidants were excluded. Pregnancy and alcohol abuse were also exclusion criteria. A participant was considered to have cardiovascular disease when she/he had been diagnosed with angina pectoris, myocardial infarction, arrhythmia, stroke, or peripheral arterial disease, or when she/he had undergone intervention procedures (balloon angioplasty, coronary bypass surgery or aortic aneurysm surgery). Of each participant demographic (age and sex), physical (height, weight, and blood pressure), and lifestyle characteristics (e.g., smoking behavior and blood donorship) and a self-reported medical history were available.

The protocol was approved by the Hospital Ethics Committee and written informed consent was obtained from all participants.

Biochemical measurements

Iron status. Serum total iron and serum total iron binding capacity (TIBC) were determined by routine clinical chemistry methods on the Hitachi 747 (Roche) and serum ferritin was measured by a solid-phase two-site chemiluminescent immunometric assay using the Immulite 2000 (Diagnostic Products, Cirrus); serum transferrin saturation was calculated as a percentage of serum total iron divided by TIBC. NTBI was measured in serum by a method using iron-sensitive fluorescence-labeled apotransferrin, as described previously [12] and recently evaluated by our group in an interlaboratory comparative study [13]. The assay has an optimal profile for reproducible measurements of a wide range of NTBI levels (range 0–7 μmol/L, within-samples CV = 4.4%). In a normal healthy population, using this assay, we found a strong correlation between serum NTBI and serum transferrin saturation ($r = 0.84$, $p < 0.001$; unpublished data).

Lipids and apolipoprotein B. Plasma cholesterol and triglycerides were determined using commercially available reagents on the Hitachi 747 analyzer (Boehringer Mannheim, Germany). High-density lipoprotein (HDL)-cholesterol was determined with the phosphotungstate/Mg^{2+} method [14]. LDL-cholesterol was calculated with the Friedewald formula. Apolipoprotein B was quantified by immunonephelometry [15].

Measures of in vivo LDL oxidation. For measurement of oxidized LDL we used a commercial non-competitive ELISA (Mercodia, Uppsala, Sweden). This assay uses monoclonal antibody 4E6 to specifically capture oxidatively modified apolipoprotein B from the sample, which is subsequently detected with an antibody to apolipoprotein B. Intra- and inter-assay coefficients of variation amounted 6% and 7%, respectively. As shown previously by our group [16] and confirmed recently by Holvoet et al. [17], duration of storage at −80 °C did not influence the amount of oxidized LDL measured, provided that the EDTA samples had been collected carefully and had not been thawed.

Circulating antibodies (IgG and IgM) to oxidized LDL were measured as described previously [18]. In short, samples were incubated in wells of microtiter plates precoated with native or oxidized LDL, and bound antibodies were detected using peroxidase-conjugated antibodies from goat specific for human IgG or IgM (Sigma–Aldrich, Steinheim, Germany). Results are expressed as means optical density values at 450 nm from duplicate measurements, and the antibody titer to oxidized LDL is calculated by subtracting the binding to native LDL from the binding to oxidized LDL. With this subtraction method our assay not only corrects for specific binding to native LDL, but also for non-specific binding of each sample to the wells of the microtiter plate. The same batch of native and oxidized LDL was used for all determinations of antibodies to oxidized LDL in this study.

Measurement of in vitro LDL oxidizability. The LDL oxidation experiments were performed as described by Esterbauer et al. [19] and modified by Kleinveld et al. [20]. Briefly, LDL was isolated from EDTA plasma supplemented with saccharose before storage at −80 °C by density gradient ultracentrifugation (40,000 rpm for 18 h at 4 °C) using a SW40 rotor (Beckman, Palo Alto, CA), and the lipid peroxidation of LDL (60 μg apolipoprotein/mL) was initiated by the addition of $CuSO_4$ to a final concentration of 18 μM at 30 °C. The kinetics of the lipid peroxidation of LDL were determined by monitoring the change of the 234 nm diene absorption in a thermostated ultraviolet spectrophotometer (Lambda 12, Perkin-Elmer GmbH, Rodgau-Jügesheim, Germany). The lipid peroxidation characteristics of LDL were determined as described previously by the lag-time (min), the peroxidation rate (nmol dienes/mg protein/min), and the amount of dienes formed during LDL lipid peroxidation (nmol/mg LDL protein) [21]. Lipid peroxidation of pooled EDTA plasma stored with saccharose at −80 °C, as assessed by these variables, was found to be stable for at least three years (data not shown) and served as control.

Vitamin E. Concentrations of vitamin E were analyzed by reversed-phase high-performance liquid chromatography [21].

Statistical analysis. Data showing normal distribution are presented as means (SD); data showing skewed distribution are presented as median (interquartile range). Differences between low and high NTBI groups were analyzed by using Student's t test for unpaired data. Variables showing significant differences between sexes were stratified by gender. All data were transformed (natural log) before analysis. A p value $\leqslant 0.05$ was considered statistically significant. Analyses were performed using SPSS 12.0.1 for Windows.

Results

Both study groups consisted of 12 males and 11 females, aged approximately 43 ± 15 years. Body mass index, waist-hip ratio, blood pressure, plasma lipid and apolipoprotein B levels, smoking status, and numbers of blood donors were not different between the two study groups (Table 1). Plasma markers of iron status and variables of in vivo and in vitro LDL oxidation are presented in Table 2. Because levels of several variables (NTBI, ferritin, and lag-time) showed significant differences between sexes, all data in the table are presented stratified by sex. Ranges of NTBI of low and high serum NTBI groups amounted 0.51–2.43 and 2.81–5.44 μmol/L for the females and

Table 1
Characteristics of 46 subjects heterozygous for the C282Y mutation in the hemochromatosis gene stratified by serum NTBI

	Low serum NTBI	High serum NTBI
Demographics		
Age (years)	43 (15)	42 (15)
Males/females[a]	12/11	12/11
Physical examination		
Body mass index (kg/m^2)	24.8 (2.9)	25.2 (4.0)
Waist/hip	0.86 (0.08)	0.85 (0.07)
Diastolic blood pressure (mm Hg)	79 (10)	79 (12)
Systolic blood pressure (mm Hg)	128 (17)	129 (17)
Plasma lipids and apolipoprotein B		
Total cholesterol (mmol/L)	5.4 (1.3)	5.2 (1.0)
Total triglycerides (mmol/L)[b]	1.30 (0.97–2.18)	1.45 (1.10–1.97)
HDL cholesterol (mmol/L)[b]	1.21 (1.02–1.40)	1.19 (1.05–1.40)
LDL cholesterol (mmol/L)	3.57 (1.11)	3.21 (0.86)
Apolipoprotein B (mg/L)	1042 (310)	951 (235)
Life style characteristics		
Smoker (present/past/never)[a]	7/5/11	8/7/8
Blood donor (yes/no)[a]	3/20	5/18

Data are presented as means (SD), unless indicated otherwise.
[a] Presented as numbers.
[b] Presented as median (interquartile range); HDL, high-density lipoprotein; LDL, low-density lipoprotein.

0.77–2.72 and 2.49–5.88 μmol/L for the males, respectively. Compared to the group with low serum NTBI, the subjects with high serum NTBI had higher levels of serum iron and transferrin saturation (13.7 ± 5.6 μmol/L and $24.6 \pm 10.1\%$ versus 25.4 ± 7.8 μmol/L and $49.0 \pm 19.1\%$, respectively, males and females combined, Table 2). Serum ferritin was higher in the high NTBI group compared to the low NTBI group only for the females (Table 2). No significant difference was observed between both groups in TIBC (Table 2). In vivo LDL oxidation was studied directly by measuring circulating oxidized LDL, and indirectly by determining plasma levels of antibodies to oxidized LDL. Both variables of in vivo LDL oxidation were not significantly different between the groups with low and high serum NTBI. Also when expressed per apolipoprotein B, no differences were observed in oxidized LDL between the two total groups (Table 2). As a measure of susceptibility to oxidative stress, we assessed the copper-induced in vitro oxidation of isolated LDL. Mean lag-phase (i.e., the time during which the diene absorption increases only weakly) was not significantly different between males or females of both study groups (Table 2). In accordance with this, mean plasma vitamin E concentration of both groups was nearly identical and LDL of both study groups contained equal amounts of vitamin E (Table 2). However, the rate of in vitro lipid peroxidation during the propagation phase and the total amount of dienes formed during oxidation of LDL from subjects with low serum NTBI were significantly higher than from subjects with high serum NTBI (14.4 ± 2.1 nmol/mg/min and 643 ± 62 nmol/mg LDL protein versus 12.9 ± 2.4 nmol/mg/min and 603 ± 70 nmol/mg LDL protein, respectively, males and females combined, Table 2).

Discussion

This is the first time oxidized LDL and NTBI are studied in one population. We report that in subjects heterozygous for the C282Y mutation and wild-type for the H63D mutation in the hemochromatosis gene, elevated serum NTBI level is not associated with altered plasma levels of oxidized LDL or lag-time of in vitro LDL oxidation. Only rate of in vitro LDL oxidation and the amount of dienes formed were slightly but significantly decreased at elevated serum NTBI. These findings suggest that NTBI-mediated increased oxidation of LDL is not underlying the reported increased cardiovascular disease risk associated with heterozygous hemochromatosis.

Previously, in two studies the relation of iron status with in vitro LDL oxidation was investigated. In one study, aggressive iron supplementation in women with low iron status improved iron status but did not affect measures of in vitro LDL oxidation (lag-time, rate of oxidation, and total dienes formed) [22]. In another study, diet composition was found to affect plasma iron in men, but no significant relations were found between any measure of iron status (including NTBI) and variables of in vitro LDL oxidation [10]. Plasma markers of in vivo LDL oxidation have not been studied before in relation to NTBI, but there are a few studies on oxidized LDL in relation to other variables of iron status [23–26]. No association was found between blood donation-induced low ferritin levels and oxidized LDL levels [26], and also among the participants of the Atherosclerosis Risk in Communities (ARIC) study, no association existed for serum ferritin with lag-time of in vitro LDL oxidation and with autoantibodies to malondialdehyde-modified LDL [24]. Weak but significant relationships were observed for serum ferritin with the cholesterol autoxidation product 7β-hydroxycholesterol in eastern Finnish men [25] and with plasma levels of oxidized LDL, normalized for serum LDL cholesterol concentration, in the male subgroup of healthy Caucasians [23]. However, serum ferritin is an inappropriate marker of free (redox-active) iron [27]. In contrast to NTBI, ferritin is hypothesized to be not available for catalyzing free radical formation. In addition, serum NTBI and serum ferritin correlate only weakly. Furthermore, serum ferritin is an acute phase reactant that is elevated by infection and may be elevated by inflammation. For these reasons, associations of markers of inflammation and in vivo LDL oxidation with NTBI differ from those with ferritin, and measurement of NTBI itself is preferred when assessing a possible association between iron status and inflammation and oxidation. Moreover, in all the studies mentioned above, iron status of the participants was normal or even subnormal. We assume that, if a relationship exists between iron status and measures of LDL oxidation, this will be most apparent when subjects with elevated serum

Table 2
Plasma markers of iron status, variables of in vivo and in vitro LDL oxidation, and vitamin E concentrations in subjects with low and high serum NTBI levels stratified by sex

Variables	Low serum NTBI		High serum NTBI		p value for total groups
	Females ($n = 11$)	Males ($n = 12$)	Females ($n = 11$)	Males ($n = 12$)	
Iron status					
NTBI (μmol/L)[a]	1.24 (0.53)	1.84 (0.47)	3.73 (0.91)[b]	3.67 (1.04)[b]	—
Iron (μmol/L)	11.6 (3.8)	15.8 (6.4)	26.7 (6.9)	24.1 (8.6)	0.00
TIBC (μmol/L)	60.7 (11.5)	54.4 (7.0)	53.7 (8.1)	52.8 (5.6)	0.13
Transferrin saturation (%)	19.6 (6.7)	29.1 (10.9)	51.0 (17.3)	47.2 (21.2)	0.00
Ferritin (μg/L)[a]	61 (29–78)	255 (126–343)	123 (47–318)[b]	156 (74–221)	—
In vivo LDL oxidation					
Oxidized LDL (U/L)	46 (29–65)	65 (41–95)	52 (42–69)	61 (43–65)	0.92
Oxidized LDL/apoB (U/mg)	0.053 (0.047–0.067)	0.052 (0.048–0.067)	0.063 (0.053–0.065)	0.059 (0.052–0.068)	0.18
IgG antibodies to oxidized LDL (OD_{450})	0.33 (0.13–0.54)	0.43 (0.25–0.69)	0.27 (0.18–0.38)	0.30 (0.19–0.54)	0.27
IgM antibodies to oxidized LDL (OD_{450})	0.62 (0.40)	0.54 (0.25)	0.62 (0.42)	0.63 (0.40)	0.83
In vitro LDL oxidation					
Lag-time (min)[a]	82.6 (8.8)	73.2 (5.7)	82.5 (8.7)	80.2 (10.3)	—
Rate of oxidation (nmol/mg/min)	14.1 (2.2)	14.7 (2.0)	12.4 (3.0)	13.3 (1.8)	0.03
Dienes formed (nmol/mg LDL protein)	640 (72)	646 (55)	596 (88)	609 (52)	0.05
Vitamin E concentration					
In plasma (μmol/L)	18.4 (5.0)	19.7 (4.8)	18.3 (7.5)	20.2 (5.5)	0.96
In LDL (μmol/g LDL protein)	10.0 (1.6)	9.6 (1.1)	9.7 (2.4)	10.5 (1.6)	0.63

TIBC, total iron-binding capacity; NTBI, non-transferrin-bound iron; LDL, low-density lipoprotein; apoB, apolipoprotein B; IgG, immunoglobulin G; IgM, immunoglobulin M; OD, optical density.
[a] Statistical analysis for females and males separately.
[b] $p < 0.05$ compared to same sex subjects of low NTBI group (t test).

iron parameters are included. Because in the present study we compared two groups of hereditary hemochromatosis heterozygotes showing clear differences in NTBI (and plasma iron and transferrin saturation), our study is more likely to detect differences in variables of LDL oxidation. Although (inter)national standardization for NTBI is still lacking, very likely NTBI levels of the subjects from the high NTBI group are supranormal. Nevertheless, neither the lag-time of in vitro LDL oxidation nor the direct and the indirect assessments of in vivo LDL oxidation (circulating oxidized LDL and autoantibodies to oxidized LDL, respectively) showed a significant difference between the groups with high and low serum NTBI. Also when normalized for apolipoprotein B, no differences were observed in circulating oxidized LDL between the two groups. It must therefore be concluded that elevated NTBI does not enhance oxidation of plasma LDL in hereditary hemochromatosis heterozygotes. Cautiousness is needed, however, when extrapolating these findings. NTBI is proposed to occur in a number of isoforms the proportion of which may depend on the disease state and on the lifestyle [28]. Therefore, we cannot exclude that a relation of NTBI with LDL oxidation does exist in other populations.

The minimal difference in rate and extent of in vitro LDL oxidation between subjects with high and low serum NTBI, that we observed in the present study, suggests that the availability of oxidizable substrates in LDL is reduced at elevated serum NTBI. The rate and extent of in vitro oxidation are determined by the availability of oxidizable substrates. At increased level, NTBI may cause a continuous low-grade oxidative pressure in such a way that molecules most prone to lipid peroxidation are constantly oxidized. As a consequence of the continuous oxidation in vivo of molecules most prone to oxidation, such as linoleic acid and arachidonic acid, LDL particles will oxidize less rapidly and produce less dienes during in vitro oxidation. This reasoning is supported by the finding that levels of F2-isoprostanes, one of the most specific measures of lipid peroxidation available, esterified in plasma lipoproteins, were increased soon after rapid infusion of iron in excess of transferrin binding capacity [29]. The lag-time, i.e., the length of the phase that precedes rapid diene production during the propagation phase, is determined by the presence and availability of antioxidants (mainly vitamin E) in LDL. We found no difference in vitamin E content of LDL from subjects with high or low serum NTBI, probably because vitamin E is regenerated by antioxidants abundantly present in plasma, and in agreement with that, we also did not find a significant difference in lag-time between these groups. Thus, the reduced rate and extent of in vitro LDL oxidation of LDL from subjects with high serum NTBI, suggests that NTBI does exert prooxidant activity towards circulating LDL particles. The antioxidant capacity of plasma, however, successfully counterbalances this oxidative insult of NTBI in vivo, since we did not find increased oxidative modification of apolipoprotein B, increased levels of autoantibodies to oxidized LDL, or decreased levels of vitamin E at elevated serum NTBI. Therefore, although our method did pick up significant differences in rate and extent of in vitro LDL oxidation between subjects with high and low serum NTBI, we believe that these alterations are clinically not relevant.

In conclusion, NTBI does not enhance LDL oxidation in hereditary hemochromatosis heterozygotes. It is still under debate whether iron is involved in atherosclerosis and CVD risk is increased at elevated iron status. The present report suggests that if this is true, it is not the result of NTBI-mediated increased oxidation of LDL. Possibly, oxidative stress-related inflammation is involved. Indications supporting this mechanism come from in vitro studies by Kartikasari et al., who very recently demonstrated that NTBI from human sera upregulates the expression of intercellular adhesion molecule-1 on human umbilical vein endothelial cells in vitro and promotes the extent of monocyte adhesion [30]. Thus, it would be very interesting to study associations between NTBI and variables of inflammation in vivo.

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