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

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Short running title: Oxidative stress and metabolic syndrome

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Abstract

Aims/Hypothesis This study was aimed at assessing oxidative stress in LDL from obese patients with metabolic syndrome (MetS) compared with LDL from type 2 diabetic patients or control volunteers, and determining their effects on platelets.

Methods The profiles of lipids, fatty acids and fatty acid oxidation products were determined in LDL isolated from plasma of MetS patients, type 2 diabetic patients and volunteers (n=10 per group). The effects of LDL isolated from these participants on platelet arachidonic acid signaling cascade and aggregation were investigated.

Results Compared with LDL from control volunteers, LDL from obese MetS and type 2 diabetic patients contained lower cholesteryl esters, higher triacylglycerols and lower ethanolamine plasmalogens levels. Proportions of linoleic acid were decreased in phosphatidylcholine and cholesteryl esters in patients' LDL. Among the markers of lipid peroxidation, oxidation products of linoleic acid (hydroxy-octadecadienoic acids) and malondialdehyde were increased by 59% and 2-fold, respectively in LDL from MetS patients and to the same extent in LDL from type 2 diabetic patients. LDL from MetS patients were as potent as LDL from type 2 diabetic patients in activating platelet arachidonic acid signaling cascade through increased phosphorylation of p38 MAPK and cytosolic phospholipase A₂, and increased thromboxane B₂ formation. LDL from patients with MetS and type 2 diabetes potentiated 3-fold and 3.5-fold respectively platelet aggregation whereas control LDL had no effects activating platelets. on Conclusions/interpretation MetS in obese patients, without or with diabetes, is associated with increased oxidative stress in LDL, which trigger platelet activation.

The protocol is registered in ClinicalTrials.gov as NCT00932087.

Keywords

Lipid peroxidation . Fatty acids . LDL. Oxidized LDL . Platelets . Metabolic syndrome . Type 2 diabetes. Obesity.

Abbreviations

BHT, butylhydroxytoluene; CE, cholesteryl esters; cPLA₂, cytosolic phospholipase A₂; DMA, dimethylacetal; HDL, high-density lipoproteins; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; HS CRP, high sensitivity C-reactive protein; MAPK, mitogen-activated protein kinase; MetS, metabolic syndrome; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; sPLA₂, secretory phospholipase A₂; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive species; TG, triacylglycerols; TLC, thin-layer chromatography; TxA₂, thromboxane A₂; TxB₂, thromboxane B₂.

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. From one hand, autoantibodies to oxidized LDL were found to be increased in type 2 diabetic patients with macrovascular disease [2]. From 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 metabolic syndrome (MetS). Some studies showed higher levels of circulating oxidized LDL [4-6] and isoprostanes [7] in individuals with MetS. Other studies did not show any difference in oxidized LDL levels [8] or markers of oxidative damage (plasma F₂-isoprostanes, HETE) [9] between MetS patients and healthy individuals. In addition, most studies focused on lipid peroxides issued from the non enzymatic peroxidation of arachidonic acid [7, 9] and 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 MetS increases the risk for the development of 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 oxidized LDL are already present in plasma from volunteers with a MetS.

Increased platelet activation occurs in type 2 diabetes [12] and MetS [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]. Moreover, *in vitro* oxidized LDL [17, 18] and LDL

from patients with type 1 [19] or type 2 diabetes [20] activate platelets *via* an increase of p38 MAPK phosphorylation and thromboxane B_2 (TxB₂) formation. To date, no data are available on the interaction between LDL from patients with MetS and platelets.

The objectives of the present study were 1) to measure different markers of oxidative stress in LDL from obese patients with MetS compared with LDL from type 2 diabetic patients or control volunteers and 2) to determine the effects of these LDL on platelets.

Methods

Patients and control volunteers.

30 men (30-75-year-old) were recruited from the Department of Endocrinology and Metabolic Diseases (Hospices Civils de Lyon). Exclusion criteria were smoking, alcohol consumption > 2 alcohol units/day, vitamin supplementation and evidence of cardiovascular complications. Patients with a metabolic syndrome were selected according to the criteria of the American Heart Association (AHA) [21] and had to fulfill three or more of the following factors : waist circumference > 102 cm, triacylglycerols (TG) > 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 should present a percentage of HbA_{1C} greater than 7% and an associated MetS. Patients treated by glitazone drugs were excluded and lipid-lowering drugs were suspended for at least 7 days before venipuncture. Control volunteers were in good health as assessed by medical history and had all above indices in the expected normal range. All volunteers gave written informed consent. The study protocol, registered in ClinicalTrials.gov as NCT00932087, was approved by the Local Ethics Committee (CCP Sud Est IV Hospices Civils de Lyon) and was in accordance with the principles of Helsinki declaration. 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 MDA in LDL.

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

Plasma sPLA₂ activity. Plasma secretory phospholipase A₂ (sPLA₂) activity was measured with a selective fluorescent substrate (Aterovax, Paris, France). Results are expressed in unit per ml of sample (U/ml), with one unit defined as the amount of sPLA₂ enzyme which catalyses the release of one nmol of product in one min. The average intra-assay coefficient of variation (CV) for individual human plasma samples was 8.46% and the average inter-assay CV was 5.24%. The minimal 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 [22]. LDL preparations were extensively dialyzed against PBS containing 1 mmol/l EDTA. LDL used for their interactions with platelets were finally dialyzed against EDTA-free PBS. The concentration of proteins was estimated using a Bradford assay [23]. Enzymatic determinations of total cholesterol and TG were processed using commercial kits (Biomérieux, Marcy l'Etoile, France). Plasma high sensitive C-reactive protein (HS CRP) was measured by nephelometry. LDL particle diameters were determined by electrophoresis of plasma in non denaturating polyacrylamide gradient gels (Lara-Spiral SA, Couternon, France).

Fatty acid compositions of lipid classes. Following the addition of internal standards and extraction with ethanol:chloroform (1:2, v/v) in the presence of BHT (50 μ mol/l), lipid classes were separated by thin-layer chromatography (TLC). Chloroform/methanol (80:8, v/v) was used to separate neutral lipids from phospholipids, and chloroform/methanol/water (63:27:4, by vol.) to separate phosphatidylcholines (PC), phosphatidylethanolamines (PE) and sphingomyelins. Neutral lipids were separated in TG and cholesteryl esters (CE) using hexane/diethylether/acetic acid (80:20:1, by vol.). The different spots were scrapped off, treated with trifluoride boron/methanol (1:1, v/v) for 90 min at 100°C. The derivatized fatty acid methyl esters and fatty dimethylacetals were extracted twice with isooctane and separated by gas chromatography using an HP 6890 gas chromatograph equipped with a SP 2380 capillary column (0.25 μ m, 30m x 0.25mm, Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID). 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 hydrolyzed with 10 mol/l KOH for 20 min at 60°C [24]. Non esterified hydroxylated fatty acids and fatty acids were first separated on an Oasis Sep-Pak cartridge column (Waters, Milford, MA) [3]. Non esterified hydroxylated fatty acids were then separated by TLC with hexane/diethylether/acetic acid (60:40:1, by vol.), extracted with methanol, separated by reversed-phase HPLC (RP-HPLC) on X Bridge C₁₈ column (3.5 μ m, 4.6 x 150 mm, Waters, Milford, MA) using a gradient solvent of acetonitrile and water (pH 3) and detected at 235 nm (for details, see a profile presented as electronic supplementary material [ESM Fig.1]).

Malondialdehyde (MDA) determination. LDL samples were mixed with thiobarbituric acid (TBA) (10 mmol/l), acetic acid and BHT (5 mmol/l) and incubated at 95°C for 60 min [25]. The TBA-MDA adducts were extracted with ethyl acetate, separated onto a Nucleosil C_{18} column (5µm, 4.6 x 250 mm) 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 RP-HPLC onto a Nucleosil C_{18} column (5µm, 4 x 150 mm) and detected fluorimetrically (excitation 295nm, emission 340nm).

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

Platelet p38 MAPK and cPLA₂ activation. Following platelet lysis, proteins (30 μg) were denatured, electrophoresed in 12% Bis-Tris gels (Bio-Rad Laboratories, Marnes-la-Coquette, France) and transferred to nitrocellulose membranes. The membranes were incubated with either 1:5000 anti-p38 MAPK or antiphospho-p38 MAPK or anti-phospho-cytosolic phospholipase A₂ (cPLA₂) polyclonal antibodies (Cell Signaling Technologies, Beverly, MA), washed, and incubated with 1:10000 goat anti-rabbit horseradish peroxidase conjugate. p38 MAPK, phospho-p38 mitogen-activated protein kinase (MAPK) and phospho-cPLA₂ were visualized by enhanced chemiluminescence, and bands were quantified by densitometry (Amersham Biosciences, Buckinghamshire,UK).

 TxB_2 measurement. Platelet TxB_2 was quantified by competitive enzyme immunoassay (Amersham Biosciences, Buckinghamshire, UK).

Platelet aggregation. Aggregation was measured in isolated platelets in a Chrono-log dualchannel aggregometer (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 subthreshold concentrations of collagen (75 ± 9 ng/ml) with continuous stirring at 1000 rpm. The subthreshold concentration of collagen was defined as the highest concentration of collagen that induced less than 15% increase in light transmission. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission 4 min after the addition of collagen.

Statistical analysis. Results are expressed as the mean \pm S.E.M. Comparisons between groups were performed using ANOVA followed by Fisher's PLSD *post hoc* test. Correlation coefficients were determined using linear regression analysis.

Results

Clinical characteristics of obese patients with MetS or diabetes and control volunteers.

The clinical and metabolic characteristics of the volunteers are summarized in Table 1. Patients with MetS fulfilled the criteria of the AHA [21]. They were either overweight or obese, had abdominal obesity, mild hypertriglyceridemia with low HDL cholesterol and were hypertensive. 2/10 MetS patients were treated against hypertension whereas 4/10 type 2 diabetic patients took anti-hypertensive medications, which could explain why the mean systolic and diastolic blood pressures were higher in the MetS group compared to the type 2 diabetic group. In addition, MetS patients showed elevated fasting insulin concentrations and a tendency to increased HOMA index, suggesting insulin resistance. Levels of HS CRP, a circulating marker of systemic inflammation, were increased similarly in MetS and type 2 diabetic patients. Type 2 diabetic patients were poorly controlled and presented a metabolic syndrome. LDL cholesterol levels were lower in patients with MetS or type 2 diabetes compared with control volunteers while plasma TG levels were higher. Healthy control volunteers had all above indices in the expected normal range.

The sPLA₂ activity was significantly increased in plasma from patients with MetS or type 2 diabetes (45.7 ± 4.3 and 51.9 ± 5.1U/ml, respectively) compared with plasma from healthy volunteers (34.4 ± 2.5 U/ml). sPLA₂ activity showed positive correlations with waist circumference ($r^2 = 0.41$, p < 0.001) and MDA concentrations in LDL (0.14, p < 0.05) and negative correlation with plasmalogen proportions ($r^2 = 0.15$, p < 0.05).

Lipid and fatty acid compositions of LDL from obese patients with MetS or diabetes compared with LDL from control volunteers.

As shown in Table 2, the concentrations of CE were lower in LDL from patients (-13% in MetS patients and -20% in type 2 diabetic patients) compared with LDL from control volunteers. By contrast, the concentrations of TG were higher in LDL from MetS (+50%) and type 2 diabetic patients (+72%). No differences in glycerophospholipid concentrations (both PC and PE) were observed between control and patients' groups. LDL particle sizes were significantly lower in patients with MetS or type 2 diabetes compared with control volunteers. The fatty acid composition of CE and PC differed between patients and control volunteers (Table 3). The proportions of PUFA decreased in CE from MetS patients while saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) proportions increased. In particular,

the proportion of linoleic acid (18:2 n-6), the main PUFA, decreased in CE and PC from MetS patients. Although palmitoleic acid (16:1 n-7) represents a minor fatty acid, its proportion increased by 70% in CE. 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 CE and PC.

Special care was taken to measure ethanolamine plasmalogens (or alkenyl,acylglycerophosphoethanolamines). They are 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 DMA (dimethyl acetal issued from the alkenyl residue) and 18:0 DMA were decreased by 22% and 23%, respectively in LDL from MetS patients compared with control LDL. In LDL from type 2 diabetic patients, 16:0, 18:0 and 18:1 DMA species were decreased by 47%, 50% and 42%, respectively. Altogether, the sum of DMA species, representative of plasmalogens, was decreased by 22% and 49%, respectively in LDL from patients with MetS or type 2 diabetes. 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 MetS or diabetes compared with LDL from control volunteers.

The concentrations of total (both free and esterified) hydroxylated fatty acids, the stable primary products of lipid peroxidation, were determined in LDL from patients and control groups. They mainly consisted of hydroxy-octadecadienoic acids (HODE) derived from linoleic acid and hydroxy-eicosatetraenoic acids (HETE) derived from arachidonic acid. 9-HODE and 13-HODE concentrations increased by 62 and 55% respectively in LDL from

MetS patients and increased 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% respectively in LDL from MetS and type 2 diabetic patients (Fig. 1c). Among HETE, 8-, 11-, 12- and 15-HETE were detected in LDL from each volunteer. There were no significant differences in the concentrations of HETE between MetS patients and control volunteers (data not shown). Only 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). The concentrations of MDA, issued from the degradation of fatty acid hydroperoxides, were significantly increased by 2-fold in patients with MetS or type 2 diabetes (Fig. 1d). The waist circumference was mildly correlated with TG and MDA levels in LDL (r² = 0.13 and 0.25, respectively, *p* < 0.05) (see [ESM] Fig.2 a and b).

The concentrations of vitamin E, the main liposoluble antioxidant, were determined in LDL. α -tocopherol concentrations did not differ between patients with MetS or type 2 diabetes (6.7 \pm 0. 5 and 6.0 \pm 0. 3 nmol/mg protein) *vs* control volunteers (5.9 \pm 0. 3 nmol/mg protein). γ tocopherol, representing 10% of α -tocopherol, was increased in LDL from MetS patients (0.9 \pm 0.1 nmol/mg protein) compared with control LDL (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).

Effects of LDL isolated from obese patients with MetS or diabetes, or control volunteers on platelet arachidonic acid signaling cascade.

To determine the effects of LDL on platelet arachidonic acid cascade, platelets from healthy donors were incubated in the absence or presence of LDL from patients or control volunteers (1 mg/ml) for 2 hours at 37°C. Phosphorylated amounts of p38 MAPK and cPLA₂, key enzymes involved in the release of arachidonic acid from membrane phospholipids, as well as concentrations of TxB_2 , the stable catabolite of the pro-aggregatory arachidonic acid

metabolite thromboxane A₂ (TxA₂), were determined. As shown in Fig. 2, the addition of LDL from control volunteers to platelets had no effects on p38 MAPK and cPLA₂ phosphorylations, compared with platelets alone. By contrast, the addition of LDL from patients with MetS or type 2 diabetes significantly increased p38 MAPK phosphorylation, by 21% and 55% respectively, and cPLA₂ phosphorylation by 23 and 24%. Compared with control platelets, the incubation of platelets with LDL from patients with MetS or type 2 diabetes significantly increased p38 MAPK phosphorylation, by 21% and 55% respectively, and cPLA₂ phosphorylation by 23 and 24%. Compared with control platelets, the incubation of platelets with LDL from patients with MetS or type 2 diabetes resulted in a 2-fold increased basal concentration of TxB₂ whereas LDL from control volunteers had no effects (Fig. 3). Platelet TxB₂ was negatively correlated with plasmalogen proportions (r² = 0.44) and positively with HODE and MDA concentrations in LDL (r² = 0.24 and 0.31, respectively, p < 0.01) (see [ESM] Fig.2 c and d).

Effect of LDL isolated from obese patients with MetS or diabetes, or control volunteers, on platelet aggregation.

To determine the effect of LDL on platelet aggregation, platelets were preincubated 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, there was no effect of LDL from control volunteers on collagen-induced platelet aggregation, compared with platelets incubated with buffer. By contrast, pre-incubation of platelets with LDL from MetS or type 2 diabetic patients resulted in a stimulation of platelet aggregation in response to subthreshold concentrations of collagen (+197% and +251%, respectively). Platelet aggregation was also potentiated by lower concentrations (50 μ g/ml) of LDL from type 2 diabetic patients (data not shown).

Discussion

The evidence for increased oxidative stress in MetS is still a matter of debate. In the present study, we provide several lines of evidence for increased oxidative stress in LDL from obese MetS patients, as assessed by different complementary indices of oxidative stress and 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 PC and CE were found in LDL from MetS 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 MetS patients, which is in agreement with a recent study showing no differences in the levels of oxidation products of arachidonic acid (total HETE and F₂-isoprostanes) in individuals with features of MetS [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 there was no dietary record in participants. 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 an oxidative stress in LDL from MetS patients was confirmed with the decreased concentrations of plasmalogens in LDL from MetS 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 PE in red blood cell membranes from hyperlipidemic patients [29]. In addition, we found that MDA, a commonly used overall marker of lipid peroxidation, described to correlate with total isoprostanes in plasma and tissues [30, 31], was increased to a similar extent in LDL from MetS and type 2 diabetic patients [32]. Although isoprostanes are recognized 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 [9, 32] or unchanged [7] in patients with MetS. Altogether, the increase of several complementary indices of oxidative stress and especially of oxidized products of linoleic acid in LDL from MetS patients gives support to the hypothesis that the MetS is associated with higher levels of circulating oxidized LDL [4-6]. In addition, compared with control LDL, LDL from patients with MetS showed higher TG and lower CE levels as well as decreased particle size, suggesting a higher percentage of smaller and denser LDL particles. Therefore, our data corroborate that the MetS 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 α -tocopherol in patients with MetS or type 2 diabetes and higher concentrations of γ -tocopherol in MetS patients. There have been discordant reports about vitamin E levels in patients with MetS or type 2 diabetes, showing either lower vitamin E concentrations in volunteers with a MetS that also consumed fewer fruits and vegetables [36] or no difference among volunteers with MetS or type 2 diabetes [37, 38]. Only one study to our knowledge reported slightly higher serum γ -tocopherol levels [38] and no changes of α -tocopherol among subjects with MetS. 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 MetS patients as in LDL from type 2 diabetic patients, suggesting that factors associated with the MetS rather than poor glycemic 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 MDA in LDL, suggesting an association between an excess of visceral fat and biochemical parameters associated with oxidative stress in LDL. It

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is also likely that obesity tightly associated with MetS may represent a major factor in the oxidative stress evidenced in LDL from obese MetS patients.

Besides, our results show that the activity of $sPLA_2$ is correlated with plasmalogens and MDA in LDL, suggesting that this enzyme catalyzes the hydrolysis of oxidized phospholipids in LDL [40]. The correlation between the activity of $sPLA_2$ and the waist circumference is in line with the association of $sPLA_2$ with MetS [41].

Regarding the functionality of LDL from patients with MetS, we show for the first time that LDL from MetS 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 cytosolic phospholipase A₂ [42], the key enzyme involved in the release of arachidonic acid from membrane phospholipids [43]. It resulted in an increased formation of TxA₂, as assessed by the increased concentration of the stable catabolite, TxB₂. 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 have no activating effects. Regarding the molecules in patients' LDL responsible for platelet activation, our data suggest the implication of lipid peroxides. LDL from patients with either obese MetS or type 2 diabetic patients showed similar increased levels of lipid peroxides and activated platelets to the same extent. In addition, correlation analyses show that platelet TxB₂ concentration is related to the concentrations of HODE and MDA in LDL, suggesting that lipid peroxides could underlie the activating effects of LDL on platelets and that glycation is not necessarily required. It is also important to take into account obesity in future studies to identify the mechanism by which oxidized LDL activate platelets.

Besides, our results obtained *ex vivo* strengthen the implication of fatty acid peroxides in platelet activation as previously demonstrated in *in vitro* studies [15, 44]. We have indeed shown that fatty acid hydroperoxides, precursors of HETE and HODE, potentiated platelet aggregation and activated platelets via the arachidonic acid signaling cascade [16]. More recently, the Podrez group showed that oxidized choline glycerophospholipids induce platelet aggregation *via* CD36 [45].

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

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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 and PM revised the manuscript. All authors approved the final version of the manuscript.

Duality of interest

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

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 Table 1. Clinical and metabolic characteristics of control volunteers and obese patients with

 MetS or type 2 diabetes.

	Control	Metabolic Syndrome	Type 2 diabetes
n (sex)	10 (M)	10 (M)	10 (M)
Age (years)	50 ± 3	$58 \pm 2*$	57 ± 3
BMI (kg/m^2)	24.3 ± 0.7	$30.7 \pm 1.4 *$	$30.8 \pm 1.1 ^{\ast\ast}$
Waist circumference (cm)	92 ± 2	$111 \pm 3^{**}$	$116 \pm 7^{**}$
Fasting glucose (mmol/l)	5.5 ± 0.2	5.6 ± 0.1	$9.0 \pm 1.0^{**}$ †
Fasting insulin (pmol/l)	41.1 ± 6.9	$68.4 \pm 12.7*$	45.4 ± 12
HOMA index	1.54 ± 0.29	2.53 ± 0.52	2.75 ± 0.92
CRP (mg/l)	0.54 ± 0.12	$3.61\pm0.82^{\ast}$	$4.82 \pm 1.52 **$
HbA _{1c} (%)	5.4 ± 0.1	5.7 ± 0.1	10.4 ± 0.9** †
HbA _{1c} (mmol/mol)	35.5 ± 0.6	38.5 ± 1.4	90.2 ± 9.8** †
Systolic blood pressure (mmHg)	121 ± 3	$144 \pm 3^{**}$	$132 \pm 6*$ †
Diastolic blood pressure (mmHg)	78 ± 4	$88\pm5^*$	77 ± 3 †
Total cholesterol (mmol/l)	6.0 ± 0.4	5.2 ± 0.3	$4.8\pm0.4*$
HDL-cholesterol (mmol/l)	1.4 ± 0.1	$1.1 \pm 0.1^{**}$	$0.9\pm0.1^{\ast\ast}$
Total cholesterol / HDL-cholesterol	4.5 ± 0.3	4.9 ± 0.3	$5.3 \pm 0.3*$
LDL-cholesterol (mmol/l)	4.1 ± 0.4	3.2 ± 0.2	$2.7 \pm 0.4^{**}$
VLDL-cholesterol (mmol/l)	0.5 ± 0.1	$0.8\pm0.1^{**}$	$1.0 \pm 0.1^{**}$ †
Triacylglycerols (mmol/l)	1.3 ± 0.1	$2.2 \pm 0.3^{**}$	$2.6\pm0.2^{\ast\ast}$
Antidiabetic treatment (%)	0	0	80
Antihypertensive treatment (%)	0	20	40
Hypolipidemic treatment (%)	0	10	20

Data are means \pm SEM (n= 8 to 10 subjects per group). * p < 0.05 and ** p < 0.01 vs LDL from control volunteers. † p < 0.05 vs LDL from MetS patients. p values were obtained by ANOVA followed by Fisher's PLSD *post hoc* test.

M, male; HOMA, HOmeostasis Model Assessment index calculated (fasting insulin x fasting glucose)/22.5; Hypolipidemic treatment was interrupted seven days before inclusion.

Table 2 Lipid classes and particle size of LDL from control volunteers and obese patientswith MetS or type 2 diabetes.

	Control	Metabolic Syndrome	Type 2 diabetes
Cholesteryl esters (nmol/mg protein)	2459 ± 118	2132 ± 61**	$1975 \pm 88^{**}$
Phosphatidylcholines (nmol/mg protein)	521 ± 17	477 ± 18	479 ± 21
Phosphatidylethanolamines (nmol/mg prot.)	17 ± 2	15 ± 1	18 ± 1
Triacylglycerols (nmol/mg protein)	140 ± 11	$210\pm19^{*}$	$241 \pm 37^{**}$
LDL particle size (nm)	25.1 ± 0.2	24.4 ± 0.2 *	24.4 ± 0.1 *

Data are means \pm SEM (n=8 to 10 subjects per group).

* p < 0.05 and ** p < 0.01 vs LDL from control volunteers. p values were obtained by

ANOVA followed by Fisher's PLSD post hoc test.

Fatty acid		Cholesteryl esters		I	Phosphatidylcholines	S
(mol%)	Control	Metabolic syndrome	Type 2 diabetes	Control	Metabolic syndrome	Type 2 diabetes
16:0	12.4 ± 0.4	$13.5\pm0.4*$	$13.7 \pm 0.3*$	33.5 ± 0.7	34.7 ± 0.4	34.3 ± 0.5
16:1n-7	2.5 ± 0.3	$4.2 \pm 0.3^{**}$	2.8 ± 0.3	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
16:1n-9	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0	0	0
18:0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	16.0 ± 0.5	15.8 ± 0.7	15.3 ± 0.7
18:1n-7	1.0 ± 0.2	0.7 ± 0.2	1.0 ± 0.2	1.3 ± 0.3	1.0 ± 0.2	1.2 ± 0.3
18:1n-9	20.6 ± 0.7	21.7 ± 0.7	22.1 ± 0.7	10.9 ± 0.5	10.9 ± 0.5	11.6 ± 0.3
18:2n-6	52.0 ± 1.2	$46.7 \pm 1.4^{**}$	$46.7 \pm 1.1^{**}$	21.8 ± 1.0	$19.2\pm0.7*$	$19.6\pm0.7*$
18:3n-3	0.4 ± 0.1	0.7 ± 0.0	0.5 ± 0.1	0	0	0
18:3n-6	0.8 ± 0.1	1.1 ± 0.1	0.8 ± 0.2	0	0	0
20:3n-6	0.8 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	3.1 ± 0.2	$3.7 \pm 0.3*$	3.3 ± 0.2
20:4n-6	6.2 ± 0.4	7.0 ± 0.6	$8.3\pm0.5^{**}$	7.2 ± 0.6	8.8 ± 0.7	$9.3 \pm 0.5^{*}$
20:5n-3	1.3 ± 0.2	1.3 ± 0.2	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.1	0.9 ± 0.2
22:5n-3	0	0	0	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
22:6n-3	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	3.2 ± 0.4	2.6 ± 0.3	2.9 ± 0.2
SFA	13.5 ± 0.5	$14.7\pm0.4^{*}$	$14.7 \pm 0.3^{*}$	50.0 ± 0.9	50.9 ± 0.7	49.9 ± 0.6
MUFA	24.4 ± 0.8	$27.1 \pm 1.0^{*}$	26.4 ± 0.5	12.9 ± 0.6	13.0 ± 0.6	13.4 ± 0.4
PUFA	62.1 ± 1.1	$58.3 \pm 1.2^{*}$	$59.0 \pm 0.7*$	37.1 ± 0.9	36.1 ± 0.8	36.7 ± 0.5

Table 3 Main fatty acid compositions of cholesteryl esters and phosphatidylcholines in LDLfrom control volunteers and obese patients with MetS or type 2 diabetes.

Data are means \pm SEM (n= 8 to 9 subjects per group). * p < 0.05 and ** p < 0.01 vs LDL from control volunteers. † p < 0.05 vs LDL from MetS patients. p values were obtained by ANOVA followed by Fisher's PLSD post hoc test. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Figure legends

Fig. 1 Indices of lipid peroxidation in LDL from control volunteers, obese patients with MetS or type 2 diabetes: fatty dimethylacetals (DMA) derived from PE plasmalogens (a), 9- and 13-HODE (b), HODE/18:2n-6 ratio (c) and MDA (d). Data are means \pm SEM (n= 7 to 10 subjects per group). LDL from MetS patients *vs* LDL from control volunteers : significantly different (*p* < 0.05) for 16:0 DMA, 18:0 DMA, DMA sum, 9-HODE, 13-HODE, HODE/18:2 n-6 and (*p* < 0.01) for MDA. LDL from type 2 diabetic patients *vs* LDL from control volunteers : significantly different (*p* < 0.01) for MDA. LDL from type 2 diabetic patients *vs* LDL from control volunteers : significantly different (*p* < 0.01) for 16:0 DMA, 18:0 DMA, 18:0 DMA, 18:0 DMA, 18:1 n-9 DMA, DMA sum and MDA ; significantly different (*p* < 0.05) for 9-HODE, 13-HODE and HODE/18:2 n-6. LDL from type 2 diabetic patients *vs* LDL from MetS patients : significantly different (*p* < 0.05) for 16:0 DMA, 18:0 DMA, 18:1 n-9 DMA, nOMA sum and MDA ; not type 2 diabetic patients *vs* LDL from MetS patients : significantly different (*p* < 0.05) for 16:0 DMA, 18:0 DMA, 18:1 n-9 DMA and DMA sum. *p* values were obtained by ANOVA followed by Fisher's PLSD *post hoc* test. HODE, hydroxyoctadecadienoic acid; MDA, malondialdehyde; DMA, dimethylacetals; PE, phosphatidylethanolamine.

Fig. 2 Effects of LDL from control volunteers, obese patients with MetS or type 2 diabetes on the phosphorylation of p38 MAPK (a) and cPLA₂ (b) in platelets. Platelets from blood donors were incubated for 2h at 37°C in the absence or presence of LDL (1 mg protein/ml) from either control volunteers, MetS patients or type 2 diabetic patients. Representative immunoblots and histograms of the normalized amount of phosphorylation of p38 MAPK and cPLA₂ are shown. Data are means \pm SEM (7 to 8 subjects per group). Platelets + LDL from MetS patients *vs* platelets incubated with buffer : significantly different (*p* < 0.05) for phospho-p38 MAPK and phospho-cPLA₂. Platelets + LDL from type 2 diabetic patients *vs* platelets incubated with buffer : significantly different (*p* < 0.01) and phospho-cPLA₂ (*p* < 0.05). Platelets + LDL from type 2 diabetic patients *vs* platelets + LDL from MetS patients : significantly different for phospho-p38 MAPK (p < 0.05). p values were obtained by ANOVA followed by Fisher's PLSD *post hoc* test. MAPK, mitogen activated protein kinase; cPLA₂, cytosolic phospholipase A₂.

Fig. 3 Effects of LDL from control volunteers, obese patients with MetS or type 2 diabetes on thromboxane B₂ concentrations in unstimulated platelets. Platelets from blood donors were incubated for 2h at 37°C in the absence or presence of LDL (1 mg protein/ml) from either control subjects, MetS patients or type 2 diabetic patients. Data, expressed as percent of control, are means \pm SEM (10 subjects per group). Thromboxane B₂ concentrations in control unstimulated platelets were 187 \pm 37 pmol/10⁹ platelets. Platelets + LDL from type 2 diabetic patients *vs* platelets incubated with buffer, and Platelets + LDL from MetS patients *vs* platelets incubated with buffer : significantly different (*p* < 0.05). *p* values were obtained by ANOVA followed by Fisher's PLSD post hoc test.

Fig. 4 Effects of LDL from control subjects, obese patients with MetS or type 2 diabetes on the aggregation of platelets induced by collagen. Platelets from blood donors were preincubated for 5 min at 37°C in the absence or presence of LDL (0.1 mg protein/ml) from either control subjects, MetS patients or type 2 diabetic patients and stimulated with subthreshold concentrations of collagen (75 \pm 9 ng/mL). Data, expressed as percent of aggregation, are means \pm SEM of 5 subjects per group. Platelets + LDL from MetS patients *vs* platelets incubated with buffer : significantly different (*p* < 0.05). Platelets + LDL from type 2 diabetic patients *vs* platelets incubated with buffer : significantly different (*p* < 0.01). *p* values were obtained by ANOVA followed by Fisher's PLSD *post hoc* test.