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Lipids and Lipoproteins

Increased Ability of LDL from Normolipidemic Type 2 Diabetic Women to Generate Peroxides

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Background: **We assessed the ability of LDL from 30 type 1 diabetic patients (18 men, 12 women), 65 type 2 diabetic patients (35 men, 30 women), and 35 controls (19 men, 16 women) to generate peroxides. The men and women in the diabetic groups were studied separately and matched for age, body mass index, duration of diabetes, glycohemoglobin, and conventional lipid characteristics according to the presence or absence of hyperlipidemia.**

Methods: **The ability of LDL to form peroxides was assessed by measuring the thiobarbituric acid-reactive substances corrected for LDL-cholesterol [ratio of malondialdehyde (MDA) to LDL-cholesterol]. LDL particle size was expressed as the ratio of LDL-cholesterol to apolipoprotein B (LDL-cholesterol/apoB).**

Results: **The MDA/LDL-cholesterol ratio was higher in type 1 and type 2 diabetic patients with hyperlipidemia than in controls. The MDA/LDL-cholesterol ratio was also higher in type 2 normolipidemic women than in controls (P <0.01). The LDL-cholesterol/apoB ratio was lower in type 2 diabetic women than in type 2 diabetic men (P <0.05). The MDA/LDL-cholesterol ratio was negatively correlated with the LDL-cholesterol/apoB ratio ($r = -0.78$, P <0.001) in hyperlipidemic type 1 (not type 2) diabetic patients. In normolipidemic type 2 diabetic patients, the MDA/LDL-cholesterol ratio was also negatively correlated with the LDL-cholesterol/apoB ratio ($r = -0.75$, P <0.001) because of the highly significant negative correlation in type 2 diabetic women ($r = -0.89$, P <0.01).**

Conclusions: **LDL from well-controlled type 2 diabetic women is smaller and more prone to form peroxides. This could explain why diabetic women are at greater risk of cardiovascular disease.**

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Diabetes is associated with increased morbidity and mortality resulting from cardiovascular disease in general and with increased risk of coronary heart disease in particular *(1)*. Diabetes also produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation *(2)*, which might be related to the increased incidence of atherosclerosis in diabetes *(3)*. The oxidation of LDL appears to be involved in the development of atherosclerosis *(4)*. The oxidation of LDL leads to alteration of the LDL apolipoprotein B (apoB)[5] recognition site and in the unregulated uptake of LDL by macrophages via the scavenger receptor. The subsequent accumulation of cholesterol-loaded macrophages (foam cells) in the subendothelium leads to the formation of fatty streaks and atherosclerotic plaques *(5)*. High blood glucose is commonly associated with increased oxidative changes in LDL *(6)*. Hyperlipidemia, which is often present in diabetes mellitus, is also associated with oxidation of LDL. However, in contrast to the general population, cardiovascular disease is equally common in both men and women suffering from diabetes mellitus, and no classical risk factor of cardiovascular disease had been linked to this observation. A previous study demonstrated that the LDL of patients with poorly controlled type 1 diabetes mellitus was unusually sensitive to oxidation *(7)*. However, it has also recently been demonstrated that women with type 1 diabetes mellitus have an increased degree of lipid per-

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[5] Nonstandard abbreviations: apo, apolipoprotein; TBARS, thiobarbituric acid-reactive substances; BMI, body mass index; HbA1c, hemoglobin A1c; and MDA, malondialdehyde.

oxidation that is independent of their blood glucose control *(8)*, although there is no information available about the situation in type 2 diabetic women. The greater cardiovascular risk in diabetic women could be related to an increased susceptibility of LDL to oxidation.

We determined the influence of diabetes mellitus, hyperlipidemia, and gender on the susceptibility of LDL to oxidation in patients with type 1 or type 2 diabetes mellitus. We assessed the ability of LDL to generate peroxides by measuring the formation of thiobarituric acid-reactive substances (TBARS) in vitro in groups of men and women with type 1 or type 2 diabetes mellitus and compared them to control groups.

Materials and Methods

PATIENTS

The study was carried out on 130 adult subjects: 30 type 1 diabetic patients (18 men and 12 women), 65 type 2 diabetic patients (35 men and 30 women), and two groups of healthy controls. One control group (n = 15; 8 men and 7 women) was matched for sex, age, and body mass index (BMI) with normolipidemic type 1 diabetic patients, and the second control group (n = 20; 11 men and 9 women) was matched for sex and age with normolipidemic type 2 diabetic patients. All controls and normolipidemic type 1 and type 2 diabetic patients were defined as normolipidemic according to the Alfediam criteria *(9)*: LDL-cholesterol <4.1 mmol/L, triglycerides <2.3 mmol/L, and HDL-cholesterol >1.04 mmol/L (women) or 0.91 mmol/L (men).

Two groups of hyperlipidemic diabetic patients were also studied: 15 type 1 diabetic patients (10 men and 5 women) and 45 type 2 diabetic patients (24 men and 21 women). The men and women in each group were studied separately, and the men and women within each group were matched for age, BMI, (duration of diabetes and glycohemoglobin for diabetic patients), and conventional lipid characteristics. All of the subjects selected had been nonsmokers for at least 1 year. None of the normolipidemic subjects was taking any drug known to influence lipid or lipoprotein metabolism (except for type 2 diabetic patients who were treated with metformin). Fifteen type 2 and four type 1 diabetic patients had hyperlipidemia and had been treated with fibrates for a long time; the history of their hyperlipidemia was not documented. None of the patients had a urinary tract infection, nondiabetic renal disease, or diabetic nephropathy.

All type 1 and type 2 diabetic patients were diagnosed according to the WHO criteria *(10)* and type 1 diabetic patients were C-peptide negative, confirmed after a 1-mg intravenous glucagon test (<0.3 nmol/L). The type 1 diabetic patients were on intensive conventional insulin therapy, with three insulin injections per day. Type 2 diabetic patients were treated by diet and/or oral hypoglycemic agents (15 mg of glibenclamide daily or/and 1700 mg of metformin daily). Oral hypoglycemic treatment had been constant for the last 3 months. All of the women with type 2 diabetes mellitus (normo- and hyperlipidemic women) and their healthy counterparts in the control groups were postmenopausal, and none of them was taking postmenopausal hormone replacement. All of the normolipidemic type 1 diabetic women and their healthy counterparts were premenopausal. All type 1 diabetic women with hyperlipidemia were postmenopausal.

The degenerative complications of diabetes were screened as follows: (*a*) retinopathy by examination of the eye fundus after maximal pupil dilation followed by fluorescein angiography; (*b*) sensory motor neuropathy by physical examination, vibration and position sense, and deep-tendon reflexes, and autonomic neuropathy by orthostatic hypotension (decreased systolic blood pressure $\geq$30 mmHg, together with decreased diastolic blood pressure $\geq$10 mmHg within 5 min); (*c*) nephropathy by measurement of plasma creatinine and evaluation for microalbuminuria and albuminuria; (*d*) arteriopathy by measurement of resting blood pressure in arms and legs measured by Doppler ultrasound and by Doppler velocimetry; (*e*) coronary artery disease by a detailed checklist of history, and routine electrocardiography.

Normolipidemic diabetic patients were followed regularly and recruited from our outpatient clinic. Hyperlipidemic diabetic patients were recruited when they were hospitalized because of poor blood glucose control and/or increased lipid profile.

The diets of hyperlipidemic patients were not assessed because it was not possible to evaluate their caloric intake or food composition before hospitalization. All other diabetic patients and controls were instructed to follow a weight-maintaining diet (15% of calories as protein, 35% as fat, and 50% as carbohydrate) taken as three main meals and two to three snacks per day. The caloric intake and food composition were measured. Dietary analyses were performed at each visit (all 4 months for type 1 diabetic patients and 6 months for type 2 diabetic patients) for all diabetic patients and involved a retrospective 5-day dietary record (including a weekend and 3 weekdays) to determine the usual food intake of the subjects before collection of blood samples. The intake of total proteins, carbohydrates, and fat; the distribution of saturated, monounsaturated, and polyunsaturated fatty acids; the ratio of polyunsaturated to saturated fatty acids; and cholesterol were assessed. Vitamin E and C consumption was assessed. None of the patients was on antioxidant supplements.

Control subjects were recruited from the Preventive Medicine Center (Vandoeuvre, France) and were in apparent good health, which was verified by clinical examination and biochemical analysis. The absence of diabetes was documented by a fasting blood glucose within the reference interval. Therefore, controls may be considered normoglycemic and not needing an oral glucose tolerance test according to the new criteria of diabetes *(11)*. All subjects gave their informed consent before participating

in the study, and the project was approved by the Ethics Committee of the Centre Hospitalier Universitaire de Nancy.

Methods

Laboratory procedures. Blood samples were taken after a 12-h overnight fast and before insulin injections or administration of hypoglycemic or hypolipidemic agents. Plasma glucose was measured by the glucose oxidase technique (Beckman Glucose Analyzer; Beckman) and hemoglobin A1c (HbA1c; reference range, 4.8–6%) was measured by HPLC on Biorex resins (Bio-Rad;). Serum total cholesterol and triglycerides were determined enzymatically (BioMerieux). HDL-cholesterol was measured (when triglycerides were <4 mmol/L) after precipitation of apoB-containing lipoproteins with phosphotungstic acid/manganese (Boehringer Mannheim). apoB was quantified by immunonephelometry (Behring Nephelometer Analyzer; Behring werke). The size of LDL particles is given as the ratio of LDL-cholesterol to apoB (LDL-cholesterol/apoB). Creatinine was measured to verify normal kidney function. Urinary albumin excretion was measured by laser nephelometry and was the mean of three 12-h overnight urine collections performed over a 3-month period.

Selective precipitation of LDL. LDL was selectively precipitated in duplicate from 60 μL of serum by adding 1 mL of BioMerieux precipitating reagent (BioMerieux) *(12, 13)* and separation by centrifugation (3000*g* for 10 min). The BioMerieux reagent does not contain interfering substances such as antioxidants, prooxidants, a high concentration of potassium bromide, or ion chelators (or peroxidizable substrates) and was discarded after centrifugation. The LDL pellet was dissolved in 600 μL of 0.015 mol/L NaOH, which does not contain interfering substances; therefore, dialysis was not needed. It is unlikely that the saturation of the LDL lipids, and thus the maximal amount of TBARS formed, was changed during the procedure. However, the initial phase of LDL oxidation (lag phase) was reduced because of the traces of detergent and alkaline hydrolysis of the LDL lipids. The cholesterol concentrations in the dissolved LDL were measured and expressed as millimoles of LDL-cholesterol per liter of serum. The selectivity of the LDL precipitant was checked with lipoproteins isolated by ultracentrifugation *(14)*. The method used has been evaluated and validated for determination of LDL-cholesterol when serum triglyceride concentrations are low by positive correlation with electrophoresis, analytical ultracentrifugation, and with the Friedewald formula *(15–18)*. Only the LDL fraction was precipitated by the BioMerieux reagent. The purity of the LDL was not checked by agarose/sodium dodecyl sulfate-polyacrylamide gel electrophoresis because all the LDL samples were apoA free. This result suggest that there was no contamination of the LDL preparations by apoA-containing lipoproteins such as VLDL or HDL. The lipoprotein (a) concentration, fatty acid composition, and antioxidant content of our preparations were not determined.

Ability of LDL to form peroxides. The LDL samples on EDTA were not stored but were used immediately to measure the lipid composition and ability to form peroxides. Phenylhydrazine was used as prooxidant to treat fresh LDL samples in a two-step procedure: (*a*) LDL solutions (200 μL) were incubated with 20 μL of 0.3 mmol/L phenylhydrazine at 37 °C for 5, 10, 15, 20, 30, 45, and 60 min to form TBARS. Blanks were obtained by incubating 200 μL of 0.015 mol/L NaOH. Like other hydrazines, phenylhydrazine oxidizes in vitro to form free radicals and peroxides that initiate lipid peroxidation. (*b*) The LDL-derived TBARS (LDL-TBARS) were estimated by adding 1 mL of 10 g/L thiobarbituric acid in 10 mL/L acetic acid, pH 3.5, to each sample and heating for 45 min at 95 °C. The samples were cooled and centrifuged 5 min at 4000*g*, and the clear pink supernatants were read in a spectrophotometer at 532 nm against the corresponding blank. The absorbances of the LDL-TBARS samples were converted to micromoles of malondialdehyde (MDA) by comparison with a calibration curve prepared from 1,1′,3,3′-tetramethoxypropane. The amount of LDL-TBARS formed under these conditions (expressed as μmol/L of serum) is taken as a measure of the capacity of LDL to form peroxidized lipoproteins. The peroxide-forming capacity of the LDL (MDA/LDL-cholesterol) is the maximum increase in TBARS over the LDL-cholesterol concentration (MDA/LDL-cholesterol).

There were three phases in TBARS formation during oxidation of LDL-lipids: (*a*) a lag phase, during which there was no absorbance and LDL lipids were resistant to oxidation; (*b*) a propagation phase, characterized by a rapid increase in TBARS (MDA) up to a maximum; and (*c*) a final phase, which began after ~20 min of incubation with phenylhydrazine, from the point at which the TBARS (MDA) concentration reached its maximum and remained constant for more than 1 h. The maximum increase in TBARS (μmol MDA/L serum), measured at 45 min, had a coefficient of variation (CV) of <5% for 20 determinations on the same sample of freshly precipitated LDL in our method *(19)*.

Statistical analysis

Data are expressed as means ± SD. The distribution of variables was tested for approximation to the gaussian distribution using the kurtosis and skewness tests. Data were compared by analysis of variance using Kruskal-Wallis test for three matched groups and the Mann–Whitney *U*-test for two matched groups. The χ^2 test was used to compare the distributions of categorical variables (incidence of micro- and macroangiopathy). The Spearman rank correlation coefficient test was used for testing correlations between variables. Analysis of covariance was performed to define significant variables to be in-

Table 1. Clinical characteristics of type 1 and type 2 diabetic patients.

	Type 1 diabetes mellitus	Type 2 diabetes mellitus	P[a]
Gender, M/F	18/12	35/30	
Age, years	41.8 ± 15.6[b]	57.6 ± 7.1[b]	<0.0001
BMI, kg/m^2	25.5 ± 3.9[b]	30.7 ± 5.9[b]	<0.0001
Diabetes duration, years	10.2 ± 9.1[b]	9.3 ± 7.5[b]	NS[c]
Microangiopathy, n	10	32	<0.001
Background retinopathy	4	15	
Neuropathy	6	17	
Macroangiopathy, n	5	41	<0.001
Hypertension	3	28	
Arteriopathy	2	9	
Coronary artery disease	0	4	
HbA1c, %	7.8 ± 1.9[b]	8.4 ± 2.1[b]	NS

[a] Statistical analysis by the Mann–Whitney *U*-test.
[b] Mean ± SD.
[c] NS, not significant.

cluded into the model of stepwise regression analysis. Nongaussian variables were log transformed before multiple linear regression analysis to identify significant independent predictors of LDL peroxidation. Statistical significance is implied by a *P* value <0.05. Statistical analysis was performed using the Statview® program (Statview V; Abacus Concepts).

Results

Clinical and laboratory characteristics of type 1 and type 2 diabetic patients

The clinical and laboratory characteristics of type 1 and type 2 diabetic patients are shown in Table 1. The type 2 diabetic patients were older and had a higher BMI than the type 1 diabetic patients, but the duration of diabetes and glycohemoglobin values were similar in type 1 and type 2 diabetic groups. Type 2 diabetic patients also had an increased incidence of micro- and macroangiopathy than the type 1 diabetic patients (*P* <0.001). This could be attributable to the difference in age, which was >15 years between the groups, and to the underestimation of the duration of diabetes usually reported in type 2 diabetic patients.

Ability to form peroxides

Type 1 and type 2 diabetic patients were compared to the respective control groups. The type 1 normolipidemic diabetic patients had MDA/LDL-cholesterol ratio significantly lower (*P* <0.001) than the controls, whereas type 1 hyperlipidemic diabetic patients had a significantly higher MDA/LDL-cholesterol ratio than the controls (Table 2A). The studies on men and women separately gave similar results. In contrast to type 1 diabetic patients, the MDA/LDL-cholesterol ratio was moderately but not significantly increased in all of the normolipidemic type 2 diabetic patients, whereas the hyperlipidemic type 2 diabetic patients had a higher MDA/LDL-cholesterol ratio than controls and the normolipidemic type 2 diabetic patients (Table 2B). Studies on men and women separately showed that both normo- and hyperlipidemic type 2 diabetic women had a higher MDA/LDL-cholesterol ratio than the controls, whereas only the hyperlipidemic type 2 diabetic men had a significantly higher MDA/LDL-cholesterol ratio than controls.

Clinical and metabolic characteristics of type 1 and type 2 diabetic patients: gender differences

There was no significant difference between the men and women in the control groups for lipids, lipoproteins, or MDA/LDL-cholesterol ratio (Tables 3 and 4).

Table 2. Peroxide-forming capacity[a] of LDL in diabetic groups and controls.

A. Capacity in controls and type 1 diabetic patients

	Controls (n = 15)	NL[b] type 1 diabetes mellitus (n = 15)	HLP type 1 diabetes mellitus (n = 15)	P
Whole group (n = 45)	34.8 ± 4.3[c]	27.5 ± 4.2[c,d]	48.4 ± 14.6[c,e,f]	<0.0001
Men (n = 26)	34.3 ± 4.5	26.2 ± 2.3[d]	41.9 ± 13.6[f]	<0.001
Women (n = 19)	35.3 ± 4.2	28.9 ± 5.6[d]	61.4 ± 3.1[e,f]	<0.01

B. Capacity in controls and type 2 diabetic patients

	Controls (n = 20)	NL-type 2 diabetes mellitus (n = 20)	HLP-type 2 diabetes mellitus (n = 45)	P
Whole group (n = 85)	34.6 ± 4.7[c]	41.5 ± 14.7[c]	52.3 ± 13.3[c,g,h]	<0.0001
Men (n = 46)	34.9 ± 4.2	32.9 ± 8.3	50.4 ± 11.5[g,h]	<0.0001
Women (n = 39)	34.2 ± 5.4	52.2 ± 13.9[i]	54.4 ± 15.1[g]	<0.001

[a] Capacity in micromoles of MDA formed after incubation with phenylhydrazine, per mole LDL-cholesterol.
[b] NL, normolipidemic; HLP, hyperlipidemic.
[c] Mean ± SD.
[d–i] Statistical analysis by the Kruskal–Wallis test for three groups and by the Mann–Whitney *U*-test for two groups: [d] *P* <0.05–0.001, NL type 1 diabetic patients vs controls; [e] *P* <0.01, HLP type 1 diabetic patients vs controls; [f] *P* <0.01–0.0001, HLP type 1 diabetic patients vs NL type 1 diabetic patients; [g] *P* <0.001–0.0001, HLP type 2 diabetic patients vs controls; [h] *P* <0.01–0.001, HLP type 2 diabetic patients vs NL type 2 diabetic patients; [i] *P* <0.01, NL type 2 diabetic patients vs controls.

Table 3. Clinical and metabolic characteristics of type 1 diabetic patients and controls by gender.

	Controls[a]		NL[b] type 1 diabetes mellitus[a]		HLP type 1 diabetes mellitus[a]	
	Men (n = 8)	Women (n = 7)	Men (n = 8)	Women (n = 7)	Men (n = 10)	Women (n = 5)
Age, years	33.0 ± 4.7	38.6 ± 4.4[c]	32.3 ± 5.8	32.6 ± 7.9	47.4 ± 17.9[d]	59.1 ± 10.6[e,f]
BMI, kg/m^2	22.7 ± 3.6	20.6 ± 2.3	23.9 ± 2.9	22.4 ± 3.1	27.2 ± 3.7	28.7 ± 2.4[e,f]
Diabetes duration, years			9.3 ± 7.4	9.3 ± 9.2	11.8 ± 12.1	9.4 ± 4.6
HbA1c, %			6.9 ± 0.6	7.4 ± 0.5	8.3 ± 2.7	8.9 ± 2.1
Total cholesterol, mmol/L	4.67 ± 0.77	4.57 ± 0.48	4.88 ± 1.41	5.33 ± 0.78	6.27 ± 0.98[d,g]	6.12 ± 1.02[e]
Triglycerides, mmol/L	0.99 ± 0.43	0.98 ± 0.31	0.69 ± 0.49	0.55 ± 0.15[h]	3.39 ± 1.81[d,g]	5.15 ± 1.88[e,f]
LDL-cholesterol, mmol/L	2.64 ± 0.66	2.33 ± 0.47	3.02 ± 1.16	2.71 ± 0.54	3.61 ± 1.18	2.87 ± 1.06
HDL-cholesterol, mmol/L	1.66 ± 0.18	1.82 ± 0.29	1.54 ± 0.32	2.07 ± 0.50	ND	ND
apo B, g/L	0.91 ± 0.07	0.82 ± 0.14	0.89 ± 0.36	0.90 ± 0.14	1.53 ± 0.39[d,g]	1.85 ± 0.41[e,f]
LDL-cholesterol/apo B	2.92 ± 0.78	2.85 ± 0.39	3.32 ± 0.52	3.01 ± 0.28	2.52 ± 1.12	1.57 ± 0.46[e,f]
MDA/LDL-cholesterol	34.3 ± 4.5	35.3 ± 4.2	26.2 ± 2.3[i]	28.9 ± 5.6[h]	41.9 ± 13.6[d]	61.4 ± 3.1[c,e,f]

[a] Data are means ± SD. Statistical analysis by the Mann–Whitney *U*-test.
[b] NL, normolipidemic; HLP, hyperlipidemic; ND, not determined (not enough data when triglyceride value was >3.42 mmol/L).
[c] Significant value ($P < 0.05$) was determined to compare values for men and women in type 1 diabetic groups and control group.
[d] $P < 0.05$–0.01, HLP type 1 diabetic men vs NL type 1 diabetic men.
[e] $P < 0.05$–0.01, HLP type 1 diabetic women vs control women.
[f] $P < 0.01$ HLP type 1 diabetic women vs NL type 1 diabetic women.
[g] $P < 0.01$–0.001, HLP type 1 diabetic men vs control men.
[h] $P < 0.05$ NL type 1 diabetic women vs control women.
[i] $P < 0.001$ NL type 1 diabetic men vs control men.

The duration of diabetes, the lipid values, and the HbA1c concentration in normolipidemic type 1 diabetic men and women were similar. Only HDL-cholesterol was higher in type 1 diabetic women than in type 1 diabetic men, but the difference was not statistically significant (Table 3). The MDA/LDL-cholesterol ratio was not statistically different in type 1 diabetic men and women. Triglyceride concentrations were lower in type 1 diabetic women than in control women ($P < 0.05$). Hyperlipidemic type 1 diabetic men and women were similar for age, BMI, duration of diabetes, lipid values, and HbA1c concentrations. Hyperlipidemic type 1 diabetic women had higher MDA/LDL-cholesterol ratios and smaller LDL particles than the hyperlipidemic type 1 diabetic men, the normolipidemic type 1 diabetic patients, or the controls (Table 3).

Type 2 diabetic men and women were similar in age, duration of diabetes, BMI, lipid values, and HbA1c con-

Table 4. Clinical and metabolic characteristics of type 2 diabetic patients and controls by gender.

	Controls[a]		NL[b] type 2 diabetes mellitus[a]		HLP type 2 diabetes mellitus[a]	
	Men (n = 11)	Women (n = 9)	Men (n = 11)	Women (n = 9)	Men (n = 24)	Women (n = 21)
Age, years	55.4 ± 11.5	57.3 ± 5.1	57.5 ± 5.3	54.6 ± 5.9	58.2 ± 8.9	58.9 ± 5.3
BMI, kg/m^2	24.2 ± 2.3	24.1 ± 1.2	29.1 ± 9.7	27.6 ± 4.5	31.9 ± 4.6[c,d]	31.7 ± 4.8[e,f]
Diabetes duration, years			12.8 ± 8.4	5.0 ± 2.3	10.4 ± 5.8	10.5 ± 5.9
HbA1c, %			7.3 ± 1.4	7.2 ± 1.9	8.7 ± 2.3	9.2 ± 1.7[f]
Total cholesterol, mmol/L	4.96 ± 0.64	5.04 ± 0.37	4.95 ± 1.01	5.31 ± 0.79	6.42 ± 0.98[c,d]	6.47 ± 0.81[e,f]
Triglycerides, mmol/L	1.01 ± 0.23	1.10 ± 0.43	1.17 ± 0.38	1.41 ± 0.19	4.44 ± 1.15[c,d]	3.85 ± 1.05[e,f]
LDL-cholesterol, mmol/L	2.58 ± 0.41	2.55 ± 0.49	3.05 ± 0.81	2.33 ± 0.81	3.46 ± 1.18[c]	3.25 ± 0.99[f]
HDL-cholesterol, mmol/L	1.99 ± 0.41	2.12 ± 0.51	1.44 ± 0.54[g]	1.32 ± 0.19[h]	ND	ND
apo B, g/L	0.98 ± 0.16	0.97 ± 0.15	1.09 ± 0.28	1.16 ± 0.21	1.88 ± 0.51[c,d]	1.74 ± 0.27[e,f]
LDL-cholesterol/apo B	2.66 ± 0.39	2.67 ± 0.60	2.89 ± 0.69	2.04 ± 0.75[i]	2.13 ± 0.57[d]	1.91 ± 0.74[e]
MDA/LDL-cholesterol	34.9 ± 4.2	34.2 ± 5.4	32.9 ± 8.3	52.2 ± 13.9[h,i]	50.4 ± 11.5[c,d]	54.4 ± 15.1[e]

[a] Data are means ± SD. Statistical analysis by the Mann–Whitney *U*-test.
[b] NL, normolipidemic; HLP, hyperlipidemic; ND, not determined (not enough data when triglyceride value was >3.42 mmol/L).
[c] $P < 0.05$–0.0001, HLP type 2 diabetic men vs control men.
[d] $P < 0.05$–0.0001, HLP type 2 diabetic men vs NL type 2 diabetic men.
[e] $P < 0.05$–0.0001, HLP type 2 diabetic women vs control women.
[f] $P < 0.05$–0.0001, HLP type 2 diabetic women vs NL type 2 diabetic women.
[g] $P < 0.01$, NL type 2 diabetic men vs control men.
[h] $P < 0.01$, NL type 2 diabetic women vs control women.
[i] Significant value ($P < 0.05$–0.01) was determined to compare values for men and women in type 2 diabetic groups and control group.

centrations (Table 4). There were no significant differences in the concentrations of plasma lipids or lipoproteins in the type 2 diabetic men and women. The MDA/LDL-cholesterol ratio was much higher in type 2 diabetic women than in type 2 diabetic men ($P < 0.01$), and the LDL-cholesterol/apoB ratio tended to be lower in type 2 diabetic women ($P < 0.05$; Table 4). The hyperlipidemic type 2 men had a higher MDA/LDL-cholesterol ratio than normolipidemic type 2 diabetic men, whereas hyperlipidemic and normolipidemic type 2 diabetic women had similar MDA/LDL-cholesterol ratios. The HDL-cholesterol of type 2 diabetic men and women was significantly lower than in respective control men and women ($P < 0.01$). The HDL concentrations of hyperlipidemic subjects were not available. Hyperlipidemic type 2 men and women were similar for age, BMI, duration of diabetes, lipid values, and HbA1c concentrations. The hyperlipidemic type 2 men and women had similar MDA/LDL-cholesterol ratios and LDL particle sizes.

Nutrients, energy, and fatty acid composition

The diets of hyperlipidemic patients were not assessed. Dietary analyses were performed only for controls and normolipidemic patients (Table 5). The three normolipidemic groups (controls, type 1 and type 2 diabetic patients) had similar energy intakes. In particular, their lipid intake (saturated, monounsaturated, and polyunsaturated) was not significantly different. However, the control group consumed less vitamin C than the diabetic groups ($P < 0.001$), and the controls consumed less complex carbohydrate than the diabetic population ($P < 0.01$). The men and women in each group had almost identical diets, including total fat intake, fat distribution (saturated, monounsaturated, and polyunsaturated), and the ratios polyunsaturated to saturated fatty acids. The dietary vitamin E was the same for all groups. Finally, there was no link between the ability of LDL to form peroxides and any dietary component.

Relationship between MDA/LDL-cholesterol ratio and clinical and laboratory characteristics

Univariate analyses of the controls and the type 1 and type 2 diabetic groups showed no significant correlation in control groups between MDA/LDL-cholesterol ratio and any clinical or laboratory characteristic. There was no correlation between the MDA/LDL-cholesterol ratio and the percentage of HbA1c in either type 1 or type 2 diabetic groups or subgroups of diabetic patients.

The MDA/LDL-cholesterol ratio of hyperlipidemic type 1 diabetic patients was positively correlated with age and triglyceride concentrations ($r = 0.83$, $P < 0.001$ for age; $r = 0.54$, $P < 0.05$ for triglyceride concentrations) and negatively correlated with LDL-cholesterol/apo B ratio ($r = -0.78$, $P < 0.001$). The triglyceride concentrations were also negatively correlated with LDL-cholesterol/apo B ($r = -0.81$, $P < 0.001$). Unlike the hyperlipidemic type 1 patients, the MDA/LDL-cholesterol ratio of hyperlipidemic type 2 diabetic patients was positively correlated only with the triglyceride concentrations ($r = 0.35$, $P < 0.05$) but not with the LDL-cholesterol/apoB ratio ($r = -0.16$, not significant; Table 6).

We found no correlation between the MDA/LDL-cholesterol ratio of normolipidemic type 1 diabetic patients and any of the characteristics studied. The MDA/LDL-cholesterol ratio was correlated with apoB only in type 2 normolipidemic diabetic men ($r = 0.74$, $P < 0.01$). The MDA/LDL-cholesterol ratio was inversely correlated with the LDL-cholesterol/apoB in type 2 normolipidemic

Table 5. Nutrients, energy, and fatty acid composition.

	Controls[a] (n = 35)	Type 1 diabetes mellitus[a] (n = 15)	Type 2 diabetes mellitus[a] (n = 20)	*P*, ANOVA
Energy, Kcal/day	2158 ± 739	2348 ± 521	2203 ± 531	NS[b]
Protein, g/day	83 ± 26	104 ± 23	112 ± 30	<0.01[c,d]
Carbohydrate, g/day	226 ± 101	260 ± 64	242 ± 60	NS
Simple	68 ± 58	41 ± 32	38 ± 35	NS
Complex	158 ± 58	219 ± 51	205 ± 47	<0.01[c,d]
Fat, g/day	102 ± 36	99 ± 29	98 ± 28	NS
PUFA	16 ± 7	19 ± 8	18 ± 6	NS
MUFA	36 ± 13	31 ± 11	29 ± 12	NS
Saturated	48 ± 16	50 ± 15	48 ± 17	NS
P:S ratio	0.33 ± 0.09	0.39 ± 0.14	0.41 ± 0.18	NS
Fiber, g/day	14 ± 7	23 ± 4	23 ± 7	<0.001[c,d]
Cholesterol, mg/day	449 ± 199	414 ± 149	389 ± 108	NS
Alcohol, cal/day	31 ± 39	53 ± 132	69 ± 101	NS
Vitamin C intake, mg/day	76 ± 57	118 ± 27	118 ± 58	<0.001[c,d]
Vitamin E intake, mg/day	7.4 ± 3.5	7.8 ± 3.4	7.2 ± 2.3	NS

[a] Data are means ± SD.
[b] NS, not significant; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; P:S, ratio of polyunsaturated to saturated fatty acids.
[c,d] Statistical analysis by the Kruskal-Wallis test: [c] $P < 0.01$, type 1 diabetes mellitus vs controls; [d] $P < 0.01$, type 2 diabetes mellitus vs controls.

Table 6. Univariate regression analysis (correlation coefficients, *r*) between LDL peroxidation and LDL size.

	Controls	Type 1 diabetes mellitus	Type 2 diabetes mellitus
Total (n = 130)	+0.13	−0.82[a]	−0.48[a]
Men (n = 72)	−0.06	−0.77[a]	−0.39[b]
Women (n = 58)	+0.34	−0.90[a]	−0.45[b]
Normolipidemic (n = 35)		−0.44	−0.75[a]
Men (n = 19)		−0.75	−0.28
Women (n = 16)		−0.22	−0.89[c]
Hyperlipidemic (n = 60)		−0.78[a]	−0.16
Men (n = 34)		−0.74[c]	+0.06
Women (n = 26)		−0.38	−0.26

[a–c] Statistical analysis by the Spearman rank correlation coefficient test: [a] $P < 0.001$; [b] $P < 0.05$; [c] $P < 0.01$.

diabetic patients ($r = -0.75$, $P < 0.01$) only because of the highly significant negative correlation between MDA/LDL-cholesterol and LDL-cholesterol/apoB in type 2 normolipidemic diabetic women ($r = -0.89$, $P < 0.01$); there was no correlation between MDA/LDL-cholesterol and LDL-cholesterol/apoB in type 2 normolipidemic diabetic men (Table 6).

stepwise regression analysis

One model of multivariate analysis was developed by stepwise regression. Analysis of covariance was also performed, leading to exclusion of age and HDL concentrations from the stepwise regression analysis because these variables were not available or statistically highly correlated ($r > 0.85$). Type of diabetes, sex, BMI, HbA1c, total cholesterol, triglycerides, apoB, and the LDL-cholesterol/apoB ratio were the independent variables. The LDL-cholesterol/apoB ratio predicted 44%, triglycerides predicted 10%, the type of diabetes predicted 3%, and sex predicted 3% of the variance of the MDA/LDL-cholesterol.

Discussion

Our results suggest that the LDL particles of normolipidemic type 2 women are more susceptible to oxidation than those of normolipidemic type 2 men or controls. The LDL of normolipidemic type 1 diabetic patients were less able to generate peroxides than controls. The LDL of hyperlipidemic type 1 and 2 diabetic patients was more susceptible to oxidation than the LDL of controls, confirming that hyperlipidemia increases the susceptibility of LDL to oxidation. However, the susceptibility to oxidation of LDL particles from normolipidemic type 2 diabetic women was similar to that of particles from hyperlipidemic type 2 diabetic patients, suggesting that other factors are involved in the process of LDL peroxidation.

Several studies have reported an increased susceptibility to lipid peroxidation in patients with diabetes mellitus *(20, 21)*. However, the influence of blood glucose control, lipid characteristics, and type of diabetes on the susceptibility of LDL to oxidation remains controversial *(2)*. Interpretation of the data could be biased by such confounding factors, particularly because plasma lipid peroxidation seems to be linked to lipid concentrations, the degree of hyperglycemia, and the plasma insulin concentration *(22, 23)*. Our data agree with previous studies showing that the LDL of type 1 diabetic patients without hyperlipidemia is not more susceptible to oxidation *(24)*. Gallou et al. *(20)* studied 117 diabetic patients (57 type 1 diabetic patients, 60 type 2 diabetic patients) and found that type 1 and type 2 diabetic patients had higher plasma TBARS than controls but that their blood glucose control was not associated with an increased susceptibility of their LDL to oxidation. But the percentages of HbA1c in the type 1 and type 2 diabetic patients were significantly different between groups in most of these studies. We agree with these results and find no relationship between LDL peroxidation and HbA1c less than 9.2% ± 1.7%. The relative incapacity of LDL from type 1 diabetic patients to form peroxides compared with the LDL of type 2 diabetic patients and controls may be linked to the low plasma triglycerides (particularly in type 1 diabetic women) and thus to increased lipolysis of triglyceride-rich lipoproteins, which is characteristic of well-controlled diabetic patients on intensive insulin therapy *(25)*.

In contrast, a study performed on 29 diabetic patients (type 1 and type 2 diabetic patients) with poor blood glucose control and increased triglycerides showed that the LDL and erythrocyte membranes of diabetic patients were very susceptible to peroxidation *(21)*. The susceptibility of LDL to peroxidation is correlated with the plasma triglyceride concentration *(12, 26)*, and our stepwise regression analysis confirms the influence of triglycerides on LDL peroxidation in diabetic patients. However, our population of hyperlipidemic diabetic patients was not homogeneous because some patients had been treated previously with hypolipidemic agents and others had hyperlipidemia because of poor glucose control.

Diets rich in monounsaturated fatty acids should not increase the amount of dense LDL and should also reduce the susceptibility of LDL and its subfractions to oxidation *(27)*. However, the amount of plasma lipid peroxidation in type 2 diabetic population seems to be similar on high monounsaturated and polyunsaturated fat diets when compared with healthy controls *(28)*. Therefore, LDL composition reflects the dietary fats *(29)*. The three normolipidemic groups (controls and type 1 and type 2 diabetic patients) had similar dietary intake. In particular, the lipid intake (saturated, monounsaturated, and polyunsaturated fatty acids) were not significantly different. The normolipidemic type 2 diabetic men and women also had similar food intake, especially total fat, fatty acid distribution (saturated, monounsaturated, and polyunsaturated), and the ratio of polyunsaturated to saturated fatty acids. The diabetic patients consumed more complex carbohydrate than the controls and more vitamin C, probably because of diet recommendations.

Our study shows that normolipidemic type 2 diabetic women have higher MDA/LDL-cholesterol ratios than type 2 diabetic men and controls. Two recent studies have suggested that the LDL from diabetic women is more susceptible to oxidation than the LDL from diabetic men. The first study examined only type 1 diabetic patients, and the LDL from type 1 diabetic women was significantly more susceptible to oxidation than the LDL from type 1 diabetic men *(8)*. In the second study, the LDL from type 2 diabetic patients was significantly more susceptible to oxidation than the LDL from nondiabetic subjects *(22)* because of a significantly greater stimulated lipid peroxidation in women than in men. However, in this study, all type 2 diabetic patients had increased triglycerides (>2.3 mmol/L), and no separate information was given on blood glucose control and lipid characteristics in type 2 diabetic men and type 2 diabetic women. We also found that the LDL from normolipidemic type 2 diabetic women has a greater ability to form peroxides in vitro, as does the LDL of hyperlipidemic type 2 diabetic patients. To our knowledge, our study is the first that demonstrates that type 2 diabetic women with good blood glucose control and no hyperlipidemia have LDL with an enhanced capacity to form peroxides. However, the factors that could control the susceptibility of LDL particles to oxidation in type 2 diabetic women remain unclear.

The LDL-cholesterol/apoB ratio reflects the preponderance of small dense LDL *(30)*, and a low LDL-cholesterol/apolipoprotein B ratio is predictive of cardiovascular death in type 2 diabetes *(31)*. There is a greater association between LDL size and diabetes in women *(32, 33)*. The in vitro susceptibility of LDL to oxidation is significantly correlated with the presence of small dense LDL particles from hypertriglyceridemic subjects *(34)*, and the plasma triglyceride concentration is closely correlated with the number of small dense LDL particles *(35, 36)*. There was a significant correlation between the MDA/LDL-cholesterol ratio and the LDL-cholesterol/apo B ratio in type 1 hyperlipidemic patients, even when the correlation coefficient was adjusted for triglyceride concentrations, which were significantly correlated with LDL-cholesterol/apoB. In addition, the LDL size was closely and negatively correlated with LDL peroxidation in type 2 normolipidemic women, suggesting that the LDL particle size could be altered independently of the lipid profile and blood glucose control *(37)*. This is confirmed by our stepwise regression analysis, which showed that LDL size explained almost 40%, but triglycerides only 10%, of the variance of LDL peroxidation.

All of the type 2 diabetic women in our study were considered postmenopausal if they had been free of menstrual cycles for the preceding year. This menopausal status of our type 2 diabetic women could influence the LDL size and consequently the susceptibility of their LDL to peroxidation in type 2 diabetic women. The penetrance of the LDL B phenotype is reduced in premenopausal females *(38)*, confirming that the LDL size is influenced by menopausal status *(39)*. It has been also demonstrated that estrogen replacement is associated with increased clearance of small dense LDL in healthy postmenopausal women *(40)*. The susceptibility of LDL to oxidation in vitro is also inhibited by 17-β estradiol *(41)*. The potency of 17-β estradiol as an antioxidant suggests that estrogen may protect against atherosclerosis by inhibiting lipoprotein oxidation. Oral estrogen replacement therapy may have antioxidative effects in postmenopausal women with coronary heart disease *(42)*, but this has not yet been demonstrated in postmenopausal women with type 2 diabetes mellitus *(43)*. Insulin resistance could also be involved in this process because although their lipid concentrations were within the reference interval, our type 2 normolipidemic women had significantly lower HDL concentrations than their control counterparts. Postmenopausal status has been characterized by decreased insulin sensitivity *(44)*, and LDL size distribution is linked to insulin sensitivity *(45, 46)*. In contrast, estrogen replacement is associated with a substantial improvement in insulin action *(47)*. Finally, postmenopausal women are at greater risk of atherosclerosis than their premenopausal counterparts *(48)*, and estrogen replacement in postmenopausal women is associated with a reduced risk of developing coronary artery disease *(49, 50)*. The current use of estrogens by postmenopausal women with diabetes was associated with a lower risk of myocardial infarction compared with those who had never used them *(51)*.

The present study therefore demonstrates that the LDL from type 2 diabetic patients, especially type 2 diabetic women, are more prone to form peroxides. Thus, the increased relative risk of coronary heart disease in type 2 diabetic women could be linked to the greater propensity of their LDL to undergo oxidation and the ability of these lipoproteins to generate peroxides even when type 2 diabetic women had fair blood glucose control and were normolipidemic. This could be attributable to the presence of small dense LDL particles in normolipidemic women, which are known to increase the risk of coronary heart disease *(52)*. Our results need to be validated with a study on more subjects, and it seems important to evaluate the effects of hormone replacement therapy on the susceptibility and size of LDL in normolipidemic type 2 diabetic women, a population at risk of cardiovascular events *(53)*.

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