

# LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets

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## Abstract

**Aims/hypothesis** This study assessed oxidative stress in LDL from obese patients with the metabolic syndrome and compared it with that in LDL from type 2 diabetic patients or control volunteers. It also determined the effect on platelets of LDL from the three groups.

**Methods** The profiles of lipids, fatty acids and fatty acid oxidation products were determined in LDL isolated from plasma of patients with the metabolic syndrome, patients with type 2 diabetes and volunteers ( $n=10$  per group). The effects of LDL from the participant groups on the platelet arachidonic acid signalling cascade and aggregation were investigated.

**Results** Compared with LDL from control volunteers, LDL from obese metabolic syndrome and type 2 diabetic patients had lower cholesteryl ester, higher triacylglycerol and lower ethanolamine plasmalogen levels. Proportions of linoleic acid were decreased in phosphatidylcholine and cholesteryl esters in LDL from both patient groups. Among the markers of lipid peroxidation, oxidation products of linoleic acid (hydroxy-octadecadienoic acids) and malondialdehyde were increased by 59% and twofold, respectively in LDL

from metabolic syndrome and type 2 diabetic patients. LDL from metabolic syndrome and type 2 diabetic patients were equally potent in activating the platelet arachidonic acid signalling cascade through increased phosphorylation of p38 mitogen-activated protein kinase and cytosolic phospholipase A<sub>2</sub>, and through increased thromboxane B<sub>2</sub> formation. LDL from patients with the metabolic syndrome and type 2 diabetes potentiated platelet aggregation by threefold and 3.5-fold respectively, whereas control LDL had no activating effects on platelets.

**Conclusions/interpretation** The metabolic syndrome in obese patients, without or with diabetes, is associated with increased oxidative stress in LDL, which triggers platelet activation.

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**Keywords** Fatty acids · LDL · Lipid peroxidation · Metabolic syndrome · Obesity · Oxidised LDL · Platelets · Type 2 diabetes

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## Abbreviations

cPLA <sub>2</sub>	Cytosolic phospholipase A <sub>2</sub>
CRP	C-reactive protein
HETE	Hydroxy-eicosatetraenoic acid
HODE	Hydroxy-octadecadienoic acid
MAPK	Mitogen-activated protein kinase
MUFA	Mono-unsaturated fatty acids
PLSD	Protected least significant difference
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
sPLA <sub>2</sub>	Secretory phospholipase A <sub>2</sub>
TxB <sub>2</sub>	Thromboxane B <sub>2</sub>

## Introduction

There is strong evidence that type 2 diabetes is associated with increased oxidative stress [1], which may contribute to the elevated risk of atherothrombosis. Oxidatively modified LDL are implicated in the pathogenesis of atherosclerosis and could be present in plasma from type 2 diabetic patients. On the one hand, autoantibodies to oxidised LDL were found to be increased in type 2 diabetic patients with macrovascular disease [2]. On the other hand, we recently showed that LDL from type 2 diabetic patients contained increased levels of lipid peroxides [3]. There is controversy on the occurrence of oxidative stress in patients with the metabolic syndrome. Some studies have shown higher levels of circulating oxidised LDL [4–6] and isoprostanes [7] in individuals with the metabolic syndrome. Other studies show no difference in oxidised LDL levels [8] or markers of oxidative damage (plasma F<sub>2</sub>-isoprostanes, hydroxy-eicosatetraenoic acid [HETE]) [9] between patients with the metabolic syndrome and healthy individuals. In addition, most studies have focused on lipid peroxides issued from the non-enzymatic peroxidation of arachidonic acid [7, 9]. Paradoxically, no data (to our knowledge) are available on circulating lipid peroxides issued from linoleic acid, although it is the main polyunsaturated fatty acid (PUFA) in LDL. Considering that the metabolic syndrome increases the risk of developing type 2 diabetes and cardiovascular diseases [10, 11], and that oxidative stress plays an important role in type 2 diabetes, it is important to determine whether oxidised LDL are already present in plasma from patients with the metabolic syndrome.

Increased platelet activation occurs in type 2 diabetes [12] and the metabolic syndrome [13, 14], and may represent a key contributing factor in the process of atherosclerosis and its thrombotic complications. Oxidative stress has been identified as one of the factors closely associated with platelet hyperactivation in diabetes [12] and lipid hydroperoxides have been shown to stimulate platelet aggregation [15, 16]. In vitro, moreover, oxidised LDL [17, 18] and LDL from patients with type 1 [19] or type 2 diabetes [20] activated platelets via an increase of p38 mitogen-activated protein kinase (MAPK) phosphorylation and thromboxane B<sub>2</sub> (TxB<sub>2</sub>) formation. To date, no data are available on how LDL from patients with the metabolic syndrome interact with platelets.

The objectives of the present study were: (1) to measure and compare different markers of oxidative stress in LDL from obese patients with the metabolic syndrome or type 2 diabetes, and from control volunteers; and (2) to determine the effects of these LDL on platelets.

## Methods

**Patients and control volunteers** We recruited 30 men (30–75 years old) from the Department of Endocrinology and Metabolic Diseases (Hospices Civils de Lyon). Exclusion criteria were smoking, alcohol consumption >2 units/day (20 ml), vitamin supplementation and evidence of cardiovascular complications. Patients with the metabolic syndrome were selected according to the criteria of the American Heart Association [21] and had to fulfil three or more of the following factors: waist circumference >102 cm, triacylglycerol >1.7 mmol/l, HDL-cholesterol <1 mmol/l, blood pressure >130/85 mmHg and fasting plasma glucose >5.6 mmol/l. Type 2 diabetic patients had to present with HbA<sub>1c</sub> greater than 7% (53 mmol/mol) and an associated feature of the metabolic syndrome. Patients treated with glitazone drugs were excluded and lipid-lowering drugs were suspended for at least 7 days before venepuncture. Control volunteers were in good health as assessed by medical history and had all above indices in the expected normal range. All participants gave written informed consent. The study protocol was approved by the Local Ethics Committee (CCP Sud Est IV, Hospices Civils de Lyon) and was in accordance with the principles of the Helsinki declaration.

**Blood collection** Venous blood was collected on EDTA. Plasma was immediately separated by centrifugation at 1,500 g for 10 min and frozen at –80°C under nitrogen in the presence of 10 µmol/l butylhydroxytoluene.

**Plasma secretory phospholipase A<sub>2</sub> activity** Plasma secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) activity was measured with a selective fluorescent substrate (Aterovax, Paris, France). Results are expressed in U/ml of sample, with 1 unit defined as the amount of sPLA<sub>2</sub> enzyme that catalyses the release of 1 nmol of product in 1 min. The average intra-assay CV for individual human plasma samples was 8.46% and the average inter-assay CV was 5.24%. The minimum detectable activity level was 10 U/ml.

**LDL isolation** LDL (1.019–1.063 g/ml) were isolated from plasma by potassium bromide stepwise ultracentrifugation (200,000 g) [22]. LDL preparations were extensively dialysed against PBS containing 1 mmol/l EDTA. LDL used to determine interactions with platelets were dialysed in a final step against EDTA-free PBS. The concentration of proteins was estimated using a Bradford assay [23]. Enzymatic determinations of total cholesterol and triacylglycerol were processed using commercial kits (Biomérieux, Marcy l’Etoile, France). Plasma high-sensitive C-reactive protein (CRP) was measured by nephelometry. LDL particle diameters were determined

by electrophoresis of plasma in non-denaturing polyacrylamide gradient gels (Lara-Spiral, Couternon, France).

**Fatty acid composition of lipid classes** Following the addition of internal standards and extraction with ethanol/chloroform (1:2, vol./vol.) in the presence of butylhydroxytoluene (50  $\mu\text{mol/l}$ ), lipid classes were separated by thin-layer chromatography. Chloroform/methanol (80:8, vol./vol.) was used to separate neutral lipids from phospholipids, and chloroform/methanol/water (63:27:4 by vol.) to separate phosphatidylcholines, phosphatidylethanolamines and sphingomyelins. Neutral lipids were separated into triacylglycerol and cholesteryl esters using hexane/diethylether/acetic acid (80:20:1 by vol.). The different spots were scraped off and treated for 90 min with trifluoride boron:methanol (1:1, vol./vol.) at 100°C. The derivatised fatty acid methyl esters and fatty dimethylacetals were extracted twice with isooctane and separated by gas chromatography using a gas chromatograph (HP 6890; Agilent Technologies, Massy, France) equipped with a capillary column (0.25  $\mu\text{m}$ , 30 m  $\times$  0.25 mm, SP 2380; Supelco, Bellefonte, PA, USA) and a flame ionisation detector. The respective concentrations of fatty acids, quantified according to the amount of appropriate internal standard, were used to calculate the concentrations of lipid classes.

**Quantification of total monohydroxylated fatty acids** Following lipid extraction, dried extracts were hydrolysed with 10 mol/l KOH for 20 min at 60°C [24]. Non-esterified hydroxylated fatty acids and fatty acids were first separated on a cartridge column (Oasis Sep-Pak; Waters, Milford, MA, USA) [3]. Non-esterified hydroxylated fatty acids were then separated by thin-layer chromatography with hexane/diethylether/acetic acid (60:40:1 by vol.), extracted with methanol, separated by reversed phase HPLC on an X Bridge C<sub>18</sub> column (3.5  $\mu\text{m}$ , 4.6  $\times$  150 mm; Waters) using a gradient solvent of acetonitrile and water (pH 3), and detected at 235 nm (for details, see profile in electronic supplementary material [ESM] Fig. 1).

**Malondialdehyde determination** LDL samples were mixed with thiobarbituric acid (10 mmol/l), acetic acid and butylhydroxytoluene (5 mmol/l), and incubated at 95°C for 60 min [25]. The thiobarbituric acid–malondialdehyde adducts were extracted with ethyl acetate, separated on to a Nucleosil C<sub>18</sub> column (5  $\mu\text{m}$ , 4.6  $\times$  250 mm; Macherey-Nagel, Düren, Germany) and detected fluorimetrically (excitation 515 nm, emission 553 nm).

**Vitamin E determination** LDL samples, containing tocol as an internal standard, were extracted twice with hexane (4 vol.)

following the addition of ethanol (1 vol.) [20]. Tocopherol isomers were separated by reversed phase HPLC on to a Nucleosil C<sub>18</sub> column (5  $\mu\text{m}$ , 4  $\times$  150 mm) and detected fluorimetrically (excitation 295 nm, emission 340 nm).

**Platelet isolation and incubation with LDL** Blood was collected at the blood bank from healthy volunteers who had not ingested any aspirin or other non-steroidal anti-inflammatory drugs in the previous 10 days. Platelets were isolated [26] and incubated for 2 h at 37°C in the presence or absence of LDL (1 mg/ml).

**Platelet p38 MAPK and cytosolic phospholipase A<sub>2</sub> activation** Following platelet lysis, proteins (30  $\mu\text{g}$ ) were denatured, electrophoresed in 12% (wt/vol.) Bis-Tris gels (Bio-Rad Laboratories, Marnes-la-Coquette, France) and transferred to nitrocellulose membranes. The membranes were incubated with anti-p38 MAPK or antiphospho-p38 MAPK or anti-phospho-cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) polyclonal antibodies (1:5,000 for all; Cell Signaling Technologies, Beverly, MA, USA), washed and then incubated with 1:10,000 goat anti-rabbit horseradish peroxidase conjugate. p38 MAPK, phospho-p38 MAPK and phospho-cPLA<sub>2</sub> were visualised by enhanced chemiluminescence and bands were quantified by densitometry (Amersham Biosciences, Little Chalfont, UK).

**TxB<sub>2</sub> measurement** Platelet TxB<sub>2</sub> was quantified by competitive enzyme immunoassay (Amersham Biosciences).

**Platelet aggregation** Aggregation was measured in isolated platelets in a dual-channel aggregometer (Chrono-log; Coulter, Margency, France) according to the method of Born [27]. Platelet suspensions were pre-incubated for 5 min at 37°C in the presence or absence of LDL (0.1 mg/ml) and stimulated with sub-threshold concentrations of collagen (75  $\pm$  9 ng/ml) while being continuously stirred at 1,000 rpm. The sub-threshold concentration of collagen was defined as the highest concentration of collagen that induced a less than 15% increase in light transmission. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission at 4 min after the addition of collagen.

**Statistical analysis** Results are expressed as the mean  $\pm$  SEM. Comparisons between groups were performed using ANOVA followed by Fisher's protected least significant difference (PLSD) post hoc test. Correlation coefficients were determined using linear regression analysis. With a statistical significance of  $p < 0.05$  and 90% power, a minimum of eight volunteers per group was required to detect a 50% difference in the concentration of malondialdehyde in LDL.

## Results

*Clinical characteristics of obese patients with the metabolic syndrome or diabetes and of control volunteers* The clinical and metabolic characteristics of the participants are summarised in Table 1. Patients with the metabolic syndrome fulfilled the criteria of the American Heart Association [21]. They were overweight or obese, had abdominal obesity, mild hypertriglycerolaemia with low HDL-cholesterol, and were hypertensive. Of ten metabolic syndrome patients, two were being treated against hypertension, whereas four of the ten type 2 diabetic patients were taking anti-hypertensive medications. This could explain why mean systolic and diastolic blood pressure were higher in the metabolic syndrome than in the type 2 diabetes group. In addition, metabolic syndrome patients showed elevated fasting insulin concentrations and a tendency to have an increased HOMA index, both suggestive of insulin resistance. Levels of high-sensitivity CRP, a circulating marker of systemic inflammation, were increased to similar degrees in metabolic syndrome and type 2 diabetic patients. The type 2 diabetic patients were poorly controlled and also had features of the metabolic syndrome. LDL-cholesterol levels were lower in patients with the metabolic syndrome or type 2 diabetes than in control volunteers, while plasma triacylglycerol levels

were higher. Healthy control volunteers had all above indices in the expected normal range.

sPLA<sub>2</sub> activity was significantly increased in plasma from patients with the metabolic syndrome or type 2 diabetes (45.7±4.3 and 51.9±5.1 U/ml, respectively), compared with plasma from healthy volunteers (34.4±2.5 U/ml). sPLA<sub>2</sub> activity showed positive correlations with waist circumference ( $r^2=0.41$ ,  $p<0.001$ ) and malondialdehyde concentrations in LDL ( $r^2=0.14$ ,  $p<0.05$ ), and a negative correlation with plasmalogen proportions ( $r^2=0.15$ ,  $p<0.05$ ).

*Lipid and fatty acid composition of LDL from obese patients with the metabolic syndrome or diabetes, compared with that of LDL from control volunteers* As shown in Table 2, the concentrations of cholesteryl esters were lower in LDL from both patient groups (−13% in metabolic syndrome, −20% in type 2 diabetic patients) than in LDL from control volunteers. By contrast, concentrations of triacylglycerol were higher in LDL from metabolic syndrome (50%) and type 2 diabetic (72%) patients. No differences in glycerophospholipid concentrations (phosphatidylcholine and phosphatidylethanolamine) were observed between control and the two patient groups. LDL particle sizes were significantly lower in patients with the metabolic syndrome or type 2 diabetes than in control volunteers.

**Table 1** Clinical and metabolic characteristics of control volunteers and obese patients with the metabolic syndrome or type 2 diabetes

Variable	Control	Metabolic syndrome	Type 2 diabetes
<i>n</i>	10	10	10
Sex	Male	Male	Male
Age (years)	50±3	58±2*	57±3
BMI (kg/m <sup>2</sup> )	24.3±0.7	30.7±1.4*	30.8±1.1**
Waist circumference (cm)	92±2	111±3**	116±7**
Fasting glucose (mmol/l)	5.5±0.2	5.6±0.1	9.0±1.0** †
Fasting insulin (pmol/l)	41.1±6.9	68.4±12.7*	45.4±12
HOMA index	0.53±0.13	0.99±0.20	1.07±0.36
CRP (mg/l)	0.54±0.12	3.61±0.82*	4.82±1.52**
HbA <sub>1c</sub> (%)	5.4±0.1	5.7±0.1	10.4±0.9** †
HbA <sub>1</sub> (mmol/mol)	35.5±0.6	38.5±1.4	90.2±9.8** †
Systolic BP (mmHg)	121±3	144±3**	132±6*†
Diastolic BP (mmHg)	78±4	88±5*	77±3†
Total cholesterol (mmol/l)	6.0±0.4	5.2±0.3	4.8±0.4*
HDL-cholesterol (mmol/l)	1.4±0.1	1.1±0.1**	0.9±0.1**
Total cholesterol:HDL-cholesterol	4.5±0.3	4.9±0.3	5.3±0.3*
LDL-cholesterol (mmol/l)	4.1±0.4	3.2±0.2	2.7±0.4**
VLDL-cholesterol (mmol/l)	0.5±0.1	0.8±0.1**	1.0±0.1** †
Triacylglycerol (mmol/l)	1.3±0.1	2.2±0.3**	2.6±0.2**
Glucose-lowering treatment (%)	0	0	80
Antihypertensive treatment (%)	0	20	40
Hypolipidaemic treatment (%)	0	10	20

Data are means ± SEM ( $n=8$  to 10 participants per group)

Hypolipidaemic treatment was interrupted 7 days before inclusion in study; the HOMA index was calculated as: fasting insulin (pmol/l) × fasting glucose (mmol/l)/400

\* $p<0.05$  and \*\* $p<0.01$  vs LDL from control volunteers;

† $p<0.05$  vs LDL from metabolic syndrome patients ( $p$  values obtained by ANOVA followed by Fisher's PLSD post hoc test)

**Table 2** Lipid classes and particle size of LDL from control volunteers and obese patients with the metabolic syndrome or type 2 diabetes

Variable	Control	Metabolic syndrome	Type 2 diabetes
Cholesteryl esters (nmol/mg protein)	2,459±118	2,132±61**	1,975±88**
Phosphatidylcholines (nmol/mg protein)	521±17	477±18	479±21
Phosphatidylethanolamines (nmol/mg protein)	17±2	15±1	18±1
Triacylglycerol (nmol/mg protein)	140±11	210±19*	241±37**
LDL particle size (nm)	25.1±0.2	24.4±0.2 *	24.4±0.1 *

Data are means ± SEM ( $n=8$  to 10 participants per group)

\* $p<0.05$  and \*\* $p<0.01$  vs LDL from control volunteers ( $p$  values obtained by ANOVA followed by Fisher's PLSD post hoc test)

The fatty acid composition of cholesteryl esters and phosphatidylcholine differed between patients and control volunteers (Table 3). The proportions of PUFA decreased in cholesteryl esters from metabolic syndrome patients, while saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA) proportions increased. In particular, the proportion of linoleic acid (18:2  $n-6$ ), the main PUFA, decreased in cholesteryl esters and phosphatidylcholine from metabolic syndrome patients. Although palmitoleic acid (16:1  $n-7$ ) represents a minor fatty acid, its proportion increased by 70% in cholesteryl esters from that group. Concerning LDL from type 2 diabetic patients, PUFA proportions decreased while those of SFA increased and

MUFA were unchanged. In particular, the proportions of linoleic acid decreased while those of arachidonic acid (20:4  $n-6$ ) increased in cholesteryl esters and phosphatidylcholine from type 2 diabetic LDL.

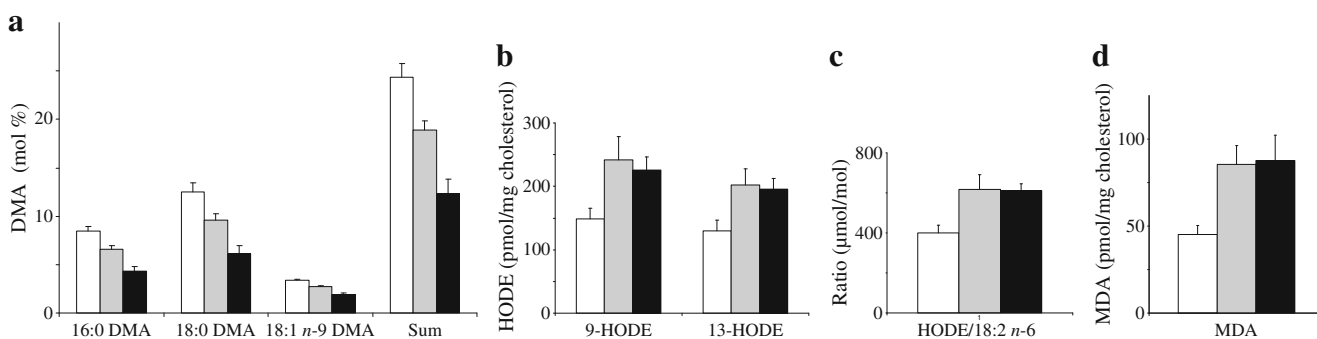
Special care was taken to measure ethanolamine plasmalogens (or alkenyl,acyl-glycerophosphoethanolamines). They constitute a glycerophospholipid subclass with a 16:0, 18:0 or 18:1 fatty chain attached at the  $sn-1$  position via a vinyl ether bond, and are more susceptible to oxidation [28]. As shown in Fig. 1a, the proportions of 16:0 dimethyl acetal issued from the alkenyl residue and of 18:0 dimethylacetal were decreased by 22% and 23%, respectively in LDL from metabolic syndrome patients compared

**Table 3** Main fatty acid compositions of cholesteryl esters and phosphatidylcholines in LDL from control volunteers and obese patients with metabolic syndrome or type 2 diabetes

Fatty acid (mol%)	Cholesteryl esters			Phosphatidylcholines		
	Type 2 diabetes	Metabolic syndrome	Control	Type 2 diabetes	Metabolic syndrome	Control
16:0	13.7±0.3*	13.5±0.4*	12.4±0.4	34.3±0.5	34.7±0.4	33.5±0.7
16:1 $n-7$	2.8±0.3	4.2±0.3**	2.5±0.3	0.3±0.1	0.5±0.1	0.3±0.1
16:1 $n-9$	0.5±0.1	0.4±0.1	0.3±0.1	0	0	0
18:0	1.0±0.1	1.0±0.1	1.0±0.1	15.3±0.7	15.8±0.7	16.0±0.5
18:1 $n-7$	1.0±0.2	0.7±0.2	1.0±0.2	1.2±0.3	1.0±0.2	1.3±0.3
18:1 $n-9$	22.1±0.7	21.7±0.7	20.6±0.7	11.6±0.3	10.9±0.5	10.9±0.5
18:2 $n-6$	46.7±1.1**	46.7±1.4**	52.0±1.2	19.6±0.7*	19.2±0.7*	21.8±1.0
18:3 $n-3$	0.5±0.1	0.7±0.0	0.4±0.1	0	0	0
18:3 $n-6$	0.8±0.2	1.1±0.1	0.8±0.1	0	0	0
20:3 $n-6$	0.9±0.1	0.9±0.1	0.8±0.0	3.3±0.2	3.7±0.3*	3.1±0.2
20:4 $n-6$	8.3±0.5** †	7.0±0.6	6.2±0.4	9.3±0.5*	8.8±0.7	7.2±0.6
20:5 $n-3$	1.0±0.2	1.3±0.2	1.3±0.2	0.9±0.2	1.1±0.1	1.1±0.3
22:5 $n-3$	0	0	0	0.4±0.1	0.4±0.2	0.4±0.2
22:6 $n-3$	0.6±0.1	0.6±0.1	0.7±0.1	2.9±0.2	2.6±0.3	3.2±0.4
SFA	14.7±0.3*	14.7±0.4*	13.5±0.5	49.9±0.6	50.9±0.7	50.0±0.9
MUFA	26.4±0.5	27.1±1.0*	24.4±0.8	13.4±0.4	13.0±0.6	12.9±0.6
PUFA	59.0±0.7*	58.3±1.2*	62.1±1.1	36.7±0.5	36.1±0.8	37.1±0.9

Data are means ± SEM ( $n=8-9$  participants per group)

\* $p<0.05$  and \*\* $p<0.01$  vs LDL from control volunteers; † $p<0.05$  vs LDL from metabolic syndrome patients ( $p$  values obtained by ANOVA followed by Fisher's PLSD post hoc test)



**Fig. 1** Indices of lipid peroxidation in LDL from control volunteers (white bars) and obese patients with metabolic syndrome (grey bars) or type 2 diabetes (black bars). **a** Fatty dimethylacetals (DMA) derived from phosphatidylethanolamine plasmalogens. **b** 9- and 13-HODE, **(c)** HODE/18:2 *n*-6 ratio and **(d)** malondialdehyde (MDA). Data are means  $\pm$  SEM ( $n=7-10$  participants per group).  $p<0.05$  for 16:0 DMA, 18:0 DMA, DMA sum, 9-HODE, 13-HODE and HODE/18:2 *n*-6, and  $p<0.01$  for malondialdehyde in LDL from metabolic

syndrome patients vs LDL from control volunteers;  $p<0.05$  for 9-HODE, 13-HODE and HODE/18:2 *n*-6, and  $p<0.01$  for 16:0 DMA, 18:0 DMA, 18:1 *n*-9 DMA, DMA sum and malondialdehyde in LDL from type 2 diabetic patients vs LDL from control volunteers;  $p<0.05$  for 16:0 DMA, 18:0 DMA, 18:1 *n*-9 DMA and DMA sum in LDL from type 2 diabetic patients vs LDL from metabolic syndrome patients.  $p$  values were obtained by ANOVA followed by Fisher's PLSD post hoc test

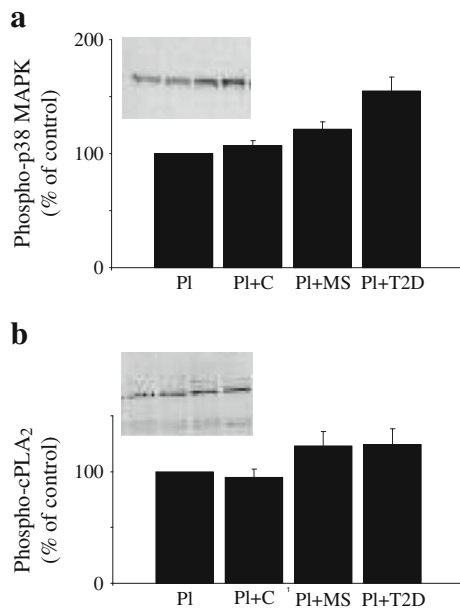
with control LDL. In LDL from type 2 diabetic patients, 16:0, 18:0 and 18:1 dimethylacetal species were decreased by 47%, 50% and 42%, respectively. Altogether, the sum of dimethylacetal species, representative of plasmalogens, was decreased by 22% and 49% in LDL from patients with the metabolic syndrome or type 2 diabetes, respectively. Interestingly, ethanolamine plasmalogen proportions were negatively correlated with waist circumference ( $r^2=0.22$ ,  $p<0.01$ ).

**Lipid peroxides and antioxidant contents of LDL from obese patients with the metabolic syndrome or diabetes, compared with those of LDL from control volunteers** The concentrations of total (free and esterified) hydroxylated fatty acids, the stable primary products of lipid peroxidation, were determined in LDL from patient and control groups. They mainly consisted of hydroxy-octadecadienoic acids (HODE) derived from linoleic acid and HETE derived from arachidonic acid. Concentrations of 9-HODE and 13-HODE increased by 62% and 55% respectively in LDL from metabolic syndrome patients and by 52% and 50% in LDL from type 2 diabetic patients (Fig. 1b). Additionally, the ratio of total HODE to linoleic acid increased by 54% and 52% in LDL from metabolic syndrome and type 2 diabetic patients, respectively (Fig. 1c). Among HETE forms, 8-, 11-, 12- and 15-HETE were detected in LDL from each participant. There were no significant differences in the concentrations of HETE between metabolic syndrome patients and control volunteers (data not shown). Only the 15-HETE isomer concentration was significantly increased by 82% in LDL from type 2 diabetic patients, compared with LDL from control volunteers ( $50.8\pm 7.6$  pmol/mg cholesterol vs  $28.0\pm 4.3$  pmol/mg cholesterol,  $p<0.05$ ). Concentra-

tions of malondialdehyde, issued from the degradation of fatty acid hydroperoxides, were significantly increased by twofold in patients with the metabolic syndrome or type 2 diabetes (Fig. 1d). Waist circumference was mildly correlated with triacylglycerol and malondialdehyde levels in LDL ( $r^2=0.13$  and  $0.25$ , respectively,  $p<0.05$ ) (ESM Fig. 2a, b).

The concentrations of vitamin E, the main liposoluble antioxidant, were determined in LDL.  $\alpha$ -Tocopherol concentrations did not differ between patients with the metabolic syndrome or type 2 diabetes ( $6.7\pm 0.5$  and  $6.0\pm 0.3$  nmol/mg protein, respectively) vs control volunteers ( $5.9\pm 0.3$  nmol/mg protein).  $\gamma$ -Tocopherol, which represents 10% of  $\alpha$ -tocopherol, was increased in LDL from metabolic syndrome patients ( $0.9\pm 0.1$  nmol/mg protein) compared with LDL from control ( $0.6\pm 0.1$  nmol/mg protein), but did not differ in LDL from type 2 diabetic patients ( $0.7\pm 0.1$  nmol/mg protein).

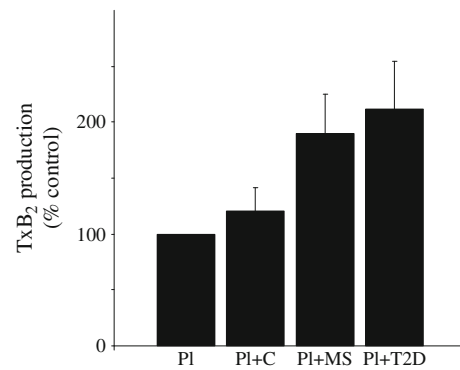
**LDL isolated from obese patients with the metabolic syndrome or diabetes, or from control volunteers: effects on platelet arachidonic acid signalling cascade** To determine the effects of LDL on the platelet arachidonic acid cascade, platelets from healthy donors were incubated for 2 h at 37°C in the absence or presence of LDL from both patient groups or control volunteers (1 mg/ml). Phosphorylated amounts of p38 MAPK and cPLA<sub>2</sub>, key enzymes involved in the release of arachidonic acid from membrane phospholipids, as well as concentrations of TxB<sub>2</sub>, the stable catabolite of the pro-aggregatory arachidonic acid metabolite thromboxane A<sub>2</sub>, were determined. As shown in Fig. 2, the addition of LDL from control volunteers to platelets had no effects on p38 MAPK and cPLA<sub>2</sub> phosphorylation, compared with platelets alone. By contrast, the addition of



**Fig. 2** Effects of LDL from control volunteers and obese patients with metabolic syndrome or type 2 diabetes on the phosphorylation of (a) p38 MAPK and (b) cPLA<sub>2</sub> in platelets. Platelets (PI) from blood donors were incubated for 2 h at 37°C in the absence or presence of LDL (1 mg protein/ml) from either control volunteers (C), metabolic syndrome patients (MS) or type 2 diabetic patients (T2D). Representative immunoblots and histograms of the normalised amount of phosphorylation of p38 MAPK and cPLA<sub>2</sub> are shown. Data are means  $\pm$  SEM ( $n=7-8$  participants per group).  $p<0.05$  for phospho-p38 MAPK and phospho-cPLA<sub>2</sub> in PI+MS vs PI;  $p<0.05$  for phospho-cPLA<sub>2</sub> and  $p<0.01$  for phospho-p38 MAPK in PI+T2D vs PI;  $p<0.05$  for phospho-p38 MAPK in PI+T2D vs PI+MS.  $p$  values were obtained by ANOVA followed by Fisher's PLSD post hoc test

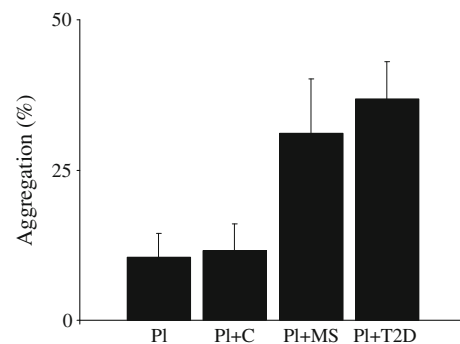
LDL from patients with the metabolic syndrome or type 2 diabetes significantly increased p38 MAPK phosphorylation, by 21% and 55% respectively, and cPLA<sub>2</sub> phosphorylation by 23% and 24% respectively. Compared with control platelets, the incubation of platelets with LDL from patients with the metabolic syndrome or type 2 diabetes resulted in a twofold increased basal concentration of TxB<sub>2</sub>, whereas LDL from control volunteers had no effects (Fig. 3). Platelet TxB<sub>2</sub> was negatively correlated with plasmalogen proportions ( $r^2=0.44$ ) and positively with HODE and malondialdehyde concentrations in LDL ( $r^2=0.24$  and  $r^2=0.31$ , respectively,  $p<0.01$ ) (ESM Fig. 2c, d).

*Effect on platelet aggregation of LDL isolated from obese patients with the metabolic syndrome or diabetes, or from control volunteers* To determine the effect of LDL on platelet aggregation, platelets were pre-incubated for 5 min with low concentrations of LDL (0.1 mg/ml) and then stimulated with sub-threshold concentrations of collagen for another 4 min. As shown in Fig. 4, LDL



**Fig. 3** Effects of LDL from control volunteers and obese patients with metabolic syndrome or type 2 diabetes on TxB<sub>2</sub> concentrations in unstimulated platelets. Platelets (PI) from blood donors were incubated for 2 h at 37°C in the absence or presence of LDL (1 mg protein/ml) from either control volunteers (C), metabolic syndrome patients (MS) or type 2 diabetic patients (T2D). TxB<sub>2</sub> concentrations in control unstimulated platelets were  $187\pm37$  pmol/10<sup>9</sup> platelets. Data, expressed as per cent of control, are means  $\pm$  SEM ( $n=10$  participants per group).  $p<0.05$  for PI+T2D vs PI and PI+MS vs PI.  $p$  values were obtained by ANOVA followed by Fisher's PLSD post hoc test

from control volunteers had no effect on collagen-induced platelet aggregation, compared with platelets incubated with buffer. By contrast, pre-incubation of platelets with LDL from metabolic syndrome or type 2 diabetic patients resulted in stimulation of platelet aggregation in response to sub-threshold concentrations of collagen (197% and 251%, respectively). Platelet aggregation was also potentiated by lower concentrations (50  $\mu$ g/ml) of LDL from type 2 diabetic patients (data not shown).



**Fig. 4** Effects of LDL from control participants and obese patients with metabolic syndrome or type 2 diabetes on the aggregation of platelets induced by collagen. Platelets (PI) from blood donors were pre-incubated for 5 min at 37°C in the absence or presence of LDL (0.1 mg protein/ml) from either control volunteers (C), metabolic syndrome patients (MS) or type 2 diabetic patients (T2D) and stimulated with sub-threshold concentrations of collagen (75 $\pm$ 9 ng/ml). Data, expressed as per cent of aggregation, are means  $\pm$  SEM of five participants per group.  $p<0.05$  for PI+MS vs PI;  $p<0.01$  for PI+T2D vs PI.  $p$  values were obtained by ANOVA followed by Fisher's PLSD post hoc test

## Discussion

The evidence for increased oxidative stress in patients with the metabolic syndrome is still a matter of debate. In the present study, we provide several lines of evidence for increased oxidative stress in LDL from obese metabolic syndrome patients, as assessed by different complementary indices of oxidative stress and by special focus on oxidation products of linoleic acid, the most abundant PUFA in LDL. Higher concentrations of total HODE and lower proportions of linoleic acid in phosphatidylcholine and cholesteryl esters were found in LDL from metabolic syndrome patients compared with control LDL. Interestingly, HODE and linoleic acid levels were modified to the same extent in LDL from type 2 diabetic patients. Conversely, there were no changes in total HETE concentrations in metabolic syndrome patients, which is in agreement with a recent study showing no differences in the levels of oxidation products of arachidonic acid (total HETE and  $F_2$ -isoprostanes) in individuals with features of metabolic syndrome [9]. This suggests that oxidation products of linoleic acid could be better markers of lipid peroxidation than those of arachidonic acid. It should be mentioned that participants did not supply a dietary record. Although we are not aware of any supplementation study reporting a concomitant decrease of linoleic acid and increase of arachidonic acid in plasma or LDL, we cannot exclude a potential effect of fat intake on plasma fatty acid composition.

The presence of oxidative stress in LDL from metabolic syndrome patients was confirmed by the decreased concentrations of plasmalogens in LDL from metabolic syndrome patients (–22%) as well as in LDL from type 2 diabetic patients (–49%), compared with control LDL. This result indicates that plasmalogens may be targets and markers of ongoing oxidative stress, and corroborates previous data showing a 20% decrease of plasmalogen phosphatidylethanolamine in erythrocyte membranes from hyperlipidaemic patients [29]. In addition, we found that malondialdehyde, a commonly used overall marker of lipid peroxidation that has been described to correlate with total isoprostanes in plasma and tissues [30, 31], was increased to a similar extent in LDL from metabolic syndrome and type 2 diabetic patients, in agreement with the enhanced serum levels of thiobarbituric acid reactive species observed in type 2 diabetic patients [32]. Although isoprostanes are recognised as reliable markers of oxidative stress *in vivo*, they derive exclusively from the free radical-induced peroxidation of arachidonic acid and their levels were either reported to be increased [7] or unchanged [9] in patients with the metabolic syndrome. Altogether, the increase of several complementary indices of oxidative stress and especially of oxidised products of linoleic acid in LDL from metabolic syndrome patients supports the hypothesis that the metabolic syndrome is associated with higher levels of

circulating oxidised LDL [4–6]. In addition, compared with control LDL, LDL from patients with the metabolic syndrome showed higher triacylglycerol and lower cholesteryl ester levels as well as decreased particle size, suggesting a higher percentage of smaller and denser LDL particles. Therefore, our data corroborate reports that the metabolic syndrome is associated with small and dense LDL particles [5, 33, 34], which are known to be more susceptible to oxidative modification [35].

Regarding the main antioxidant in LDL, we found similar concentrations of  $\alpha$ -tocopherol in patients with the metabolic syndrome or type 2 diabetes, and higher concentrations of  $\gamma$ -tocopherol in metabolic syndrome patients. There have been discordant reports about vitamin E levels in patients with metabolic syndrome or type 2 diabetes, showing either lower vitamin E concentrations in participants who had a feature of the metabolic syndrome and also consumed fewer fruits and vegetables [36], or no difference among participants with the metabolic syndrome or type 2 diabetes [37, 38]. Only one study to our knowledge has reported slightly higher serum  $\gamma$ -tocopherol levels [38] and no changes of  $\alpha$ -tocopherol among participants with the metabolic syndrome. Our results obtained *ex vivo* suggest that tocopherols may have been spared by molecules (such as plasmalogens) serving as primary targets of free radicals during LDL oxidation [39].

It is worth noting that oxidative stress was enhanced to a similar extent in LDL from metabolic syndrome and from type 2 diabetic patients, suggesting that factors associated with the metabolic syndrome, rather than poor glycaemic control, are key factors associated with oxidative modification of LDL. In agreement with this statement, our results show correlations between waist circumference and plasmalogens or malondialdehyde in LDL, suggesting an association between an excess of visceral fat and biochemical variables associated with oxidative stress in LDL. It is also likely that obesity tightly associated with the metabolic syndrome may represent a major factor in the oxidative stress seen in LDL from obese metabolic syndrome patients.

Beside the above, our results show that the activity of sPLA<sub>2</sub> is correlated with plasmalogens and malondialdehyde in LDL, suggesting that this enzyme catalyses the hydrolysis of oxidised phospholipids in LDL [40]. The correlation between the activity of sPLA<sub>2</sub> and waist circumference is in line with the association of sPLA<sub>2</sub> with the metabolic syndrome [41].

Regarding the functionality of LDL from patients with metabolic syndrome, we show for the first time that LDL from metabolic syndrome patients activate platelets and prime collagen-induced platelet aggregation. The activation occurs through an increased phosphorylation of p38 MAPK, the stress kinase responsible for the phosphorylation of cPLA<sub>2</sub> [42], the key enzyme involved in the release of arachidonic acid from membrane phospholipids [43]. It resulted in



increased formation of thromboxane A<sub>2</sub>, as assessed by the increased concentration of the stable catabolite, TxB<sub>2</sub>. We confirm that LDL isolated from type 2 diabetic patients activate platelets through the arachidonic acid cascade [20] and give new evidence for a priming effect of LDL on platelet aggregation, whereas LDL from control volunteers had no activating effects. As for the question of which molecules in LDL from the two patient groups are responsible for platelet activation, our data suggest that lipid peroxides may be implicated. LDL from obese patients with metabolic syndrome or type 2 diabetes showed similarly increased levels of lipid peroxides and activated platelets. In addition, correlation analyses show that the platelet TxB<sub>2</sub> concentration is related to concentrations of HODE and malondialdehyde in LDL, suggesting that lipid peroxides could underlie the activating effects of LDL on platelets and that glycation is not necessarily required. In future studies it will be important to take into account obesity in order to identify the mechanism by which oxidised LDL activate platelets.

In addition, our results obtained *ex vivo* strengthen the notion that fatty acid peroxides are implicated in platelet activation as previously demonstrated in *in vitro* studies [15, 44]. Indeed, we have previously shown that fatty acid hydroperoxides, precursors of HETE and HODE, potentiated platelet aggregation and activated platelets via the arachidonic acid signalling cascade [16]. More recently, the Podrez group showed that oxidised choline glycerophospholipids induce platelet aggregation via CD36 [45].

Altogether, our results support the idea that: (1) oxidative stress is increased in LDL from obese metabolic syndrome patients with or without type 2 diabetes; and (2) *in vivo* oxidatively modified LDL from metabolic syndrome patients activate control platelets to the same extent as LDL from type 2 diabetic patients. Considering that the metabolic syndrome often precedes the occurrence of diabetes [46] and that cardiovascular complications occur frequently at the onset of type 2 diabetes, our data suggest that oxidative stress in metabolic syndrome is an early promoter of premature atherothrombosis [47]. The identification of specific targets of oxidative stress in the metabolic syndrome, such as linoleic acid-containing lipids, might provide opportunities to selectively slow down some specific lipid peroxidation processes in LDL at the stage of the metabolic syndrome and to prevent the associated platelet hyperactivation.

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**Contribution statement** CC, RC, ML and PM conceived and designed the study. CCA, MM and PM recruited the patients. RC and AS acquired data. All authors interpreted data. CC wrote the manuscript and RC, ML, PM, AS, MG, CCA and MM revised the manuscript critically. All authors approved the final version of the manuscript.

**Duality of interest** The authors fully declare that there is no duality of interest associated with this manuscript.

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