

L. Perségol · B. Vergès · M. Foissac ·  
P. Gambert · L. Duvillard

## Inability of HDL from type 2 diabetic patients to counteract the inhibitory effect of oxidised LDL on endothelium-dependent vasorelaxation

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**Abstract** *Aims/hypothesis:* In healthy normolipidaemic and normoglycaemic control subjects, HDL are able to reverse the inhibition of vasodilation that is induced by oxidised LDL. In type 2 diabetic patients, HDL are glycosylated and more triglyceride-rich than in control subjects. These alterations are likely to modify the capacity of HDL to reverse the inhibition of vasodilation induced by oxidised LDL. *Subjects and methods:* Using rabbit aorta rings, we compared the ability of HDL from 16 type 2 diabetic patients and 13 control subjects to suppress the inhibition of vasodilation that is induced by oxidised LDL. *Results:* Oxidised LDL inhibited endothelium-dependent vasodilation (maximal relaxation [E<sub>max</sub>]=58.2±14.6 vs 99.3±5.2% for incubation without any lipoprotein,  $p<0.0001$ ). HDL from control subjects significantly reduced the inhibitory effect of oxidised LDL on vasodilatation (E<sub>max</sub>=77.6±12.9 vs 59.5±7.7%,  $p<0.001$ ), whereas HDL from type 2 diabetic patients had no effect (E<sub>max</sub>=52.4±20.4 vs 57.2±18.7%, NS). HDL triglyceride content was significantly higher in type 2 diabetic patients than in control subjects (5.3±2.2 vs 3.1±1.4%,  $p<0.01$ ) and was highly inversely correlated to E<sub>max</sub> for oxidised LDL+HDL in type 2 diabetic patients ( $r=-0.71$ ,  $p<0.005$ ). *Conclusions/interpretation:* In type 2 diabetes mellitus, the ability of HDL to counteract the inhibition of endothelium-dependent vasorelaxation induced by oxidised

LDL is impaired and is inversely correlated with HDL triglyceride content. These findings suggest that HDL are less atheroprotective in type 2 diabetic patients than in control subjects.

**Keywords** HDL · Oxidised LDL · Triglyceride · Type 2 diabetes mellitus · Vasodilatation

**Abbreviations** apo: apolipoprotein · *d*: density · eNOS: endothelial NO synthase · NO: nitric oxide · ox-LDL: oxidised LDL · PAF-AH: platelet-activated factor-acetyl hydrolase · PON: paraoxonase · TBS: Tris buffer saline · TG: triglyceride

### Introduction

Type 2 diabetic patients present a significantly higher risk for atherosclerosis than nondiabetic subjects. The quantitative and qualitative lipoprotein abnormalities frequently observed in these patients are likely to play a central role in the premature development of atherosclerotic lesions. Quantitative abnormalities observed in type 2 diabetic patients are hypertriglyceridaemia and decreased plasma HDL cholesterol concentration. The main qualitative abnormalities consist of the glycation and a triglyceride (TG) enrichment of the different lipoprotein subclasses [1, 2].

HDL particles have multiple antiatherosclerotic properties. One of them is their beneficial effect on endothelium-dependent vasorelaxation. The intravenous administration of reconstituted HDL either to hypercholesterolaemic men or to heterozygotes deficient in ATP-binding cassette A1, both of whom had initially impaired endothelium-dependent vasorelaxation, restored normal vasorelaxation [3, 4]. More particularly, ex vivo experiments on isolated strips of rabbit thoracic aorta showed that HDL are able to counteract the inhibitory effect of oxidised LDL (ox-LDL) on endothelium-dependent vasorelaxation [5].

Endothelium dysfunction, a predictor of cardiovascular events [6], has been observed in patients with type 2 diabetes mellitus [7, 8]. Due to qualitative modifications,

L. Perségol · B. Vergès · M. Foissac ·  
P. Gambert · L. Duvillard (✉)  
INSERM U498, Hôpital du Bocage,  
BP 77908, 21079 Dijon Cédex, France  
e-mail: laurence.duvillard@chu-dijon.fr  
Tel.: +33-380293646  
Fax: +33-380293661

L. Perségol · B. Vergès · M. Foissac ·  
P. Gambert · L. Duvillard  
University of Burgundy,  
IFR 100, Dijon, France

B. Vergès  
Department of Endocrinology and Metabolic Diseases, CHU,  
Bocage Hospital,  
Dijon, France

HDL from type 2 diabetic patients may be less protective against impairments of endothelium-dependent vasorelaxation than HDL from healthy subjects. To test this hypothesis, we investigated whether HDL from type 2 diabetic patients show the same ability as HDL from normolipidaemic and normoglycaemic control subjects to protect endothelium-dependent vasorelaxation from the inhibitory effect of ox-LDL. In order to study the effect of HDL separately, our work was performed on isolated rings of rabbit aorta, a previously validated model [5, 9]. In addition we measured the activity of paraoxonase (PON), an HDL-associated enzyme with an anti-oxidant role due to its ability to hydrolyse some oxidised lipids.

## Subjects and methods

### Subjects

We included 16 type 2 diabetic patients and 13 normolipidaemic and normoglycaemic control subjects in this protocol, which was approved by the Ethics Committee of Dijon University Hospital, with written informed consent obtained from each subject before the study. Type 2 diabetic and control subjects were not taking any anti-oxidants or any drugs that could affect lipid metabolism except hypoglycaemic drugs for type 2 diabetic patients. Of the diabetic patients, three patients were being treated by sulfonylureas, four by glinides, two by metformin, three by sulfonylureas and metformin, two by sulfonylurea and acarbose, and two by sulfonylureas, metformin and acarbose. No type 2 diabetic subject was insulin-treated or taking thiazolidinediones. Diabetes duration was  $9.13 \pm 6.85$  years. Three diabetic patients had microalbuminuria  $>30$  mg/l, one had retinopathy and two had neuropathy. Nine diabetic patients had high blood pressure and five had cardiovascular disease or macroangiopathy. Nine diabetic patients were taking one or several of the following drugs: angiotensin converting enzyme inhibitors, beta blocking drugs, calcium channel blockers, antithrombotic drugs. Metabolic syndrome was excluded in control subjects according to the criteria of the National Cholesterol Education Program Adult Treatment Panel III report (NCEP-ATP III) [10].

### Chemical compounds

Acetylcholine, arterenol hydrochloride, sodium nitro-prusside, butylated hydroxytoluene and EDTA were purchased from Sigma Chemical (Saint Quentin Fallavier, France).

### Preparation of lipoproteins

The different lipoprotein subclasses were isolated from serum or plasma by sequential flotation ultracentrifugation, according to their density ( $d$ ). The LDL ( $1.019 < d < 1.063$  g/ml) used to form ox-LDL were prepared in a 70.Ti rotor on an ultracentrifuge (XL-90; Beckman, Palo Alto, CA, USA) by sequential flotation ultracentrifugation from 170 ml of fresh citrated plasma drawn from normolipidaemic donors. Densities of plasma were adjusted by adding potassium bromide. After a 15-hour run at 184,000 g, the top fraction of the sample containing lipoproteins with  $d < 1.019$  g/ml was removed. Finally, LDL were prepared from the infranatant adjusted to a 1.063 g/ml density by a 24-hour run at 149,000 g. HDL ( $1.063 < d < 1.21$  g/ml) of control and type 2 diabetic subjects were prepared from 20 ml of EDTA plasma in a 70.Ti rotor on an ultracentrifuge (Beckman). After a 20-hour run at 184,000 g, the top fraction of the sample containing lipoproteins with  $d < 1.063$  g/ml, was removed. Finally, HDL were prepared from the infranatant adjusted to a 1.21 g/ml density by a 24-hour run at 184,000g. The different lipoprotein fractions were dialysed overnight against a 10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4 buffer (TBS). To avoid oxidation, native lipoproteins were supplemented with EDTA (final concentration, 200  $\mu$ mol/l) and butylated hydroxytoluene (final concentration, 20  $\mu$ mol/l) and kept at 4°C.

### Oxidative modification of LDL

The oxidation of LDL was performed by incubating freshly prepared LDL, adjusted to a 1.2 g/l final concentration of proteins in TBS, with a copper sulfate solution (final concentration, 5  $\mu$ mol/l) for 24 h at 37°C. At the end, oxidation was stopped by adding EDTA (final concentration, 200  $\mu$ mol/l) and butylated hydroxytoluene (final concentration, 20  $\mu$ mol/l) and ox-LDL were kept at 4°C [9].

### Serum and HDL biochemical measurements

Glycaemia was measured on a Vitros analyser (Ortho Clinical Diagnostics, Rochester, NY, US). Total and HDL cholesterol, TG, proteins, apolipoprotein (apo)AI and apoB were quantitated on a Dimension analyser with dedicated reagents (Dade Behring, Newark, DE, USA). LDL-cholesterol was calculated using the Friedewald formula. Fructosamine, free cholesterol and phospholipids were measured on the same analyser with Wako reagents (Wako, Neuss, Germany) for free cholesterol and phospholipids, and ABX Diagnostics reagents (Montpellier, France) for fructosamine. HbA<sub>1c</sub> was performed by high performance liquid chromatography on a Variant II (Bio-Rad, Richmond, CA, US) (normal range: 4.0–6.0%).

## Measurement of paraoxonase activity

PON activity was measured as already described in fresh HDL samples within 2 days of isolation [11]. It was assayed in tubes containing Tris-HCl buffer (150 mmol/l NaCl, 1 mmol/l CaCl<sub>2</sub>, pH 7.4) and 100 µg proteins of HDL (final volume 800 µl). The reaction was initiated by adding paraoxon (1 mmol/l) and the absorbance monitored at 412 nm for 1 h. The amount of 4-nitrophenol formed was calculated from the molar extinction coefficient (128,00 mol<sup>-1</sup> cm<sup>-1</sup>). PON activity was expressed as nmol of 4-nitrophenol formed per minute and mg of apoAI, under the experimental conditions.

## Vasoreactivity on rabbit aorta rings

Vasoreactivity experiments were performed on rabbit (Charles River, L'Arbresle, France) aorta rings as previously described [9]. Our protocol has been approved by the local Ethics Committee for animals at Dijon University. The principles of laboratory animal care were followed. Aortic rings were first contracted by 0.3 µmol/l arterenol hydrochloride, a concentration giving 75% of the maximal contraction. Then the rings were relaxed by cumulative additions of acetylcholine in the 1 nmol/l to 0.01 mmol/l concentration range. After wash-out and a 30-min recovery period, aortic rings were incubated for 2 h either with ox-LDL or HDL or both. The concentration of proteins in the organ bath (native or oxidised lipoproteins) was constantly 1 g/l for each lipoprotein subclass, as used in many previous studies conducted with rabbit aortic rings [5, 9, 12]. At the end of the incubation period, aortic segments were again contracted with arterenol hydrochloride and progressively relaxed with acetylcholine or with sodium nitroprussiate. Sodium nitroprussiate, a nitric oxide (NO) donor, was used to check that the observed defects in vasodilatation were endothelium-dependent.

The maximal relaxation (E<sub>max</sub>) induced by acetylcholine and expressed as a percentage of the contraction to arterenol hydrochloride (0.3 µmol/l) was determined from experimental data. To limit variations of results due to intrinsic properties of vessels, experiments were performed in quadruplicate on aorta rings from four different rabbits, and results were averaged.

## Statistical analysis

Statistical analysis was performed using Statview statistic software. Data are expressed as mean±SD. The comparison of data from type 2 diabetic subjects and controls was performed by the Mann-Whitney *U* test. The maximal relaxation values obtained in two different conditions and the same experiment were compared by the nonparametric Wilcoxon matched-pair test. Correlation coefficients were performed by simple linear regression analysis. We also performed a multiple regression analysis in which the dependent variable was the maximal vasorelaxation and the

independent variables were HDL TG percentage, HDL fructosamine:protein ratio, plasma TG, age, sex, diabetes duration and PON. A *p* value <0.05 was considered to be statistically significant.

## Results

### Clinical and biochemical characteristics

The clinical and biochemical characteristics of type 2 diabetic and control subjects are shown in Table 1. Each of our control subjects had a fasting glycaemia <6.10 mmol/l. We recruited 16 type 2 diabetic patients with a broad range of glycaemic control, i.e. with fasting glycaemia at 4.66 to 18.57 mmol/l and HbA<sub>1c</sub> of 6.0 to 13.3%. Total plasma and LDL cholesterol did not differ between the two groups. Compared to the control group, plasma TG level was 1.8-fold higher in the type 2 diabetic group (*p*<0.05), whereas serum HDL cholesterol and apoAI concentrations were 26% (*p*<0.01) and 14% (*p*<0.05) lower, respectively.

### HDL composition

Table 2 illustrates the composition of HDL in the two groups of subjects. Compared to control subjects, type 2 diabetic patients exhibited an 1.7-fold higher TG proportion in HDL (*p*<0.01), whereas other lipid components, total proteins and apoAI were not significantly different. The HDL TG:apoAI ratio was 1.5-fold higher (*p*<0.05) in type 2 diabetic patients than in control subjects and the HDL fructosamine:total protein ratio was 2.7-fold higher (*p*<0.005). PON activity was not statistically different between the two groups.

**Table 1** Clinical and biochemical characteristics of type 2 diabetic and control subjects

	Control subjects (n=13)	Type 2 diabetic patients (n=16)
Age (years)	57±13	63±13
Sex (male/female)	7/6	8/8
BMI (kg/m <sup>2</sup> )	23.6±1.4	30.5±5.4 <sup>c</sup>
Waist circumference (cm)	83.4±9.1	108.5±13.3 <sup>c</sup>
Fasting glycaemia (mmol/l)	5.48±0.55	9.19±4.20 <sup>b</sup>
HbA <sub>1c</sub> (%)	ND	9.3±3.0
Total cholesterol (mmol/l)	5.68±0.78	5.49±1.49
HDL-cholesterol (mmol/l)	1.50±0.35	1.11±0.30 <sup>a</sup>
LDL-cholesterol (mmol/l)	3.62±0.70	3.42±1.12
TG (mmol/l)	1.18±0.57	2.08±1.50 <sup>b</sup>
Apo AI (g/l)	1.46±0.23	1.25±0.29 <sup>b</sup>
Apo B (g/l)	1.03±0.28	1.09±0.32

Values are expressed as mean±SD

ND, Not determined

<sup>a</sup>*p*<0.05; <sup>b</sup>*p*<0.005; <sup>c</sup>*p*<0.001

**Table 2** HDL composition in control and type 2 diabetic subjects

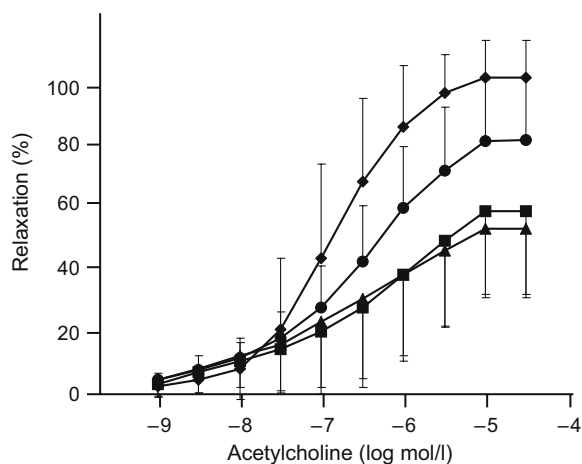
	Control subjects (n=13)	Type 2 diabetic patients (n=16)
Protein (%)	49.6±6.2	51.5±4.7
Phospholipid (%)	21.4±3.7	18.5±3.5
Free cholesterol (%)	2.8±0.5	2.7±0.4
Cholesteryl ester (%)	23.2±2.6	22.9±4.6
TG (%)	3.1±1.4	5.3±2.2 <sup>b</sup>
ApoAI (%)	38.5±5.3	42.5±10.3
TG/apoAI (mmol/g)	0.084±0.040	0.124±0.052 <sup>a</sup>
Fructosamine/total proteins (µmol/mg)	2.39±0.42	6.48± 3.25 <sup>c</sup>
PON activity (nmol min <sup>-1</sup> mg <sup>-1</sup> apoAI)	1.39±0.89	1.31±0.80

Values are expressed as mean±SD

HDL composition is expressed as the percentage of total HDL mass  
<sup>a</sup> $p<0.05$ ; <sup>b</sup> $p<0.01$ ; <sup>c</sup> $p<0.005$

### Oxidised LDL influence on endothelium-dependent vasorelaxation

In accordance with previous data, pre-incubation of isolated arterial segments with ox-LDL inhibited the endothelium-dependent relaxation induced by acetylcholine, the maximal relaxation to acetylcholine decreasing from 99.3±5.2% in segments incubated with Krebs solution to 58.2±14.6% in segments treated with ox-LDL ( $p<0.0001$ ) (Fig. 1).



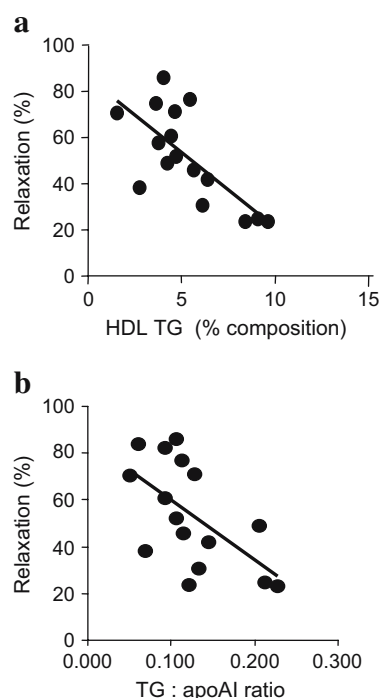
**Fig. 1** Influence of HDL from type 2 diabetic and control subjects on the inhibitory effect of ox-LDL on endothelium-dependent vasorelaxation in rabbit aorta rings. The graph shows mean concentration–response curves for acetylcholine in aortas contracted with 0.3 µmol/l arterenol hydrochloride and pre-incubated for 2 h either with ox-LDL alone (1 g protein/l) (squares), or with ox-LDL and HDL from control subjects (circles), or with ox-LDL and HDL from type 2 diabetic patients (triangles) or in Krebs buffer (diamonds). Values represent means±SD

### Ability of HDL to counteract the inhibitory effect of oxidised LDL on endothelium-dependent vasodilation

Compared to the maximal relaxation obtained in segments pre-incubated in Krebs buffer, no significant difference was observed between segments pre-incubated with HDL from control subjects or from type 2 diabetic subjects ( $E_{max}=92.8±4.0$  vs  $99.3±5.2$  and  $92.4±8.4$  vs  $99.3±5.2\%$ , respectively) (data not shown). The maximal relaxation of rabbit aortic rings was significantly greater after preincubation with HDL from control subjects+ox-LDL than after preincubation with ox-LDL alone ( $E_{max}=77.6±12.9$  vs  $59.5±7.7\%$ ,  $p<0.01$ ) (Fig. 1). In contrast, the addition of HDL from type 2 diabetic patients to ox-LDL did not modify the maximal relaxation, compared to incubation with ox-LDL ( $E_{max}=52.4±20.4$  vs  $57.2±18.7\%$ , NS) (Fig. 1).

### Linear regression analysis

In type 2 diabetic patients the  $E_{max}$  obtained after co-incubation of the vessels with ox-LDL and HDL ( $E_{max}$  [ox-LDL+HDL]) was strongly and negatively correlated with the percentage of TG in HDL and the HDL TG:apo AI ratio ( $r=-0.71$ ,  $p<0.005$  and  $r=-0.60$ ,  $p<0.05$ , respectively) (Fig. 2a,b). In control subjects, such a correlation was not observed ( $r=0.138$ , NS).  $E_{max}$  (ox-LDL+HDL) was not



**Fig. 2** Correlation between **a** HDL-TG and **b** TG:apoAI ratio, and the maximal endothelium-dependent relaxation on rabbit aortic rings after preincubation with HDL from type 2 diabetic subjects and oxidised LDL.  $r=-0.71$ ,  $p=0.0019$  (a);  $r=-0.60$ ,  $p=0.0147$  (b)

correlated with the percentage in HDL-free or esterified cholesterol, phospholipids or proteins, either in type 2 diabetic patients or in control subjects. No significant correlation was observed between the HDL fructosamine:total protein ratio or PON activity and Emax (ox-LDL+HDL) in type 2 diabetic patients or controls. In multiple regression analysis, including HDL TG percentage, the HDL fructosamine:protein ratio, plasma TG, age, sex, diabetes duration, and PON, the only parameter that was significantly correlated to Emax was HDL TG percentage ( $r=-0.91$ ,  $p=0.0134$ ).

## Discussion

In the present study, we demonstrated for the first time that the ability of HDL to protect the vessel from ox-LDL-induced inhibition of endothelium-dependent vasorelaxation is impaired in type 2 diabetic patients. Moreover, the ability of HDL to counteract the inhibitory effect of ox-LDL on vasorelaxation is inversely correlated with HDL TG content. In contrast, no significant correlation was observed between the HDL fructosamine:total protein ratio or PON activity and Emax (ox-LDL+HDL) in type 2 diabetic patients or control subjects.

The inhibitory effect of ox-LDL and some of their specific compounds such as derivatives of cholesterol oxidised in position 7 or lysophosphatidylcholine on endothelium-dependent vasorelaxation is mainly related to a decreased bioavailability of NO [13–17]. HDL are likely to counteract the inhibitory effect of ox-LDL on endothelium-dependent vasorelaxation both by a direct and an indirect way. HDL inhibit the LDL oxidation process by removing lipid peroxides and oxidised derivatives of cholesterol from LDL via cholesteryl ester transfer protein [18]. The two HDL-associated enzymes, PON and platelet-activated factor-acetyl hydrolase (PAF-AH) hydrolyse oxidised cholesterol or hydroperoxides [19–21]. Furthermore, HDL prevent the depletion in free cholesterol of endothelial cell caveolae induced by ox-LDL, thus allowing eNOS to stay in caveolae, where it can be activated by vasodilatory ligands of G protein-coupled receptors [22]. On the other hand, HDL can stimulate NO production. eNOS activity can be upregulated by HDL through regulation of phosphorylation sites [23]. Different signalling pathways are likely to be involved in this process. First, the binding of HDL to scavenger receptor BI has been demonstrated to activate Akt and mitogen-activated protein kinases, which in turn phosphorylate eNOS [24]. Secondly, the binding of HDL to the lysophospholipid receptor S1P<sub>3</sub> activates Ca<sup>2+</sup>-calmodulin-dependent kinases and Akt with consecutive activation of eNOS [25]. Finally, HDL can increase the expression of the gene encoding eNOS [26].

The inability of HDL from type 2 diabetic patients to counteract the inhibitory effect of ox-LDL on endothelium-

dependent vasodilatation indicates that one or several of the HDL protective effects detailed above are deficient.

In previously published works, HDL from patients with metabolic syndrome or type 2 diabetes mellitus were less protective against LDL oxidation than HDL from healthy subjects, and the antioxidative capacity of HDL was inversely correlated with plasma and HDL TG [11, 27, 28]. The lesser antioxidant capacity of HDL from type 2 diabetic patients is likely to contribute to the lesser ability of these particles to counteract the inhibitory effect of ox-LDL on endothelium-dependent vasorelaxation. The mechanisms underlying the decreased antioxidant capacity of HDL from type 2 diabetic patients are not well understood. However, TGs appear to play an important role, since a strong inverse correlation was found between the content of HDL in TGs and their antioxidant properties in several studies [11, 27, 28]. Moreover, it has recently been demonstrated that the replacement of cholesteryl esters by TGs in HDL abrogates the HDL-mediated protection against LDL oxidation [28].

PON and PAF-AH activities have inconstantly been found to be decreased and related to HDL antioxidant capacity in type 2 diabetic patients [11, 27–29]. Interestingly, in type 2 diabetic patients, the antioxidant capacity of HDL can be decreased, whereas PON and PAF-AH activities are similar to those measured in controls [11], indicating that in type 2 diabetes mellitus other factors than the decrease of PON and PAF-AH activities are likely to impair HDL antioxidant activity. Thus, in our study, a normal PON activity in HDL from type 2 diabetic patients does not preclude impairment of the antioxidant capacity of these HDL.

During the few last years it was found that HDL could also be oxidised [30]. It is noteworthy that we observed no impairment of Emax with HDL from type 2 diabetic patients alone and that Emax was quite similar to that measured with HDL from control subjects, indicating that HDL from our type 2 diabetic patients were not able to decrease NO bioavailability by themselves, and that initially they were probably not highly oxidised.

The inability of HDL from type 2 diabetic patients to counteract the inhibitory effect of ox-LDL on endothelium-dependent vasorelaxation could also be related to a lesser capacity to induce NO synthesis. Experiments on reconstituted HDL particles demonstrated that changes to the HDL content in TG modulate the conformation of apoAI and the expression of epitopes all along the molecule [31, 32]. Therefore, the TG enrichment of HDL particles usually observed in type 2 diabetic patients could modify apoAI conformation and decrease the binding of HDL particles to the cell surface receptors implicated in the signalling pathways that activate eNOS. This hypothesis needs to be confirmed by further experimentation.

The fact that we recruited patients with a wide range of glycaemic control allowed us to search for a possible relationships between HDL glycation and HDL vasorelaxant effect. We did not observe any correlation between

these two parameters, which suggests that HDL glycation does not play an important role in the lesser vasorelaxant effect of HDL from type 2 diabetic patients, compared to the TG enrichment.

A limitation of our study is that we did not measure PAF-AH and lecithin cholesterol acyltransferase activities. However, in previous studies performed in type 2 diabetic patients or insulin-resistant subjects no correlation could be found between the activity of these two enzymes and the decreased antioxidant properties of HDL [10, 26, 27]. Therefore, it is unlikely that we could have explained the decreased vasorelaxant effect of HDL from type 2 diabetic patients by a decrease in PAF-AH and lecithin cholesterol acyltransferase activities.

In conclusion, the present study demonstrates that the ability of HDL from type 2 diabetic patients to counteract the inhibitory effects of ox-LDL on endothelium-dependent vasorelaxation is impaired, and is highly inversely correlated with the TG content of HDL. As endothelial dysfunction is likely to favour the early development of atherosclerotic lesions, these findings provide further evidence that HDL in patients with type 2 diabetes mellitus has a lower atheroprotective effect than that in control subjects; they also provide further evidence for the deleterious effect of the hypertriglyceridaemia frequently associated with this disease.

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