Electronegative low density lipoprotein subform is increased in patients with short-duration IDDM and is closely related to glycaemic control

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Summary We evaluated the effect of improving glycaemic control with intensive insulin therapy on LDL susceptibility to oxidation, electronegative LDL proportion, and LDL subfraction phenotype in a group of 25 patients with short-duration insulin-dependent diabetes mellitus (IDDM); 25 matched healthy control subjects were also studied. LDL susceptibility to oxidation was measured by continuous monitoring of conjugated diene formation. Electronegative LDL was isolated by anion exchange chromatography, and quantified as percentage of total LDL. Six LDL subfractions were isolated by density gradient ultracentrifugation and phenotype A or B classified as the quotient (LDL1-LDL3)/(LDL4-LDL6). Compared to the control group, IDDM subjects with poor glycaemic control showed higher electronegative LDL (19.03 ± 10.09 vs $9.59 \pm 2.98\%$, $p < 0.001$), similar LDL subfraction phenotype and lower susceptibility to oxidation (lag phase 45.6 ± 8.8 vs 41.2 ± 4.7 min, $p < 0.05$). After three months of intensive insulin therapy, HbA_{1c} decreased from 10.88 ± 2.43 to $5.69 \pm 1.54\%$ ($p < 0.001$), and electronegative LDL to $13.84 \pm 5.15\%$ ($p < 0.05$). No changes in LDL susceptibility to oxidation or LDL subfraction phenotype were observed. Electronegative LDL appeared significantly correlated to HbA_{1c} and fructosamine ($p < 0.01$ and $p < 0.001$) only in poorly controlled IDDM patients. These findings suggest that high electronegative LDL in IDDM subjects is related to the degree of glycaemic control, and could therefore be due to LDL glycation rather than to LDL oxidation or changes in LDL subfraction phenotype. [Diabetologia (1996) 39: 1469–1476]

Keywords Insulin-dependent diabetes mellitus, intensive insulin therapy, glycaemic control, electronegative LDL, LDL subfraction phenotype, LDL oxidation.

Atherosclerosis is the major cause of death in the diabetic population. Hyperglycaemia per se is an independent risk factor for the development of cardiovascular disease, in spite of the coexistence of other known risk factors, such as hyperlipidaemia and hypertension [1,2]. Elevated concentrations of low density lipoproteins (LDL) are also a major risk factor in the general population [3,4]. However, LDL levels are usually normal in both insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes (NIDDM) [2, 5].

It has been postulated that the increased cardiovascular risk for diabetic patients could be related, among other factors, to LDL qualitative modifications such as oxidation and glycation. These modifications impair LDL cellular catabolism, leading to loss of affinity for LDL receptors in fibroblasts, increased accumulation of cholesteryl esters in macrophages, and immunological responses [6, 7]. In

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Abbreviations: IIT, Intensive insulin therapy; LDL(–), electronegative LDL subform; FPLC, fast protein liquid chromatography; PBS, phosphate buffered saline; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus.

addition, it has been reported that glycation and oxidation processes are closely related, and potentially accelerated by each other [8]. Some authors have measured plasma lipid peroxides in human diabetes [9, 10] and proposed that increased lipid peroxides are usually related to underlying atherosclerosis. A higher autoantibody titre against oxidized and glycated LDL has recently been reported in NIDDM patients when compared to control subjects [11]. Several groups have reported increased susceptibility to oxidation of LDL isolated from diabetic patients [12–19], although other authors disagree [20, 21]. In particular, studies carried out in IDDM subjects that showed higher susceptibility to oxidation [15–18] have been performed in patients with diabetes of more than 10 years duration.

Other qualitative LDL changes, such as prevalence of small, dense LDL particles have been described in patients with enhanced cardiovascular risk [22], including both IDDM and NIDDM [23–25]. The predominance of small, dense LDL subfractions (named phenotype B) is associated with relatively increased concentrations of plasma triglyceride, which is usually present in poorly controlled diabetic subjects. Increased atherogenicity of small LDL may be related, among other factors, to its increased susceptibility to oxidation when compared to that of large, buoyant LDL particles [26].

On the other hand, the presence of a plasma circulating electronegative LDL form (named LDL$^-$) has been described, and suggested that it was the result of a peroxidative process. These particles had a higher content of lipoperoxides, cholesterol oxides, lower content of α-tocopherol, and higher electrophoretic motility [27–29]. In addition, loss of affinity of these particles for the LDL receptor in fibroblasts has also been observed [27]. Other authors [30] have also reported the presence of an oxidation-labile LDL subfraction in plasma, with negative electrical charge. However, the oxidative origin of the electronegative LDL has recently been questioned [31]. The origin and relevance of this circulating modified LDL are not clear, but its increased plasma concentration has been related to high atherosclerosis risk [32].

As far as we know, the effect of improved glycaemic control on the LDL subfraction distribution has only been evaluated in very short-term studies (2 weeks)[23, 24], and no data are available on the LDL susceptibility to oxidation and the proportion of electronegative LDL. In this investigation, we have evaluated the effect of 3 months of intensive insulin therapy (IIT) achieving blood glucose optimization on the aforementioned parameters. For this purpose, we studied a group of patients with short-duration IDDM, without chronic complications in order to avoid pathogenic factors other than hyperglycaemia.

Subjects and methods

Patients. We included 25 patients (20 males and 5 females) with IDDM in the study. They were recruited from the diabetes clinic on the basis of poor glycaemic control [glycated haemoglobin (HbA$_{1c}$) > 8 %, non-ketoacidosis]. IDDM was defined according to the National Diabetes Data Group criteria [33]. All patients older than 30 years showed positivity for islet-cell antibodies. Mean age was 28.1 ± 10.3 years (range: 14–47), and mean body mass index (BMI) 21.6 ± 3.15 kg/m^2 (range: 17.2–29.8). Known diabetes duration range was 0–20 months, with 19 of 25 being new-onset diabetic patients, not previously treated. None had chronic diabetic complications and 32 % were smokers.

We also recruited 25 age-sex matched healthy control subjects (20 males and 5 females) from the hospital staff as control group. Their mean age was 28.7 ± 10.0 years (range:16–49), BMI 22.8 ± 2.95 kg/m^2 (range:17.8–29.4) and 28 % were smokers. BMI had remained stable for at least 1 year previous to their inclusion in the study. None of the patients or control subjects were taking drugs (other than insulin) or vitamins, or had any disease known to influence lipoprotein metabolism.

All patients were included in an intensive insulin therapy programme with regular insulin before main meals, and intermediate insulin before dinner or at bedtime. Patients were instructed observe an isocaloric diet, providing 50–55 % carbohydrate and 30–35 % fat. All of them were given a specific diabetes education programme and visited the outpatient unit every 2–4 weeks. Assessment was carried out at baseline and after 3 months of intensive insulin therapy.

The study was approved by the hospital ethics committee, and all patients and control subjects gave their informed consent.

Blood glucose analysis. Glucose was determined by a standarized automated method adapted to a Hitachi 747 autoanalyzer (Hitachi, Hitachi-Boehringher Mannheim, Mannheim, Germany). Fructosamine was determined by a standard colourimetric method (Boehringer Mannheim, Mannheim, Germany) (normal range: 205–285 µmol/l). HbA$_{1c}$ was determined by HPLC [Hi-AutoA1c HA-8121 Analyzer (Dic-Kioto, Kioto, Japan)], normal range: 3.7–5.5 %.

Lipid analysis. Lipoproteins were quantified by the combined ultracentrifugation-precipitation method, as recommended by the *Lipid Research Clinics Laboratory* [34]. Cholesterol and triglyceride concentrations from EDTA-plasma and lipoprotein fractions were determined by enzymatic methods (Boehringer Mannheim) adapted to an RA-XT autoanalyzer (Bayer, Leverkusen, Germany). LDL composition was calculated from plasma isolated LDL (densities 1025–1050 g/l). Total and free cholesterol, triglyceride, phospholipid (enzymatic method, Wako, Wako Chemicals GmbH, Neuss, Germany) and protein [35] content were expressed as percentage of total LDL mass.

LDL oxidation studies

1. Isolation of native LDL particles. Plasma aliquots from venous blood drawn in 7.5 % EDTA-containing vacutainer tubes, were isolated by centrifugation at 1500 *g* for 15 min at 4 °C, and stored at –80 °C until processed (before 16 weeks). After thawing plasma, native LDL (density range 1025–1050 g/l) was isolated by sequential ultracentrifugation [36]. In order to avoid oxidative modification of lipoproteins, ultracentrifugation was

performed at 4 °C with KBr solutions containing 1 mmol/l EDTA. LDL was dialysed extensively against degassed 10 mmol/l Tris-HCl buffer pH 7.4, containing 1 mmol/l EDTA, and stored at 4 °C in the dark.

2. Identification of the electronegative LDL subform. Electronegative subform of LDL was isolated from total LDL by chromatography in an anion exchange column (Mono Q 5/5) with a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Uppsala, Sweden), as described [37, 38], with additional minor modifications in order to shorten the duration of NaCl gradient process. Briefly, LDL was eluted at 1 ml/min for 10 min by a linear gradient of 0–0.1 mol/l NaCl, followed by a multistep gradient procedure: 10–18 min 0.2 mol/l NaCl, 18–24 min 0.3 mol/l NaCl, 24–29 min 1 mol/l NaCl, and 29–35 min 0 mol/l NaCl. Two LDL forms, a major form named LDL$^{(+)}$ (elution at 0.2 mol/l NaCl), and a minor form named LDL$^{(-)}$ (elution at 0.3 mol/l NaCl), differing in their electrical charges, were identified at 280 nm and their relative proportion quantified by peak integration.

3. "In vitro" LDL susceptibility to oxidation. LDL was dialysed against phosphate buffer saline (PBS) pH 7.4 by gel filtration chromatography in a G-25 Sephadex column (Pharmacia), diluted to a concentration of 50 mg of protein/l, and incubated with 2.5 μmol/l $CuSO_4$ at 30 °C. Conjugated diene formation of an aliquot of LDL was continuously monitored at 234 nm for 3 h, according to Esterbauer et al. [39], although using a different Cu + + /protein ratio (50 vs 16.6), as described above, in a Biochrom 4060 spectrophotometer equipped with a seven-position cell changer (Pharmacia LKB). Lag phase time (expressed as min) and diene rate formation (maximal curve slope expressed as Δabs/min) were measured. Pre- and post-treatment samples corresponding to the same IDDM patient and a sample from a control subject were assayed in the same batch. In our laboratory we have obtained an intra-assay imprecision of 2.88 % for the lag phase time and 3.08 % for the diene rate formation. Our inter-assay imprecision was 6.00 % for the lag phase time and 14.7 % for the diene rate formation. In order to study the viability of freezing plasma we studied 12 samples before and after 3 months of freezing at –80 °C, and no statistical significant differences were found (fresh plasma lag phase time 45.4 ± 5.0 min; frozen plasma lag phase time 45.6 ± 5.9 min).

LDL subfraction distribution. LDL subfractions were isolated from aliquots of frozen plasma-EDTA at –80 °C according to the method of Griffin et al. [40] with some modifications (37 000 rev/min during 20 h at 20 °C). Subfraction isolation also differed from this method. Briefly, six LDL subfractions LDL1 (density range 1.020–1.026 g/ml), LDL2 (1.026–1.030), LDL3 (1.030–1.036), LDL4 (1.036–1.043), LDL5 (1.043–1.051) and LDL6 (1.051–1.056), were isolated by aspiration of 0.8 ml aliquots. In order to assess the lack of mixture of the different subfractions, potassium concentration in each aliquot was determined, and the corresponding density calculated. Cholesterol, triglyceride, phospholipid and protein content of each subfraction was determined as described above. LDL subfraction mass was calculated by the sum of each constituent. Results were expressed as the percent contribution of each subfraction to the total LDL mass, and the ratio [(LDL1 + LDL2 + LDL3)/(LDL4 + LDL5 + LDL6)] was calculated. According to our previous results [38], we classified LDL subfraction phenotype A when the ratio exceeded 1.8, B when it was below 1.1, and AB when it ranged from 1.1 to 1.8. Thus, we considered small dense LDL particles when predominant density was higher than the cut-off value of 1.036 g/ml, similar to that reported by other authors [41–43]. Pre- and

post-treatment samples corresponding to the same IDDM patient and the sample corresponding to one control subject were assayed in the same batch. Results obtained in our laboratory showed that freezing only modified the subfraction ratio by 8.7 % without modifying phenotype assignation.

Statistical methods. Statistical differences between both periods of glycaemic control for IDDM patients were assessed by using paired Student's *t*-test when the variables were normally distributed, and non-parametric test (Wilcoxon *t*-test) for variables without normal distribution. Unpaired Student's *t*-test and Mann-Whitney U-test were used to compare the diabetic patients with the control group. Fisher's exact probability test evaluated differences in LDL phenotype distribution (phenotype A vs phenotype AB + B). Association between variables was tested by linear correlation (*r*). In all cases, a probability (*p*) less than 0.05 was considered as statistically significant. All data are expressed as mean ± SD.

Results

Table 1 shows clinical characteristics, glycaemic control parameters and plasma lipid levels of diabetic and control subjects. Diabetic patients during poor glycaemic control (before IIT) presented an abnormal lipoprotein profile evidenced by higher plasma levels of triglyceride, VLDL cholesterol (cVLDL) and LDL cholesterol (cLDL), and lower HDL cholesterol (cHDL) concentration than the control group. Table 2 shows the LDL susceptibility to oxidation, LDL$^{(-)}$ proportion and LDL subfraction ratio for control group and diabetic patients before and after blood glucose optimization with IIT. The percentage of LDL$^{(-)}$ in poorly controlled IDDM was significatively higher than in the control group (19.0 ± 10.1 vs 9.59 ± 2.98 %, $p < 0.001$). However, LDL from poorly controlled IDDM patients showed a significant ($p < 0.05$) enhancement on the resistance to oxidative modification, when compared with control subjects, as indicated by the longer lag phase time of conjugated diene formation (45.6 ± 8.81 vs 41.2 ± 4.71 min). The rate of conjugated diene formation did not differ between IDDM and control groups. In addition, LDL susceptibility to oxidation and the percentage of LDL$^{(-)}$ did not differ between smokers and non-smokers and were not significant in either IDDM or control subjects (data not shown). Concerning LDL composition (Table 3), LDL particles from IDDM patients before IIT showed a higher percentage of triglyceride (7.68 ± 4.09 vs 5.65 ± 1.11 %, $p < 0.001$) and a lower percentage of polar lipids (free cholesterol: 12.8 ± 1.45 vs 14.6 ± 1.32 % $p < 0.001$; phospholipid: 31.3 ± 1.38 vs 32.2 ± 0.59 %, $p < 0.01$, respectively) than control subjects. With regard to LDL subfraction distribution phenotype, no differences ($p = 0.18$) were observed between the diabetic patients and non-diabetic subjects (Fig. 1), with clear predominance of phenotype A in both groups (84 and 96 %, respectively). Three

Table 1. Clinical characteristics and parameters of glycaemic control and lipoprotein profile of control subjects and IDDM patients before and after intensive insulin therapy (IIT)

	Control subjects	IDDM patients	
		Before IIT	After IIT
Age (years)	28.7 ± 10.0	28.1 ± 10.3	
BMI (kg/m^2)	22.83 ± 2.90	21.59 ± 3.15	21.8 ± 5.11
Glucose (mmol/l)	4.48 ± 0.38	10.30 ± 4.75^c	$7.48 \pm 2.36^{b, e}$
Fructosamine (μmol/l)	ND	518.0 ± 142.8	287.6 ± 58.9^f
HbA$_{1c}$ (%)	ND	10.88 ± 2.43	5.69 ± 1.54^f
Total cholesterol (mmol/l)	4.52 ± 0.77	5.39 ± 2.52	4.47 ± 0.92
Triglycerides (mmol/l)	0.72 ± 0.32	2.05 ± 3.61^b	$0.97 \pm 0.56^{a, d}$
cVLDL (mmol/l)	0.30 ± 0.12	0.70 ± 1.74	0.29 ± 0.25
cLDL (mmol/l)	2.76 ± 0.69	3.51 ± 1.09^b	2.80 ± 0.69^e
cHDL (mmol/l)	1.46 ± 0.41	1.11 ± 0.33^a	1.37 ± 0.33^e

Data are mean ± SD

ND , not determined; [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ vs control subjects; [d] $p < 0.05$; [e] $p < 0.01$; [f] $p < 0.001$ vs IDDM before IIT

Table 2. LDL susceptibility to oxidation (lag phase time and conjugated diene rate formation) electronegative LDL proportion (LDL$^{(-)}$), and LDL subfraction ratio [(LDL1 + LDL2 + LDL3) / (LDL4 + LDL5 + LDL6)] of IDDM patients before and after IIT, and control subjects

	Control subjects	IDDM patients	
		before IIT	after IIT
Lag phase time (min)	41.2 ± 4.7	45.6 ± 8.8^a	43.4 ± 5.8
Dienes rate formation (Δabs/min)	0.0241 ± 0.003	0.0246 ± 0.005	0.0240 ± 0.004
% LDL$^{(-)}$	9.59 ± 2.98	19.03 ± 10.08^c	$13.85 \pm 5.15^{b, d}$
LDL subfraction ratio	3.19 ± 1.30	2.90 ± 1.15	2.90 ± 0.99

Data are mean ± SD

[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ vs control subjects; [d] $p < 0.05$ vs IDDM before IIT

Table 3. LDL composition (expressed as percentage of LDL mass) of control subjects and IDDM patients before and after intensive insulin therapy (IIT)

	Control subjects	IDDM patients	
		before IIT	after IIT
Total cholesterol	46.34 ± 1.39	45.72 ± 3.88	45.04 ± 2.21^a
Esterified cholesterol	31.75 ± 2.13	32.82 ± 3.69	31.94 ± 2.76
Free cholesterol	14.59 ± 1.32	12.89 ± 1.45^c	13.09 ± 1.63^c
Triglyceride	5.65 ± 1.11	7.68 ± 4.09^c	7.70 ± 2.35^c
Phospholipid	32.17 ± 0.59	31.31 ± 1.38^b	32.23 ± 1.74^d
Protein	15.80 ± 0.91	15.32 ± 1.51	15.01 ± 2.04

Data are mean ± SD

[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ vs control subjetcs; [d] $p < 0.005$ vs IDDM before IIT

diabetic patients and one control subject displayed phenotype AB. Only one diabetic subject with high plasma cholesterol and triglyceride concentrations showed phenotype B.

After 3 months of IIT, glycaemic control improved significantly (Table 1). This improvement of glycaemic control was accompanied by normalization of lipoprotein concentrations, which became similar to those of the control subjects. Briefly, total plasma cholesterol decreased by 17%, triglyceride 53% ($p < 0.05$), cVLDL 59%, and cLDL 20% ($p < 0.01$), whereas cHDL increased by 33% ($p < 0.01$). All of these changes were greater than those attributed to intraindividual biological variation of these lipidic constituents regardless of statistical significance [44]. After improvement of glycaemic control with IIT, the percentage of LDL$^{(-)}$ also decreased significantly (from 19.0 ± 10.1% to 13.8 ± 5.15%, $p < 0.05$), but still remained higher ($p < 0.01$) than the control group. Figure 2 shows a representative chromatogram of the LDL elution profile obtained from one IDDM patient before and after IIT. In contrast, LDL susceptibility to oxidation (expressed as lag phase time and rate of conjugated diene formation) did not change (Table 2), although significant differences compared to the control group disappeared. LDL composition (Table 3) did not change after IIT, except for a trend towards normalization of the
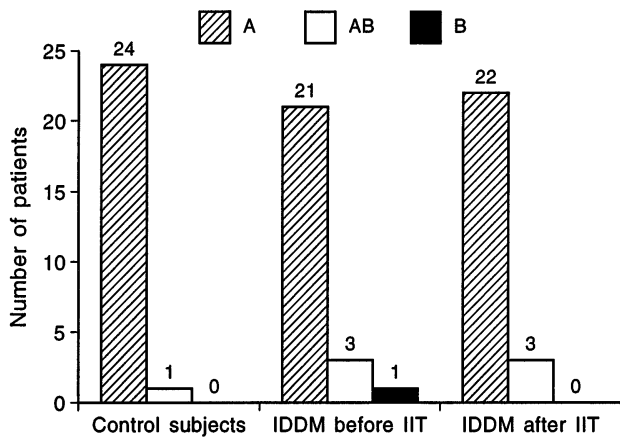
Fig. 1 LDL subfraction phenotype of control subjects and IDDM patients before and after intensive insulin therapy (IIT). No statistical differences were observed ($p = 0.18$, Fisher's exact probability test). The LDL subfraction pattern was classified as A when the subfraction ratio $[(LDL1 + LDL2 + LDL3)/(LDL4 + LDL5 + LDL6)]$ exceeded 1.8, as B when the ratio was below 1.1, and as AB when the ratio was between 1.1 and 1.8
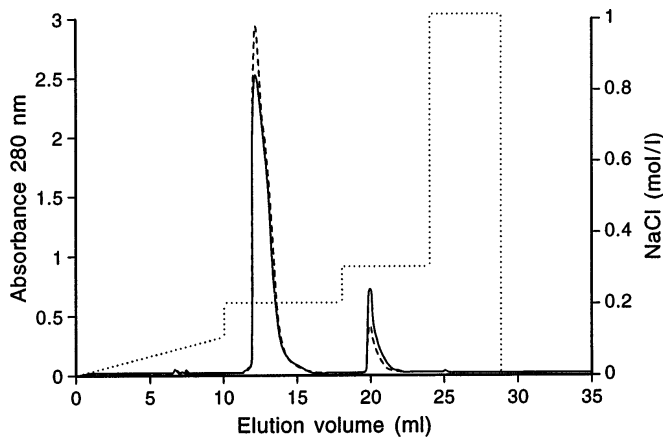
Fig. 2 Representative chromatographic profile of LDL from an IDDM patient before IIT (*continuous line*) and after IIT (*dashed line*). NaCl gradient (right axis, *dotted line*) was formed with buffer A (10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 7.4) and buffer B (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 1 mol/l NaCl, pH 7.4), as indicated in Materials and Methods. Most of the LDL (LDL +) eluted with 0.2 mol/l NaCl between 12–15 ml. LDL$^{(-)}$ eluted with 0.3 mol/l NaCl between 19–21 ml

percentage of phospholipids (from 31.3 ± 1.38 to $32.2 \pm 1.74\%$, $p < 0.005$). LDL subfraction phenotype also remained unchanged for all diabetic patients showing phenotype A or AB. However, following the marked improvement in glycaemic control and the dramatic diminution of plasma cholesterol (from 16.2 to 4.19 mmol/l) and triglyceride (from 18.4 to 0.69 mmol/l), the single patient with phenotype B changed it to phenotype A.

The proportion of LDL$^{(-)}$ in IDDM patients before IIT, positively correlated with HbA$_{1c}$ ($r = 0.535$, $p < 0.01$) and fructosamine levels ($r = 0.639$,

$p < 0.0005$), as well as with total plasma triglyceride ($r = 0.657$, $p < 0.0005$), percentage of LDL triglyceride ($r = 0.599$, $p < 0.001$) and LDL protein ($r = 0.571$, $p < 0.001$), and negatively correlated with that of LDL cholesterol ($r = -0.761$, $p < 0.0005$) and LDL phospholipid ($r = -0.421$, $p < 0.05$). All these statistically significant associations disappeared after IIT. On the other hand, lag phase time, in poorly controlled IDDM patients positively correlated with HbA$_{1c}$ ($r = 0.401$, $p < 0.05$) and fructosamine levels ($r = 0.545$, $p < 0.01$). Concerning LDL composition, lag phase time showed a positive correlation with the percentage of LDL triglyceride ($r = 0.626$, $p < 0.0005$) and a negative one with that of LDL cholesterol ($r = -0.559$, $p < 0.01$) and LDL phospholipid ($r = -0.412$, $p < 0.05$). After optimization of glycaemic control, all significant correlations disappeared. In addition, the percentage of LDL$^{(-)}$ and lag phase time were highly correlated ($r = 0.519$, $p < 0.005$) in diabetic patients before IIT, but this strong relationship disappeared after the marked improvement of HbA$_{1c}$. In the control group, the single significant correlation observed between the investigated parameters was a positive correlation between total plasma triglycerides and LDL$^{(-)}$ ($r = 0.412$, $p < 0.05$).

Discussion

Considerable effort has been focused on identifying the leading factors for diabetic vascular complications. Qualitative modification of LDL is strongly implicated as a major factor in the current hypothesis of the pathogenesis of atherosclerosis [6]. Since increased atherosclerotic risk in the diabetic population is not always associated with increased lipoprotein concentration, and qualitative modifications of LDL particles play a central role in the development of arteriosclerotic lesions, we have evaluated the effect of marked improved glycaemic control by IIT on LDL susceptibility to oxidation, LDL$^{(-)}$ proportion, and LDL subfraction phenotype in a group of patients with IDDM of short-duration.

In accordance with others [45], quantitative abnormalities of lipoprotein profile found in poorly controlled IDDM were reversed by marked improvement of glycaemic control. All the variations observed in the lipoprotein profile (including those not showing statistical differences), were above the intra-individual variations described for reference subjects [44]. Concerning qualitative alterations, two findings were found to be of interest: decreased LDL susceptibility to oxidation and higher proportion of LDL$^{(-)}$ in IDDM patients during the stage of poor glycaemic control.

Regarding LDL susceptibility to oxidation, previously reported results by other authors are controversial. In most cases, LDL from NIDDM patients is

more susceptible to oxidation [12–14, 19, 46], probably reflecting the well-known diminished resistance to oxidation of small LDL particles [26] which is increased in these patients [25]. Regarding IDDM subjects, most studies [15–18] were performed in patients with diabetes of long duration (10 years or more) and displayed increased LDL susceptibility to oxidation when compared to control subjects. Those studies describing decreased [20] or no difference [21, 47] in LDL susceptibility to oxidation between IDDM and control subjects, did not report the duration of the disease. Our results showing decreased susceptibility to oxidation of LDL from IDDM patients when compared to control subjects, were not due to differences in LDL subfraction phenotype. Discrepancies between the present results and those showing an increased susceptibility to oxidation of LDL in IDDM, could be attributed to differences in the recruited groups and also in biochemical procedures. In this investigation, the diabetic population mainly comprised new onset patients, while in other studies [15–18], diabetes had lasted for at least 10 years. Longer diabetes duration could contribute to increasing the LDL susceptibility to oxidation. In this regard it has been reported [10, 48] that increased plasma lipid peroxidation in diabetes may not reflect the consequences of high blood glucose, but may be related to underlying complications, in particular atherosclerosis developed during long-duration diabetes. In these circumstances, structural proteins from the vascular wall become modified by advanced glycation end-products, resulting in enhanced trapping of lipoproteins, which could contribute to prolong their half-life in plasma [49] and, thus, facilitate atherogenic modifications. This hypothesis is supported by the recent findings of Walzem et al. [50] who observed a relationship between the susceptibility to oxidation of lipoproteins and their permanence in the blood-stream. As the present study was conducted in IDDM of short-duration without chronic complications, it might be assumed that LDL susceptibility to oxidation was not affected by underlying atherosclerosis. On the other hand, in vitro glycated LDL has been described as more resistant to oxidative modification than native LDL [13]. This may contribute to explain the longer lag phase time observed in our study in IDDM patients with respect to control subjets. It could be hypothesized that LDL glycation in patients with short-duration IDDM, in the absence of chronic complications, would make LDL particles more resistant to oxidative stress. Further studies should be developed using in vivo glycated LDL isolated from both short and long-duration IDDM subjects, in order to clarify these findings.

For several years, different authors have described the presence of a minor plasma circulating LDL with increased negative charge, which has been described as a mildly oxidized particle. Its role in the development of atherosclerosis remains unclear, although it has some atherogenic characteristics [27–30, 37, 38], and has been related to increased atherosclerosis risk [32]. Our group previously described an increase of LDL$^{(-)}$ proportion in trained subjects after heavy aerobic exercise [38], possibly as a consequence of increased of free radical production during exercise, in association to enhanced susceptibility to oxidation of the LDL. In the present work, some distinctive features between poorly controlled IDDM patients and the control group or IDDM patients after IIT were observed. First, the strikingly high LDL$^{(-)}$ proportion positively correlated with parameters of glycaemic control, plasma triglyceride and lag phase time. Secondly, the lag phase time was longer and positively correlated with parameters of glycaemic control. Furthermore, significant differences on lag phase time between poorly controlled IDDM and the control group disappeared after IIT. Finally, the LDL$^{(-)}$ proportion diminished after IIT, and all statistically significant correlations among parameters disappeared. These findings suggest that possibly the increased proportion of LDL$^{(-)}$ in poorly controlled IDDM patients could be a consequence of glycation rather than a consequence of lipoperoxidation. Supporting these data, we have observed that in vitro glycated LDL by the method of Steinbrecher and Witzum [51] elutes at the same ionic strength (0.3 mol/l NaCl) than LDL$^{(-)}$ (unpublished observations), as other authors have described [52, 53]. However, other modifications affecting LDL charge, as desialylation [54] cannot be discarded as relevant in diabetic patients. Further studies should be developed to elucidate the origin of the negative charge of LDL$^{(-)}$ in diabetic subjects.

One of the alterations in LDL composition confirmed in patients with poorly controlled IDDM, was the triglyceride enrichment [23, 55]. LDL was also depleted of free cholesterol and phospholipid. After improving blood glucose control, only the phospholipid percentage of LDL particles increased towards normalization, but the other alterations persisted. The depletion in free cholesterol observed in the IDDM group contrasts with the increased resistance to oxidation of LDL particles, as free cholesterol has been suggested to act as a protective factor against free radical induced oxidation. However, other factors such as antioxidant vitamins and/or fatty acid composition modulate the response of LDL to an oxidative stress [56]. Furthermore, these compositional modifications are probably too subtle to be reflected as a significant change on the LDL susceptibility to oxidation. The lack of complete normalization of lipoprotein compositional abnormalities even after improving glycaemic control, has already been described by other authors [55, 57] and it may be due to the non-complete normalization of glycaemic control achieved, or to the non-physiological

portal/peripheral insulin levels obtained by subcutaneous administration [57].

In conclusion, high proportion of LDL$^{(-)}$ subform and compositional abnormalities in LDL particles are present in poorly controlled patients with IDDM of short-duration, in the absence of chronic complications, and appear to be dependent on the degree of glycaemic control. Near-normalization of glycaemic control improves these alterations, without changes in the LDL subfraction phenotype. Increased LDL$^{(-)}$ proportion might be a consequence of LDL glycation rather than LDL oxidation. Furthermore, the duration of diabetes seems to be of outstanding importance to evaluate qualitative characteristics of LDL, such as susceptibility to oxidation and/or subfraction phenotype. Future research should clarify the LDL$^{(-)}$ origin, and whether it may play a pathogenic role in the development of atherosclerosis in diabetic patients.

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