

## Impaired Oxidant/Antioxidant Status and LDL-Fatty Acid Composition Are Associated with Increased Susceptibility to Peroxidation of LDL in Diabetic Patients

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**Abstract.** This study was carried out to determine the relationships between oxidant/antioxidant status, *in vitro* LDL oxidizability and LDL-fatty acid composition in diabetes mellitus. Plasma total antioxidant capacity (oxygen radical absorbance capacity, ORAC) and LDL-cholesteryl ester fatty acids were investigated in type 1 and type 2 diabetic subjects with and without complications. The degree of LDL oxidation was determined by the measurement of hydroperoxide levels before and after *in vitro* peroxidative stress with CuSO<sub>4</sub>. ORAC values were decreased in diabetic subjects who showed high basal hydroperoxide levels. Oxidizability of LDL in these subjects was higher than in control subjects and it was unrelated to LDL-fatty acid composition. However, in type 2 diabetic subjects with complications, alterations in LDL-fatty acid composition were associated with their enhanced oxidative susceptibility. LDL-fatty acid alterations might be an additional factor that influences LDL oxidizability especially in type 2 diabetes. In conclusion, diabetes mellitus is associated with enhanced oxidative stress and defective antioxidant/oxidant balance regardless the type of diabetes and presence of complications.

**Key words:** Antioxidant status — Diabetes mellitus — Fatty acids — LDL

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**Abbreviations:** apo B, apolipoprotein B; APC, allophycocyanin; HbA1C, glycosylated hemoglobin; HDL, high density lipoprotein; HDL-C, HDL-cholesterol; LDL, low density lipoproteins; LDL-C, LDL-cholesterol; ORAC, oxygen radical absorbance capacity; PUFA, polyunsaturated fatty acids; TG, triglycerides.

## Introduction

Diabetes mellitus is associated with increased oxidative stress and free radical production (Giugliano et al. 1995; Bonnefont-Rousselot et al. 2000). Increased production of reactive oxygen species as well as reduced antioxidant defence mechanisms have been suggested to play a role in type 1 and type 2 diabetic patients (Bonnefont-Rousselot et al. 2000; Gokkusu et al. 2001). Oxidative stress plays an important role in the etiology of diabetic complications such as atherosclerosis, a major cause of morbidity and mortality in these patients (Baynes 1991; Vanderjagt et al. 2001). The following mechanisms are thought to be involved in the increased oxidative stress in diabetes mellitus: hyperglycemia, oxygen free radical generation due to nonenzymatic protein glycosylation, autooxidation of glucose and glycation products, and changes in antioxidant defence systems (Bonnefont-Rousselot et al. 2000; Baynes 1991; Inouye et al. 1999; Courderot-Masuyer et al. 2000). Lipid alterations and oxidizability of lipoproteins have been also considered as contributory factors to oxidative stress in diabetes mellitus (Semenkovich and Heinecke 1997; Holvoet and Collen 1998). Beaudoux et al. (1995) have shown an increased susceptibility of LDL to *in vitro* oxidation in both types of diabetes mellitus. However, other investigators have not confirmed this observation and have reported either unaltered (Jenkins et al. 1996) or decreased (Feillet et al. 1998) oxidizability of LDL isolated from diabetic patients. Such discrepancies could be due to the heterogeneity of diabetic populations, glycemic control and the presence or absence of vascular complications (Bonnefont-Rousselot et al. 2000). A relationship between the amount of polyunsaturated fatty acids (PUFA) in LDL and susceptibility of LDL to oxidation has been demonstrated (Dimitriadis et al. 1995; Prescott et al. 1999). However, the relationships between LDL oxidizability, fatty acid composition and oxidant/antioxidant status in diabetes mellitus are still not clear. As lipid peroxidation leads to increased atherogenicity of LDL, antioxidant status should have a major impact not only on the rate of LDL oxidation but also on development of atherosclerosis (Thomas 2000).

Determination of oxidant/antioxidant balance *in vivo* is a very complex task. Markers of lipid peroxidation such as thiobarbituric acid reactive substances and hydroperoxides levels were elevated in both types of diabetes (Bonnefont-Rousselot et al. 2000). Among the nonenzymatic antioxidant defence systems, serum vitamin C and E, and reduced glutathione are the most important agents in diabetes. Because of the relative difficulty in measuring each antioxidant separately, some assays have been designed to measure the overall capacity of plasma sample to scavenge oxygen radicals (oxygen radical absorbance capacity, ORAC) (Cao et al. 1993).

ORAC has been found to be a good index of oxidative stress in diabetes mellitus (Courderot-Masuyer et al. 2000; Therond et al. 2000). However, it is less clear whether markers of oxidative stress are high in diabetic subjects with long-term complications. We have previously determined vitamin C and E levels and activities of erythrocyte antioxidant enzymes (superoxide dismutase, glutathione reductase and peroxidase, catalase) in type 1 and 2 diabetic subjects with and without complications, i.e. hypertension, coronary artery disease and renal failure (Merzouk et al. 2003). Our previous results supported altered antioxidant status in diabetes mellitus regardless to various complications. However, the oxidant status was not investigated and then ORAC would be a better index of oxidant/antioxidant balance in these patients. Indeed, there is inadequate evidence that oxidant/antioxidant balance is related to LDL susceptibility to oxidation and that enhanced LDL oxidizability is related to its fatty acid composition in diabetic patients. To extend our previous findings, the present study was conducted to evaluate ORAC, hydroperoxide levels, the *in vitro* LDL oxidizability and its fatty acid composition in type 1 and 2 diabetic subjects with or without complications. This investigation was aimed at assessing whether increased oxidative stress in diabetes mellitus is associated with increased LDL oxidizability and fatty acid alterations, and whether these relationships are related to diabetic complications.

## Materials and Methods

### Subjects

A total of 38 type 1 and 39 type 2 diabetic patients were recruited in the Department of Diabetics, University Hospital of Tlemcen (Algeria). Medical records were screened by specialist physicians. The type 1 or type 2 diabetic patients were divided into two groups: group I consisted of patients without complications (sixteen patients for each type of diabetes); group II consisted of patients with complications. Complications in the diabetic patients included coronary artery disease in 12 type 1 and 13 type 2 diabetic patients, diagnosed by clinical symptoms of angina pectoris, electrocardiogram examination or documented myocardial infarction, and renal failure in 10 patients for each type of diabetes, evaluated by significant renal impairment such as abnormal creatinine or macroalbuminuria.

All diabetic patients received insulin (type 1 diabetes), or oral hypoglycemic agents like sulphonylureas or metformin (type 2 diabetes). In addition, diabetic patients with renal failure were treated by angiotensin converting enzyme inhibitors. Diabetic patients with coronary artery disease were treated by calcium antagonists. No subjects had received lipid lowering medications.

Two groups of control subjects were selected. Controls and diabetic subjects were matched with respect to age and body mass index. All individuals were non smokers. None had taken vitamin supplements. The characteristics of patients are given in Table 1. The study was approved by the ethical committee of the Tlemcen

**Table 1.** Characteristics of the patients included in the study

	Type 1			Type 2		
	Control	Without complications	With complications	Control	Without complications	With complications
Number	20	16	22	20	16	23
M/F ratio	11/9	7/9	10/12	10/10	8/8	12/11
Age (years)	28.2 ± 1.8	32.1 ± 1.5	30.8 ± 1.8	55.0 ± 1.6	56.3 ± 2.6	57 ± 2.5
BMI (kg/m <sup>2</sup> )	21.1 ± 1.6	22.9 ± 1.5	23.7 ± 1.3	28.0 ± 1.6	25.3 ± 1.7	24.0 ± 1.6
Duration of disease (years)	–	9.0 ± 0.6	10.2 ± 1.4	–	8.4 ± 1.0	12.3 ± 1.2
Fasting glucose (mmol/l)	4.8 ± 0.3	8.4 ± 0.9*	10.1 ± 1.1*	5.2 ± 0.5	9.5 ± 0.6*	11.5 ± 0.8*
HbA1C (%)	5.1 ± 0.3	7.8 ± 0.5*	9.5 ± 0.6*	4.9 ± 0.4	8.7 ± 0.5*	10.5 ± 0.7*

Values are means ± SEM. BMI, body mass index; \* significantly different ( $p < 0.05$ ) compared to control subjects.

University Hospital (Algeria) and informed written consent was obtained from all the subjects.

### **Blood samples**

Fasting venous blood samples were collected by venipuncture into heparinized tubes. Plasma was obtained by centrifugation at  $2000 \times g$  for 15 min at room temperature, and was used immediately for the determination of glucose, glycosylated hemoglobin (HbA1C). For lipids, lipoproteins and ORAC assays, plasma was left stored at  $-20^{\circ}\text{C}$  up to one week.

### **Laboratory methods**

#### *Plasma determination*

HbA1C levels were determined by isolab column chromatography (Kaplan et al. 1982). Plasma glucose was determined by glucose oxidase method using a glucose analyzer (Beckman Instruments, Fullerton, CA, USA). Plasma triglyceride (TG) and total cholesterol and HDL-cholesterol (HDL-C) contents were determined by using enzymatic methods, according to the instructions furnished with the kit (Boehringer, Mannheim, Germany). LDL-apo B concentrations were performed by immunoelectrophoresis using monoclonal antibodies and ready to use plates (Hydragel SEBIA Kit, Issy Les Moulineaux, France).

*Scavenging capacity of plasma*

Plasma ORAC was measured according to Cao et al. (1993). A fluorescent protein, allophycocyanin (APC) was used in this assay (Courderot-Masuyer et al. 2000). ORAC employs the oxidative loss of the intrinsic fluorescence of APC. APC fluorescence decay shows a lag or retardation in the presence of antioxidants, related to the antioxidant capacity of the sample. The reaction mixture (2 ml) contained a final concentration of 37.5 nmol/l APC in 75 mmol/l phosphate buffer (pH 7.0) at 37°C in the absence (blank) or presence of 20  $\mu$ l of trolox (1  $\mu$ mol/l) or plasma, respectively. The reaction was initiated by the introduction of 9  $\mu$ mol/l of  $\text{CuSO}_4$  and 0.3%  $\text{H}_2\text{O}_2$  as redox catalysts. This reaction was followed spectrophotometrically by the decrease in fluorescence at 651 nm emission and 598 nm excitation, using a spectrofluorometer SFM25 Kontron. Trolox was used as a reference antioxidant for calculating the ORAC values, with one ORAC unit defined as the net protection area provided by 1  $\mu$ mol/l final concentration of trolox. ORAC value of the samples was calculated as:  $\text{ORAC} = (\text{A sample} - \text{A blank}) / (\text{A trolox} - \text{A blank})$ , A refers to the area under the quenching curve of APC.

*LDL isolation*

Plasma LDL ( $d < 1.063$ ) fraction was separated by sequential ultracentrifugation in a Beckman ultracentrifuge (model L5-65, 65 Ti rotor), using sodium bromide for density adjustment, according to Havel et al. (1955). LDL fraction was dialyzed against 0.15 mol/l NaCl and 1 mmol/l disodium EDTA, pH 7.4, at 4°C in spectra/por 2 dialysis tubing (spectrum Medical Industries, Los Angeles, CA, USA). LDL-C and TG were then determined using the enzymatic method. LDL-protein content was determined according to Lowry et al. (1951).

*LDL susceptibility to in vitro oxidative stress*

LDL fraction was diluted to a final concentration of 200  $\mu$ g/ml protein using PBS. LDL samples were incubated at 37°C for 18 h with a freshly prepared  $\text{CuSO}_4$  solution added to a final concentration of 10  $\mu$ mol/l as reported by Rabini et al. (1999) and Nourooz-Zadeh et al. (1996). The degree of lipoprotein oxidation was determined by the measurement of hydroperoxide levels before and after the peroxidative stress.

Hydroperoxides were measured by the ferrous ion oxidation-xylenol orange assay (Fox2) in conjunction with a specific ROOH reductant, triphenylphosphine (TPP), according to the method of Nourooz-Zadeh et al. (1996). Fox2-reagent was obtained as the commercially available material from pierce (kit Peroxoquant methanol-compatible formulation, Rockford, IL, USA).

This method is based on the principle of the rapid peroxide-mediated oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  under acidic conditions. The latter, in the presence of xylenol orange, forms a  $\text{Fe}^{3+}$ -xylenol orange complex which can be measured spectrophotometrically at 560 nm. Hydroperoxide content in the samples was determined as a function of the mean absorbance difference of samples with and without elimination

of ROOH by TPP. Calibration was done with standard peroxides such as hydrogen peroxide. LDL susceptibility to oxidation is measured as differential hydroperoxide content before and after incubation with  $\text{CuSO}_4$ .

#### *Fatty acid analysis*

For the determination of the fatty acid content in LDL, lipids were extracted by the method of Folch et al. (1957). LDL-cholesteryl esters were isolated by thin layer chromatography. After saponification with NaOH/methanol 0.5 N, fatty acids were transmethylated by boron trifluoride/methanol (14%) at 80°C for 15 min. Fatty acid methyl esters were analyzed by gas liquid chromatography, using a Becker gas chromatograph (Spiral-RD, Couternon, France) equipped with a 50 m capillary glass column packed with carbowax 20M (Becker instruments, Downers grove, IL, USA). Methyl ester (17:0) was used as an internal standard. Identification of different fatty acids was performed by comparison of relative retention times with those of commercial standards. Areas were calculated with an ENICA 21 integrator (DELSI Instrument, Suresnes, France).

#### *Statistical analysis*

Data are expressed as mean  $\pm$  SEM. Statistical analysis was carried out using Statistica (version 4.1, Statsoft, Paris, France). The significance of the differences between two groups was determined by Student's *t*-test. Multiple comparisons were performed using ANOVA followed by the least significant difference test.  $p < 0.05$  was considered to represent statistical significance.

### **Results**

#### *HbA1C, plasma glucose, cholesterol and triglyceride concentrations*

Significant differences were found between diabetic and control subjects for plasma glucose and HbA1C levels which were high in all diabetic patients regardless to complications (Table 1). However, the highest glucose concentrations were apparent in diabetes mellitus (type 1 and 2) associated with complications. HDL-C amounts were lower only in type 1, and 2 diabetes with complications compared to control subjects (Table 2). Plasma TG levels were significantly increased in all type 2 and type 1 diabetes with complications. LDL-C, LDL-TG and apo B amounts were significantly higher in type 1 and 2 diabetic subjects with complications compared to control subjects (Table 2).

#### *Plasma ORAC*

Scavenging capacity of plasma was lower in diabetic patients compared to control subjects regardless type of diabetes and the presence of complications (Table 3). Moreover, type 1 and 2 diabetic patients with complications showed lower ORAC values than diabetic subjects without complications.

**Table 2.** Lipid and lipoprotein levels in control and diabetic subjects

	Type 1			Type 2		
	Control	Without complications	With complications	Control	Without complications	With complications
Total cholesterol (mmol/l)	5.6 ± 0.9	6.0 ± 0.7	6.6 ± 0.9	5.5 ± 0.5	6.2 ± 0.8	6.6 ± 0.8
TG (mmol/l)	1.3 ± 0.5	1.2 ± 0.6	2.8 ± 0.5*	1.4 ± 0.6	2.1 ± 0.5*	2.8 ± 0.5*
HDL-C (mmol/l)	2.1 ± 0.4	1.8 ± 0.5	1.1 ± 0.5*	1.8 ± 0.6	1.7 ± 0.2	1.4 ± 0.5*
LDL-C (mmol/l)	3.0 ± 0.6	3.5 ± 0.5	5.0 ± 0.4*	3.6 ± 0.3	3.9 ± 0.7	5.6 ± 0.9*
LDL-TG (mmol/l)	0.7 ± 0.06	0.8 ± 0.09	1.05 ± 0.1*	0.74 ± 0.06	0.8 ± 0.08	1.08 ± 0.10*
LDL-apo B (g/l)	0.75 ± 0.15	0.8 ± 0.15	1.28 ± 0.10*	0.8 ± 0.08	0.93 ± 0.14	1.26 ± 0.12*

Values are means ± SEM. HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; LDL-TG, LDL triglycerides; LDL-apo B, LDL apolipoprotein B. Statistical analysis was performed by employing ANOVA followed by the least significant difference test. \* significantly different (< 0.05) compared to control subjects.

#### *In vitro oxidation of LDL*

The basal hydroperoxide levels were significantly increased in all diabetic patients compared with controls (Table 3). Significantly increased hydroperoxide levels were found in Cu<sup>2+</sup>-catalyzed oxidized LDL with respect to nonoxidized LDL both in patients and controls. The *in vitro* oxidative stress induced by Cu<sup>2+</sup> incubation resulted in a significantly higher increase in hydroperoxide levels in LDL from diabetic patients than in LDL from controls, regardless the type of diabetes or complications.

#### *LDL-cholesteryl ester fatty acid composition*

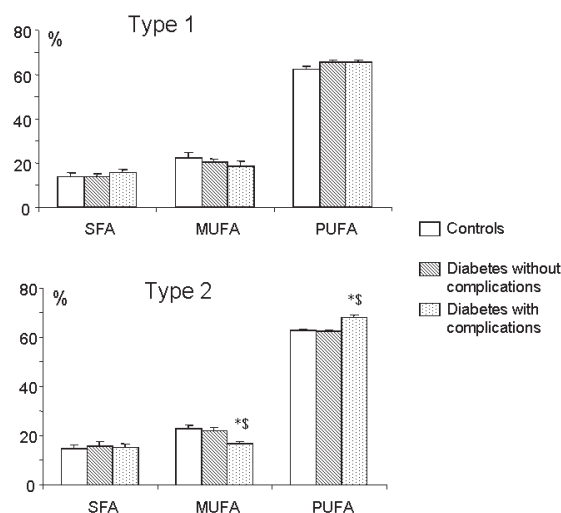
There were no significant differences in the fatty acid composition of cholesteryl ester in LDL isolated from type 1 diabetic subjects and their respective controls (Fig. 1). However, a significant increase in PUFA contents with a significant decrease in monounsaturated fatty acids levels were observed in LDL of type 2 diabetic patients with complications compared with other groups.

**Table 3.** Plasma oxygen radical absorbance capacity (ORAC) values, Copper-induced *in vitro* oxidation of low density lipoprotein (LDL) isolated from control and diabetic subjects

	Type 1			Type 2		
	Control	Without complications	With complications	Control	Without complications	With complications
ORAC (arbitrary units)	2.56 ± 0.11	1.45 ± 0.13*	1.22 ± 0.14*\$	2.25 ± 0.12	1.36 ± 0.18*	0.98 ± 0.15*\$
Basal oxidation hydroperoxide (μmol/l)	3.58 ± 0.56	6.1 ± 0.70*	6.82 ± 0.81*	4.26 ± 0.63	6.15 ± 0.54*	7.21 ± 0.83*
Oxidation with CuSO <sub>4</sub> hydroperoxide (μmol/l)	10.75 ± 0.82	18.65 ± 0.72*	21.84 ± 1.55*\$	9.64 ± 0.57	19.65 ± 1.75*	23.56 ± 0.93*\$

Values are means ± SEM. The level of ORAC was determined as described in Materials and Methods. LDL oxidisability was measured as differential hydroperoxide content before and after incubation with CuSO<sub>4</sub> as described in Materials and Methods. Statistical analysis was performed by employing ANOVA followed by the least significant difference test. \* significantly different ( $p < 0.05$ ) compared to control subjects; \$, significantly different ( $p < 0.05$ ) compared to diabetics without complications.





**Figure 1.** Cholesteryl ester fatty acid composition of LDL was determined as described in Materials and Methods. Values are means  $\pm$  SEM of 38 type 1 diabetics (16 without and 22 with complications), 39 type 2 diabetics (16 without and 23 with complications) and 40 controls. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. Statistical analysis was performed by employing ANOVA followed by the least significant difference test. \* significantly different ( $p < 0.05$ ) compared to control subjects. § significantly different ( $p < 0.05$ ) compared to diabetics without complications.

## Discussion

Diabetes mellitus is a global health problem, and its incidence is growing in Algeria. Recent evidences suggest that diabetic patients are under oxidative stress and that complications of diabetes seem to be mediated by oxidative stress (Giugliano et al. 1995; Bonnefont-Rousselot et al. 2000). However, the relationships between free radical production, antioxidant levels, lipoprotein oxidation, fatty acid composition, glycemic control and the presence or absence of diabetic complications are still unclear.

Our data revealed that the total antioxidant activity (ORAC) was decreased in the plasma of type 1 and 2 diabetics regardless any complications, in favor of an oxidative stress in such patients. These results are in agreement with previous studies (Courderot-Masuyer et al. 2000; Therond et al. 2000; Pieri et al. 2001). Previous data have demonstrated a strong association between poor glycemic control and the depletion of protective antioxidant defence in diabetes mellitus (Bonnefont-Rousselot et al. 2000; Merzouk et al. 2003; Brownlee 1994; Maxwell et al. 1997). In our study, all diabetic patients were poorly controlled, as indicated by their high glucose and HbA1C levels. The inverse correlation between HbA1C and ORAC was consistent with the idea that chronic hyperglycemia may increase oxidative

stress. In addition, the reduction of ORAC was associated with the increase of hydroperoxide levels in these patients, regardless type of diabetes and complications. Hydroperoxides were measured as a marker of lipid peroxidation. A number of studies have assessed elevated levels of hydroperoxides in plasma from both type 1 and 2 diabetic patients (Nourooz-Zadeh et al. 1997; Berg et al. 1998). High hydroperoxide levels in our diabetic patients could result from their hyperglycemia. Regarding the individual antioxidants, we have previously observed unaltered plasma levels of vitamin C, whereas plasma vitamin E contents were decreased in diabetic patients, regardless type of diabetes and complications (Merzouk et al. 2003). In addition, we have shown that erythrocyte superoxide dismutase, glutathione reductase and peroxidase, and catalase activities were also reduced in these diabetic patients (Merzouk et al. 2003). It has been reported that oxidative stress is induced by both the increases in free radicals and disturbance of the free radical scavenging system in diabetes mellitus (Cao et al. 1993; Sinclair 1993). Altogether, our previous and present data provide convincing evidence for decreased antioxidant defence and altered antioxidant/oxidant balance in diabetic patients, irrespective to their complications.

In our study, increased LDL oxidizability was observed in diabetic patients, regardless of type of diabetes and complications, in agreement with other studies (Beaudeau et al. 1995; Tsai et al. 1994; Liguori et al. 2001). Previous studies provide evidence for the role of LDL glycation in its increased *in vitro* oxidizability (Bonfont-Rousselot et al. 2000). It is then possible that poor glycemic control may account for the elevated LDL oxidizability in our diabetic patients. However, our findings show that in diabetes, even in the absence of complications, depletion in antioxidant defenses might also be responsible for LDL increased oxidizability. In the presence of complications, lipid alterations were seen and could influence the susceptibility of LDL to oxidation. Diabetic patients with complications had higher plasma TG, LDL-C, LDL-TG, apo B and lower HDL-C concentrations than those without complications and controls, in agreement with previous reports (Ginsberg 1991; Dullaart 1995). Oxidation of LDL induces LDL-C accumulation (Holvoet and Collen 1998; Witztum 1994). High levels of cholesterol activate thrombocytes and cause the release of substances that activate phospholipase A<sub>2</sub>. Hence, accumulated arachidonic acid may be metabolized to leukotrienes, thromboxanes, prostaglandins and malonaldehyde. During this metabolism, oxygen radicals may be produced, and under insufficient antioxidant capacity, these radicals may also trigger lipid peroxidation, increasing susceptibility of LDL to oxidation. Hypertriglyceridemia induced changes in LDL size and LDL oxidizability (Feillet et al. 1998; Kondo et al. 2001). As HDL inhibits the oxidative modification of LDL (Klimov et al. 1993; Sangvanich et al. 2003), its reduction in diabetic patients could influence the susceptibility of LDL to oxidation.

The oxidizability of LDL is also dependent on the presence of oxidizable lipids, i.e., PUFA. Positive correlations were found between PUFA levels and the susceptibility of LDL to oxidation (Balkan et al. 2002). However, in our study, despite increased *in vitro* oxidation of LDL isolated from all diabetic patients, LDL-

cholesteryl ester fatty acids were not altered except in type 2 diabetic patients with complications. In these patients, an increase in polyunsaturated fatty acid contents and a reduction in monounsaturated fatty acid levels were detected in LDL, in agreement with other studies (Salomaa et al. 1990; Pelikanova et al. 1991a,b, 2001). It has been postulated that LDL with an increase in polyunsaturated fatty acids is more easily oxidizable (Dimitriadis et al. 1995). Thus, in complicated type 2 diabetes mellitus, fatty acid alterations might be an additional factor that may influence LDL oxidizability.

In conclusion, the present study demonstrates increased lipid peroxidation and reduced total antioxidant activity in favor of an oxidative stress in both type 1 and 2 diabetes mellitus, with or without complications. These abnormalities are closely related to poor glycemic control. LDL-fatty acid composition appeared to play little in determining the susceptibility of LDL to oxidation which seemed to be related to glycemic control and reduced antioxidant defence. Improvement of glycemic control is then a beneficial factor to decrease oxidative stress and LDL atherogenicity in diabetic patients.

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