# Glycoxidized HDL, HDL enriched with oxidized phospholipids and HDL from diabetic patients inhibit platelet function

Quang Huy Lê, Meddy El Alaoui, Evelyne Véricel, Bérénice Ségrestin, Laurent Soulère, Michel Guichardant, Michel Lagarde, Philippe Moulin, Catherine Calzada

# Glycoxidized HDL, HDL enriched with oxidized phospholipids and HDL from diabetic patients inhibit platelet function

Quang Huy Lê[1], Meddy El Alaoui[2], Evelyne Véricel[1], Bérénice Ségrestin[3], Laurent Soulère[2], Michel Guichardant[1], Michel Lagarde[1], Philippe Moulin[1,3] and Catherine Calzada[1]

[1] Université de Lyon, Institut National de la Santé et de la Recherche Médicale (Inserm) UMR 1060, Laboratoire CarMeN, UMR 1397 Institut National de la Recherche Agronomique (Inra), Institut National des Sciences Appliquées de Lyon (INSA-Lyon), IMBL, 69621 Villeurbanne, France;

[2] Laboratoire de chimie organique et bioorganique, Institut de Chimie et Biochimie Moléculaires et Supramoléculaires (ICBMS), UMR 5246 Centre National de la Recherche Scientifique (CNRS), INSA-Lyon, Villeurbanne, France;

[3] Fédération d'Endocrinologie, Hospices Civils de Lyon, 69677 Lyon Bron, France.

**Abbreviated title:** Anti-aggregatory effects of glycoxidized HDL in type 2 diabetes.

**Keywords:** High density lipoproteins; platelets; hydroxy-octadecadienoic acid; oxidized phospholipids; diabetes.

Word count : 3567 words

1 Table and 5 figures

**Corresponding author:**

Dr. Catherine Calzada
Inserm UMR.1060 / Université de Lyon
« Cardiovasculaire, Métabolisme, diabétologie, et Nutrition » (CarMeN)
INSA-Lyon, IMBL, Bât. Louis Pasteur, 20 avenue Albert Einstein
69621 Villeurbanne, France
Tel: +33472438479 Fax: +33472438524
catherine.calzada@insa-lyon.fr

**Disclosure statement:** The authors have nothing to disclose.

**ABSTRACT**

**Context:** High-density lipoproteins (HDL) possess atheroprotective properties including anti-thrombotic and antioxidant effects. Very few studies relate to the functional effects of oxidized HDL on platelets in type 2 diabetes (T2D).

**Objective:** The objective of our study was to investigate the effects of *in vitro* glycoxidized HDL, and HDL from T2D patients on platelet aggregation and arachidonic acid signaling cascade. At the same time, the contents of hydroxylated fatty acids were assessed in HDL.

**Results:** Compared to control HDL, *in vitro* glycoxidized HDL had decreased proportions of linoleic (LA) and arachidonic (AA) acids in phospholipids and cholesteryl esters, and increased concentrations of hydroxy-octadecadienoic acids (9-HODE and 13-HODE) and 15-hydroxy-eicosatetraenoic acid (15-HETE), derived from LA and AA respectively, especially hydroxy derivatives esterified in phospholipids. Glycoxidized HDL dose-dependently decreased collagen-induced platelet aggregation by binding to SR-BI. Glycoxidized HDL prevented collagen-induced increased phosphorylation of platelet p38 MAPK and cytosolic phospholipase $A_2$, as well as intracellular calcium mobilization. HDL enriched with oxidized phospholipids, namely PC(16:0/13-HODE) dose-dependently inhibited platelet aggregation. Increased concentrations of 9-HODE, 13-HODE and 15-HETE in phospholipids (2.1, 2.1 and 2.4-fold increase respectively) were found in HDL from patients with T2D, and these HDL also inhibited platelet aggregation *via* SR-BI.

**Conclusions:** Altogether, our results indicate that *in vitro* glycoxidized HDL as well as HDL from T2D patients inhibit platelet aggregation, and suggest that oxidized LA-containing phospholipids may contribute to the anti-aggregatory effects of glycoxidized HDL and HDL from T2D patients.

**Abbreviations:**

AA, arachidonic acid; BHT, butylated hydroxytoluene; CE, cholesteryl esters; $cPLA_2$, cytosolic phospholipase $A_2$; DHA, docosahexaenoic acid; DMA, dimethylacetal; EPA, eicosapentaenoic acid; HDL, high-density lipoproteins; HEDE, hydroxy-eicosadienoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; LA, linoleic acid; LDL, low-density lipoproteins; MDA, malondialdehyde; $NaBH_4$, sodium borohydride; PC, phosphatidylcholine; PL, phospholipids; PRP, platelet-rich plasma; PUFA, polyunsaturated fatty acids; SR-BI, scavenger receptor BI; TAG, triacylglycerols; TBA, thiobarbituric acid; TLC, thin-layer chromatography; $TxA_2$, thromboxane $A_2$, T2D, type 2 diabetes.

## INTRODUCTION

There is growing evidence that HDL composition determines its functional properties besides the levels of HDL cholesterol (1). HDL are highly heterogeneous particles consisting of two hundred individual molecular lipid species and a hundred of proteins (2,3). Amongst lipids, the presence of oxidizable polyunsaturated fatty acids (PUFAs) substrates renders these particles sensitive to oxidative attack. In particular, HDL are the major carriers of primary and terminal end-products of non-enzymatic lipid peroxidation such as lipid hydroperoxides (4) and isoprostanes (5) in human blood plasma. HDL also remove seeding molecules from LDL such as hydroperoxy-octadecadienoic and hydroperoxy-eicosatetraenoic acids (6). Oxidatively modified HDL have been detected *in vivo* (7) in the intima of atherosclerotic plaques in human abdominal aortae (8) and in patients with type 2 diabetes (T2D) (9), however the molecular mechanisms involved were not investigated compared to the extensive studies on oxidized LDL in atherogenesis. Functional properties of oxidized HDL in T2D, a disease associated with increased risk for atherothrombosis and oxidative stress, have been investigated but gave contradictory results. Most studies showed that HDL might lose their protective properties in T2D (10,11) while other studies provided evidence for beneficial effects such as increased cholesterol efflux from macrophages in patients with T2D (12,13). Studies on the effects of oxidatively modified HDL on platelet functions are few and contradictory, reporting either a stimulation (14) or inhibition (15) of platelet aggregation by *in vitro* oxidized HDL. To clarify this issue in the frame of T2D, we undertook two approaches. On one hand, HDL from control subjects were modified *in vitro* by glycoxidation in order to mimic changes occurring in HDL particles of T2D patients, and their effects on platelet activation were determined and compared to those induced by unmodified HDL. On the other hand, HDL from T2D patients were compared to HDL from healthy control subjects as far as platelet aggregation was concerned. We hypothesized that some elective lipid peroxides in HDL phospholipids (PL) might exert anti-aggregating properties.

## SUBJECTS AND METHODS

### *Study subjects*

Eight T2D patients (5 men and 3 women, aged 66 ± 3.5 years) and eight healthy subjects (6 men and 2 women, aged 25 ± 2.9 years) were included. The patients had poorly controlled diabetes (fasting glycemia : 8.7 ± 1.4 mmol/L ; glycated hemoglobin $HbA_{1C}$ : 8.3 ± 0.6%, 67 mmol/mol). They had mild hypertriglyceridemia (triglycerides: 1.9 ± 0.4 mmol/L), normal LDL-cholesterol (2.4 ± 0.2 mmol/L) and low HDL-cholesterol (1.1 ± 0.1 mmol/L). The patients were recruited at the Department of Endocrinology and Metabolic Diseases, Cardiovascular Hospital, Lyon Bron. The protocol was approved by the Local Ethics Committee (CPP Sud-Est IV, Hospices Civils de Lyon) and the study was conducted in accordance with the principles of the Helsinki declaration. Written informed consent was obtained from each of the participants.

### *Isolation of HDL by ultracentrifugation*

Blood was collected on EDTA and HDL were immediately isolated from plasma by potassium bromide stepwise ultracentrifugation (16). HDL were extensively dialyzed against PBS (pH 7.35) in the presence of 1 mmol/L EDTA. The concentration of proteins was estimated using a Lowry assay (17).

### *Preparation of HDL modified by glycoxidation*

Glycoxidized HDL consisted of HDL incubated with 50 mmol/L glucose for 5 days at 37°C, dialyzed in PBS to remove excess glucose, and treated with 5 µmol/L $CuCl_2$ for 1 day at 37°C. Control HDL were prepared by incubating native HDL in the presence of butylated hydroxytoluene (BHT) (5µmol/L) and EDTA (1mmol/L) for 6 days at 37°C. All HDL were finally dialyzed against EDTA-free PBS just before their interaction with platelets.

### *Platelet isolation*

Venous blood was collected on citrate-phosphate-dextrose (19.6 mmol/L citric acid, 89.4 mmol/L sodium citrate, 16.1 mmol/L $NaH_2PO_4$, 128.7 mmol/L dextrose, pH 5.6) from healthy volunteers who

had not ingested any aspirin or anti-inflammatory drugs in the previous ten days. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 200g for 15 min at 20°C. Platelets were isolated as previously described (18).

***Synthesis of 1-palmitoyl, 2-(13-hydroxy-octadecadienoyl)-sn-glycero-3-phosphocholine***

1-palmitoyl,2-(13(S)-hydroperoxy-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/13(S)-HpODE) was enzymatically synthesized from 1-palmitoyl,2-linoleoyl-sn-glycero-3-phosphocholine (PLPC or PC(16:0/18:2), Cayman Chemicals, Ann Arbor, MI, USA) following incubation of PC(16:0/18:2) with soybean 15-lipoxygenase (type V) in the presence of deoxycholate for 30 min under a continuous flux of oxygen (19). Phosphatidylcholine hydroperoxide formed was reduced by sodium borohydride ($NaBH_4$) into phosphatidylcholine hydroxide. 1-palmitoyl,2-(13(S)-hydroxy-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/13-HODE)) concentration was determined spectrophotometrically by UV absorbance at 235 nm and its purity was checked by HPLC.

***Synthesis of 1-palmitoyl,2-(15(S)-hydroxy-eicosadienoyl)-sn-glycero-3-phosphocholine***

Firstly, 1-palmitoyl,2-eicosadienoyl-sn-glycero-3-phosphocholine PC(16:0/20:2n-6) was chemically synthesized. To a solution of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (0.2 mmol), eicosa-11,14-dienoic acid (20:2n-6) (0.08 mmol) was added in anhydrous chloroform (1 mL). A solution of freshly recrystallized 4-Pyrrolidinopyridine (PPyr) (20) (0.09 mmol) and dicyclohexylcarbodiimide (DCC) (0.09 mmol) in 1 mL of chloroform was then added drop wise (21). After 40 h, the reaction mixture was concentrated and the product was then purified by flash chromatography on silica gel (chloroform/methanol/$H_2O$, 65:35:4, by vol.). Fractions containing the product were further purified by ion exchange chromatography using Amberlyst resin (chloroform/methanol/$H_2O$, 65:35:4, by vol.) to give PC(16:0/20:2). 1-palmitoyl,2-(15(S)-hydroperoxy-eicosadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/15(S)-HpEDE) was then enzymatically synthesized from PC(16:0/20:2) as described above and reduced into PC(16:0/15(S)-HEDE), and used as an internal standard in relevant analyses.

***In vitro enrichment of HDL with oxidized phospholipids***

To prepare HDL enriched with oxidized PL, native HDL were incubated with dried PC(16:0/13-HODE) for 24 hours at 37°C in a shaking bath. Unbound PL were removed by dialysis in PBS and using PD-10 desalting columns. Similar procedure was applied to prepare HDL enriched with PC(16:0/18:2).

***Characterization of HDL***

*Fatty acid compositions of lipid classes*

Fatty acid methyl esters and fatty dimethylacetals were analyzed by gas chromatography as detailed in the Supplemental Methods.

*Quantification of monohydroxylated fatty acids*

Hydroxylated fatty acids were separated and quantified by reverse-phase HPLC according to the amount of appropriate internal standards, PC(16:0/15-HEDE) and 15-HEDE. For the detailed procedure, please refer to Supplemental Methods.

*Stereochemical analysis of hydroxylated fatty acids*

Optical isomers of HODE and HETE were separated by chiral phase HPLC as detailed in the Supplemental Methods.

*Malondialdehyde (MDA) determination*

Overall lipid peroxidation was assessed by quantitation of thiobarbituric acid (TBA)-MDA adducts by reverse-phase HPLC with fluorimetric detection. For details, please refer to Supplemental Methods.

*Vitamin E determination*

Tocopherol isomers were separated by reverse-phase HPLC and measured fluorimetrically containing tocol as an internal standard. For details, please refer to Supplemental Methods.

***Platelet aggregation***

Aggregation was measured in isolated platelets in a Chrono-log dual-channel aggregometer (Coulter, Margency, France) according to the method of Born (22). Platelet suspensions were pre-incubated for 5 min at 37°C in the presence or absence of different preparations of HDL and then stimulated with threshold concentrations of collagen (Nycomed, Linz, Austria) with continuous stirring at 1000 rpm. The threshold concentration of collagen was defined as the concentration of collagen that induced approximately a 60% 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.

***Platelet p38 MAPK and cytosolic phospholipase $A_2$ activation***

Chemiluminescent Western blotting detection of p38 MAPK, phospho-p38 MAPK and phospho-$cPLA_2$ was performed as described in the Supplemental Methods.

***Determination of intracellular $Ca^{2+}$ concentrations***

Intracellular calcium concentrations were measured fluorimetrically in Fura-2-loaded platelets as described in the Supplemental Methods.

***Statistical analysis***

Results are expressed as the means ± SEM. Comparisons between groups were performed using paired Student t-test.

**RESULTS**

**Characterization of glycoxidized HDL compared to control HDL**

*Fatty acid composition of phospholipids and cholesteryl esters (CE)*

Following *in vitro* glycoxidation, proportions of the main PUFAs, linoleic (LA, 18:2n-6) and arachidonic (AA, 20:4n-6) acids, significantly decreased in PL and CE of glycoxidized HDL compared to control HDL (Table). In PL from glycoxidized HDL, the proportions of LA, AA,

eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids decreased by 54%, 91%, 56% and 91% respectively. The proportions of 16:0 dimethylacetals (DMA), 18:0 DMA and 18:1 n-9 DMA issued from the alkenyl residue of alkenyl,acyl-glycero-phosphoethanolamine (ethanolamine plasmalogens) decreased in PL from glycoxidized HDL compared to those from control HDL. By contrast, proportions of palmitic (16:0), stearic (18:0) and oleic (18:1n-9) acids increased in modified HDL. In CE, glycoxidation of HDL also led to decreased proportions of LA, AA and EPA, by 53%, 92% and 86% respectively, and increased proportions of palmitic, stearic and oleic acids.

*Vitamin E concentration*

The concentrations of alpha-tocopherol strongly decreased in glycoxidized HDL compared to control HDL (0 in glycoxidized HDL vs. 2.04 ± 0.28 nmol/mg HDL protein in control HDL, n=5, $P<0.001$). Gamma-tocopherol concentrations decreased by 75% in glycoxidized HDL (0.08 ± 0.01 in glycoxidized HDL vs. 0.32 ± 0.02 nmol/mg HDL protein in control HDL, n=5, $P<0.001$).

*MDA concentration*

The concentrations of MDA, a marker of overall lipid peroxidation, were more than 10-fold higher in glycoxidized HDL compared to control HDL (1.93 ± 0.32 nmol/mg protein in glycoxidized HDL vs. 0.18 ± 0.13 nmol/mg protein in control HDL, n=4, $P<0.01$).

*Concentrations of hydroxylated fatty acids in lipid classes*

The concentrations of the stable primary products of PUFA peroxidation, 13-HODE and 9-HODE, derived from LA, and 15-HETE, derived from AA, were assessed in HDL. In control HDL, most hydroxylated fatty acids were present in CE, followed by PL and TAG, while most of them were present in PL of glycoxidized HDL (Figure 1). HODE concentrations increased strongly in PL of glycoxidized HDL compared to those of unmodified HDL (Figures 1A and 1B). 13-HODE concentrations tended to increase (1.8-fold) and 9-HODE concentrations significantly increased in CE from glycoxidized HDL. In TAG, 13-HODE concentrations increased in glycoxidized HDL (2.7-fold) and 9-HODE concentrations tended to increase (1.8-fold) compared to control HDL. 15-HETE

concentration increased in PL from glycoxidized HDL (8.5-fold) while 15-HETE concentration tended to increase in CE and TAG from glycoxidized HDL compared to control HDL (Figure 1C).

**Effects of control and glycoxidized HDL on platelet activation**

Compared to platelets incubated with collagen, pre-incubation of platelets with glycoxidized HDL for 5 min at 37°C resulted in a dose-dependent inhibition of collagen-induced platelet aggregation with near complete inhibition at 100 µg/mL (Figure 1D). Control HDL had no effect at 25 µg/mL but significantly inhibited collagen-induced platelet aggregation at concentrations greater than or equal to 50 µg/mL. Because scavenger receptor SR-BI binds modified HDL and is expressed in platelets (23), its involvement in the mechanism of action of HDL on these cells was sought. Pre-incubation of platelets with anti-SRBI blocking antibody for 5 min at 37°C fully prevented the inhibitory effects of glycoxidized HDL on collagen-induced platelet aggregation (Figure 1D). Pre-incubation of platelets with anti-SRBI also prevented the inhibition of collagen-induced platelet aggregation in presence of control HDL (aggregation rate: 65 % in platelets incubated with anti-SRBI antibody and 100 µg/ml control HDL vs. 12% in platelets incubated with 100 µg/ml control HDL). Anti-SRBI blocking antibody had no effect on collagen-induced platelet aggregation, and pre-incubation with non-immune isotype control antibody did not reduce the inhibitory effect of glycoxidized HDL on platelet aggregation (data not shown).

To determine the effects of glycoxidized HDL on key enzymes involved in the release of AA from membrane PL, phosphorylation of p38 MAPK and $cPLA_2$ was determined in platelet suspensions incubated in the absence or presence of HDL for 5 min and further stimulated with collagen for 4 min. As shown in Figures 2A and 2B, addition of collagen to platelets resulted in increased amounts of phosphorylated p38 MAPK and $cPLA_2$. Pre-incubation of collagen-stimulated platelets with glycoxidized HDL fully prevented the collagen-induced increased phosphorylation of both enzymes.

Since $Ca^{2+}$ is a key second messenger downstream of most signaling pathways and is essential for the translocation of $cPLA_2$ to membranes, the effects of control and glycoxidized HDL on the kinetics of collagen-induced $Ca^{2+}$ release were determined (Figure 2C). Addition of collagen to Fura2-AM loaded platelets resulted in a rapid and transient increase in intracellular $Ca^{2+}$ levels. Glycoxidized HDL

inhibited collagen-induced intracellular $Ca^{2+}$ increase in platelets by 47% while control HDL decreased it by 32%. Pre-incubation of platelets with anti-SRBI blocking antibody for 5 min at 37°C alleviated the inhibitory effects of glycoxidized HDL on phosphorylation of p38 MAPK and $cPLA_2$, and $Ca^{2+}$ mobilization (data not shown).

**Effects of HDL enriched with 1-palmitoyl,2-(13-hydroxy-octadecadienoyl)-sn-glycero-3-phosphocholine on platelet aggregation**

To determine the role of oxidized LA-containing PL for anti-aggregating effects of HDL, HDL were pre-loaded with two different concentrations of PC(16:0/13-HODE) prepared by 15-lipoxygenation of LA esterified to phosphatidylcholine, followed by chemical reduction with $NaBH_4$ (Figure 3A). As expected, higher concentrations of 13-HODE, close to those found in glycoxidized HDL, were present in enriched HDL compared to control HDL, while concentrations of 9-HODE were similar in control and enriched HDL, confirming the absence of non enzymatic lipid peroxidation during the preparation of enriched HDL particles. As shown in Figure 3B, HDL enriched with PC(16:0/13-HODE) inhibited collagen-induced platelet aggregation. The more HDL were enriched with PC(16:0/13-HODE), the more platelet aggregation was inhibited. HDL enriched with native PC(16:0/18:2) had no significant effect on collagen-induced platelet aggregation compared to platelets incubated with control HDL and stimulated with collagen (data not shown).

**Effects of glycoxidized HDL obtained from patients with T2D on platelet aggregation**

The concentrations of hydroxylated fatty acids were assessed in HDL PL from poorly controlled T2D patients and control subjects. 13-HODE and 9-HODE concentrations were two-fold higher in HDL PL from T2D patients compared to those in control subjects. 15-HETE concentration was 2.4-fold higher in patient HDL PL compared to its concentration in control HDL (Figure 4A). To determine whether hydroxylated fatty acid products originated from enzymatic or non enzymatic lipid peroxidation, chiral phase HPLC was carried out on each isomer. Concerning hydroxylated linoleic acid metabolites, 9(R)-HODE and 9(S)-HODE enantiomers were equally present in HDL from T2D patients (51% 9(S)-HODE and 49% 9(R)-HODE) suggesting that the great majority of 9-HODE originated from non-

enzymatic lipid peroxidation. 13-HODE consisted of 78% S isomer and 22% R isomer indicating the involvement of enzymatic lipid peroxidation, presumably by 15-/ω6-lipoxygenase, together with non-enzymatic lipid peroxidation in the formation of 13-HODE. 15-HETE comprised 84% S and 16% R isomer suggesting again the involvement of 15-lipoxygenase in the oxidation of AA. By comparison, unmodified HDL from control healthy subjects contained equal ratios of 9(R/S)-HODE, 60% S isomer and 40% R isomer for 13-HODE and 15-HETE, suggesting that the lipoxygenation was a minor process compared to what occurred in T2D.

The pre-incubation of platelets with HDL from T2D patients resulted in an inhibition of collagen-induced platelet aggregation compared with control platelets incubated with collagen (Figure 4B). The inhibitory effect of patients HDL on collagen-induced platelet aggregation was stronger than the one induced by control HDL because 50 µg/mL patients HDL inhibited collagen-induced platelet aggregation by 63% whereas 50 µg/mL control HDL inhibited it by 28%. To establish the role of oxidized PL in the anti-aggregating effects of HDL, HDL were enriched with PC(16:0/13-HODE) to obtain similar amounts of hydroxylated fatty acid as those found in T2D HDL (Figure 4C). PC(16:0/13-HODE) enriched HDL inhibited collagen-induced platelet aggregation to a similar extent as patient HDL (Figure 4D).

## DISCUSSION

The present results indicate that HDL modified by glycoxidation inhibited platelet aggregation *via* SR-BI in a dose-dependent manner and displayed higher anti-aggregatory potency than control HDL. Regarding the platelet signaling cascade involved in the mechanism of action of HDL, we present new data showing that *in vitro* glycoxidized HDL prevented both the collagen-induced increased phosphorylation of p38 MAPK, the stress kinase responsible for the phosphorylation of $cPLA_2$ (24) and that of $cPLA_2$, the key enzyme involved in the release of AA from membrane PL (25) which constitutes a rate-limiting step in the biosynthesis of biologically active eicosanoids. Supporting our *in vitro* results, we show for the first time that HDL from T2D patients also inhibited platelet aggregation *via* SR-BI at concentrations as low as 50 µg/mL. Our results are in line with our previous results reporting anti-aggregatory properties of severely oxidized HDL isolated from patients with

316 abetalipoproteinemia, an orphan metabolic disease characterized by the absence of apolipoprotein B-
317 containing lipoproteins (26), and extend them in T2D, a common and growing disease known to be
318 associated with chronic oxidative stress (27). T2D patients, with (28) or without cardiovascular
319 complications (29), show platelet hyperactivation evidenced by increased platelet adhesion and
320 aggregation as well as increased thromboxane $A_2$ production. Oxidatively modified lipoproteins
321 present in plasma from T2D patients may represent important contributing factors modulating platelet
322 activation, as shown with oxidized LDL from T2D patients which activate platelets (30). Decreased
323 plasma levels of HDL in T2D patients lead to lower levels of oxidized HDL, and it is likely that the
324 anti-aggregatory effects of HDL from T2D may be reduced and might indirectly contribute to platelet
325 hyperactivation in T2D. So far, few studies have investigated the effects of oxidized HDL on platelet
326 function. While it has been shown that hypochlorite-oxidized HDL may stimulate platelet aggregation
327 *via* CD36 (31), HDL oxidatively modified by copper sulfate or myeloperoxidase strongly inhibit
328 agonist-induced platelet activation and aggregation *via* SR-BI (15). Besides effects of HDL on platelet
329 function, oxidative modification of HDL (as a result of *in vitro* oxidation or diabetes) may decrease
330 cholesterol efflux capacity (32,33), impair their capacity to protect LDL from oxidation (10) and alter
331 their anti-inflammatory properties (34). Nevertheless, a few studies reported beneficial effects of HDL
332 modified by oxidative tyrosylation on the efflux of cholesterol in fibroblasts and macrophages (35).
333 Altogether, these discrepant results might be related to differences in the extent and type of HDL
334 oxidation which may determine the binding of HDL to either SR-BI or CD36 platelet receptors and the
335 capacity of HDL to inhibit or stimulate platelet aggregation by binding either to SR-BI or CD36
336 respectively (36). Our results are also in agreement with studies demonstrating that copper-oxidized
337 HDL showed better binding to platelet SR-BI than native HDL (15), and showing that oxidized HDL
338 were more effective competitors than native HDL in cultured endothelial cells (8). In addition, it has
339 been reported that the abundance of human SR-BI was reduced on the surface of platelets from
340 patients with atherosclerotic disease and that its expression level correlated negatively with platelet
341 aggregation (23), which might impair the anti-aggregatory properties of HDL in T2D patients.
342

We also establish the contribution of PL esterified with oxidized LA, especially PC(16:0/13-HODE), in the anti-aggregatory properties of glycoxidized HDL. First, HODEs and 15-HETE were strongly increased in PL from *in vitro* glycoxidized HDL. It was associated with decreased proportions of LA and AA in this class of lipids, increased MDA levels and vitamin E consumption. Increased concentrations of HODE and 15-HETE, originating from both enzymatic and non enzymatic lipid peroxidation, were also observed in HDL PL from T2D patients compared to control HDL. It should be underlined that the concentrations of HODEs and HETE in HDL PL were negatively associated with collagen-induced platelet aggregation ($r = -0.65$, $P=0.004$). This confirms PL esterified with oxidized LA as important biologically active components supporting protective effects of HDL against platelet aggregation. Second, *in vitro* enrichment of HDL in phosphatidylcholine carrying out 13(S)-HODE inhibited platelet aggregation whereas enrichment of HDL with non-oxidized phosphatidylcholine had no significant effect on platelet aggregation. Similar increases of 13-HODE, 9-HODE and 15-HETE have been recently described in HDL total lipids from patients with diabetes compared with those from patients without diabetes (37) but this is the first evidence of increased content of hydroxylated fatty acids in the phospholipid class of HDL from T2D patients correlated with atheroprotective activities of HDL. Moreover, the association of higher levels of oxidized PL in HDL and lower response to aggregation are in line with the Cardiovascular Risk in Young Finns study suggesting that an elevated cardiovascular risk profile was associated with lower oxidized HDL lipids levels (38). Due to their localization at the surface of HDL particles, it is conceivable that PL are more accessible targets of free radicals compared to CE and TAG localized in the core of HDL, and are more likely to interfere with platelets following SR-BI interaction. In addition, the structure of lipoproteins in the physiological conditions might increase the preferential oxidation of linoleate into HODE over cholesterol as shown in human plasma (39). A limitation of our study is that lipidomic analyses were limited to HODE and HETE species, including their stereo-isomers. The contribution of other oxidized lipid species should be explored in order to decipher the molecules involved in the protective role of glycoxidized HDL on platelet aggregation, although inhibition of platelet aggregation by exogenous PC(16:0/13-HODE) was very similar to that observed by endogenous ones in glycoxidized HDL as well as in HDL from DT2 patients.

We conclude from our experiments that, as far as platelet aggregation is concerned, the ability of HDL to decrease platelet aggregation is not impaired in glycoxidized HDL, but even increased compared to control HDL. Supporting our *in vitro* results, we also show that glycoxidized HDL from T2D patients retain their anti-aggregating properties. We show that the HDL content of hydroxylated fatty acids esterified in PL could contribute to the inhibitory effects of glycoxidized HDL on platelet aggregation and associated signaling pathways through SR-BI (Figure 5). Our consistent findings challenge the concept of the systematic detrimental effects of oxidized lipids on atherothrombosis.

**ACKNOWLEDGEMENTS**

The authors thank Inserm for financial support. Quang Huy Lê is funded by the French Ministry of Education and Research for his PhD thesis. Meddy El Alaoui thanks the "Région Rhône-Alpes" for a PhD grant. Catherine Calzada is supported by the CNRS. The authors are grateful to all participants for their cooperation and to the nurses at Hospices Civils de Lyon for expert blood drawing.

**REFERENCES**

1. Marsche G, Saemann MD, Heinemann A, Holzer M. Inflammation alters HDL composition and function: implications for HDL-raising therapies. Pharmacol Ther 2013; 137:341-351.

2. Davidsson P, Hulthe J, Fagerberg B, Camejo G. Proteomics of apolipoproteins and associated proteins from plasma high-density lipoproteins. Arterioscler Thromb Vasc Biol. 2010; 30:156-163.

3. Kontush A, Lhomme M, Chapman MJ. Unraveling the complexities of the HDL lipidome. J Lipid Res 2013; 54:2950-2963.

4. Bowry VW, Stanley KK, Stocker R. High density lipoprotein is the major carrier of lipid hydroperoxides in human blood plasma from fasting donors. Proc Natl Acad Sci USA 1992; 89:10316-10320.

5. Proudfoot JM, Barden AE, Loke WM, Croft KD, Puddey IB, Mori TA. HDL is the major lipoprotein carrier of plasma F2-isoprostanes. J Lipid Res 2009; 50:716-722.

6. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian A, Fonarow GC, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: Steps 2 and 3. J Lipid Res 2000; 41:1495-1508.

7. Nakano T, Nagata A. Immunochemical detection of circulating oxidized high-density lipoprotein with antioxidized apolipoprotein A-I monoclonal antibody. J Lab Clin Med 2003; 141:378-384.

8. Nakajima T, Origuchi N, Matsunaga T, Nakajima T, Origuchi N, Matsunaga T, Kawai S, Hokari S, Nakamura H, Inoue I, Katayama S, Nagata A, Komoda T. Localization of oxidized HDL in atheromatous plaques and oxidized HDL binding sites on human aortic endothelial cells. Ann Clin Biochem 2000; 37:179-186.

9. Ueda M, Hayase Y, Mashiba S. Establishment and evaluation of 2 monoclonal antibodies against oxidized apolipoprotein A-I (apoA-I) and its application to determine blood oxidized apoA-I levels. Clin Chim Acta 2007; 78:105-111.

10. Nobécourt E, Jacqueminet S, Hansel B, Hansel B, Chantepie S, Grimaldi A, Chapman MJ, Kontush A. Defective antioxidative activity of small dense HDL3 particles in T2D: relationship to elevated oxidative stress and hyperglycaemia. Diabetologia 2005; 48:529-538.

11. Sorrentino SA, Besler C, Rohrer L, Meyer M, Heinrich K, Bahlmann FH, Mueller M, Horváth T, Doerries C, Heinemann M, Flemmer S, Markowski A, Manes C, Bahr MJ, Haller H, von Eckardstein A, Drexler H, Landmesser U. Endothelial-vasoprotective effects of high-density lipoprotein are impaired in patients with T2D mellitus but are improved after extended-release niacin therapy. Circulation 2010; 121:110-122.

12. Low H, Hoang A, Forbes J, Thomas M, Lyons JG, Nestel P, Bach LA, Sviridov D. Advanced glycation end-products (AGEs) and functionality of reverse cholesterol transport in patients with T2D and in mouse models. Diabetologia 2012; 55:2513-2521.

13. de Vries R, Groen AK, Perton FG, Dallinga-Thie GM, van Wijland MJ, Dikkeschei LD, Wolffenbuttel BH, van Tol A, Dullaart RP. Increased cholesterol efflux from cultured fibroblasts to plasma from hypertriglyceridemic T2D patients: roles of pre [beta]-HDL, phospholipid transfer protein and cholesterol esterification. Atherosclerosis 2008; 196:733-741.

14. Assinger A, Schmid W, Eder S, Eder S, Schmid D, Koller E, Volf I. Oxidation by hypochlorite converts protective HDL into a potent platelet agonist. FEBS Lett 2008; 582:778–784.

15. Valiyaveettil M, Kar N, Ashraf, M.Z, Byzova, TV, Febbraio, M, Podrez, EA. Oxidized high-density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI. Blood 2008; 111:1962-1971.

16. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955; 34:1345-1353.

17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-275.

18. Lagarde M, Bryon PA, Guichardant M, Dechavanne M. A simple and efficient method for platelet isolation from their plasma. Thromb Res 1980; 17:581-588.

19. Januel C, El Hentati FZ, Carreras M, Arthur JR, Calzada C, Lagarde M, Véricel E. Phospholipid-hydroperoxide glutathione peroxidase (GPx-4) localization in resting platelets, and compartmental change during platelet activation. Biochim Biophys Acta 2006; 1761:1228-1234.

20. Schmidpeter A, Luber J. Spirophosphoranes from Acylhydrazines and Phosphorus(III)Compounds. Angew Chem Int Ed Engl 1972; 11:306-307.

21. Duclos RI. Synthesis of 1-palmitoyl-2-hexadecyl-sn-glycero-3-phosphocholine (PHPC). Chem Phys Lipids 1993; 66:161-170.

22. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194:927-929.

23. Imachi H, Murao K, Cao W, Tada S, Taminato T, Wong NC, Takahara J, Ishida T. Expression of human scavenger receptor B1 on and in human platelets. Arterioscler Thromb Vasc Biol 2003; 23:898-904.

24. Börsch-Haubold AG, Kramer RM, Watson SP. Phosphorylation and activation of cytosolic phospholipase A2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. Eur J Biochem 1997; 245:751-759.

25. Kramer RM, Sharp JD. Structure, function and regulation of Ca2+-sensitive cytosolic phospholipase A2. FEBS Lett 1997; 410:49-53.

26. Calzada C, Véricel E, Colas R, Guillot N, El Khoury G, Drai J, Sassolas A, Peretti N, Ponsin G, Lagarde M, Moulin P. Inhibitory effects of in vivo oxidized high-density lipoproteins on platelet aggregation: evidence from patients with abetalipoproteinemia. FASEB J 2013; 27:2855-2861.

27. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 2004; 53:119-124.

28. Ferroni P, Basili S, Falco A, Davì G. Platelet activation in type 2 diabetes mellitus. J Thromb Haemost. 2004; 2:1282-1291.

29. Véricel E, Januel C, Carreras M, Moulin P, Lagarde M. Diabetic patients without vascular complications display enhanced basal platelet activation and decreased antioxidant status. Diabetes 2004; 53:1046-1051.

30. Colas R, Sassolas A, Guichardant M, Cugnet-Anceau C, Moret M, Moulin P, Lagarde M, Calzada C. LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets. Diabetologia 2011; 54:2931-2940.

31. Assinger A, Koller F, Schmid W, Zellner M, Babeluk R, Koller E, Volf I. Specific binding of hypochlorite-oxidized HDL to platelet CD36 triggers proinflammatory and procoagulant effects. Atherosclerosis 2010; 212:153-160.

32. Nagano Y, Arai H, Kita T. High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. Proc Natl Acad Sci USA 1991; 88:6457-6461.

33. Cavallero E, Brites F, Delfly B et al Abnormal reverse cholesterol transport in controlled type II diabetic patients. Studies on fasting and postprandial LpA-I particles. Arterioscler Thromb Vasc Biol 1995; 15:2130-2135.

34. Morgantini C, Natali A, Boldrini B, Imaizumi S, Navab M, Fogelman AM, Ferrannini E, Reddy ST. Anti-inflammatory and antioxidant properties of HDLs are impaired in type 2 diabetes. Diabetes 2011; 60:2617-2623.

35. Francis GA, Mendez AJ, Bierman EL, Heinecke JW. Oxidative tyrosylation of high density lipoprotein by peroxidase enhances cholesterol removal from cultured fibroblasts and macrophage foam cells. Proc Natl Acad Sci USA 1993; 90:6631-6635.

36. Van Der Stoep M, Korporaal SJ, Van Eck M. High-density lipoprotein as a modulator of platelet and coagulation responses. Cardiovasc Res 2014; 103:362-371.

37. Morgantini C, Meriwether D, Baldi S, Baldi S, Venturi E, Pinnola S, Wagner AC, Fogelman AM, Ferrannini E, Natali A, Reddy ST. HDL lipid composition is profoundly altered in patients with type 2 diabetes and atherosclerotic vascular disease. Nutr Metab Cardiovasc 2014; 24:594-599.

38. Kresanov P, Ahotupa M, Vasankari T, Kaikkonen J, Kähönen M, Lehtimäki T, Viikari J, Raitakari OT. The associations of oxidized high-density lipoprotein lipids with risk factors for atherosclerosis: the Cardiovascular Risk in Young Finns Study. Free Radic Biol Med 2013; 65:1284-1290.

39. Yoshida Y, Niki E. Relative susceptibilities of linoleates and cholesterol to oxidation assessed by total hydroxyoctadecadienoic acid and 7-hydroxycholesterol. J Oleo Sci 2008; 57:407-414.

**Table. Fatty acid composition of phospholipids and cholesteryl esters in control and glycoxidized HDL.**

| Fatty acid | Phospholipids | | Cholesteryl esters | |
|---|---|---|---|---|
| (mol %) | Control HDL | Glycoxidized HDL | Control HDL | Glycoxidized HDL |
| 16:0 | 34.6 ± 2.1 | 48.9 ± 2.6 *** | 14.9 ± 1 | 28.7 ± 2.7 *** |
| 18:0 | 18.7 ± 1.2 | 26 ± 1.5 *** | 2.6 ± 0.4 | 7.2 ± 2.5 * |
| 18:1n-9 | 8 ± 0.5 | 10.6 ± 0.7 * | 19.4 ± 1 | 27.2 ± 1.9 ** |
| 18:2n-6 | 14.8 ± 1.6 | 6.9 ± 0.8 *** | 44.5 ± 1.3 | 20 ± 3.4 *** |
| 20:4n-6 | 8.8 ± 0.8 | 0.8 ± 0.2 *** | 7.3 ± 0.5 | 0.6 ± 0.3 *** |
| 20:5n-3 | 0.9 ± 0.1 | 0.4 ± 0.2 * | 0.7 ± 0.2 | 0.1 ± 0.1 * |
| 22:6n-3 | 3.2 ± 0.2 | 0.3 ± 0.1 *** | 1.9 ± 0.4 | 1.3 ± 0.5 |
| DMA sum | 2.1 ± 0.8 | 0.53 ± 0.5 *** | | |

Results, expressed as mol % of main fatty acids, are means ± SEM of 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control HDL. Dimethylacetal (DMA) sum corresponds to the sum of 16:0 DMA, 18:0 DMA and 18:1 n-9 DMA.

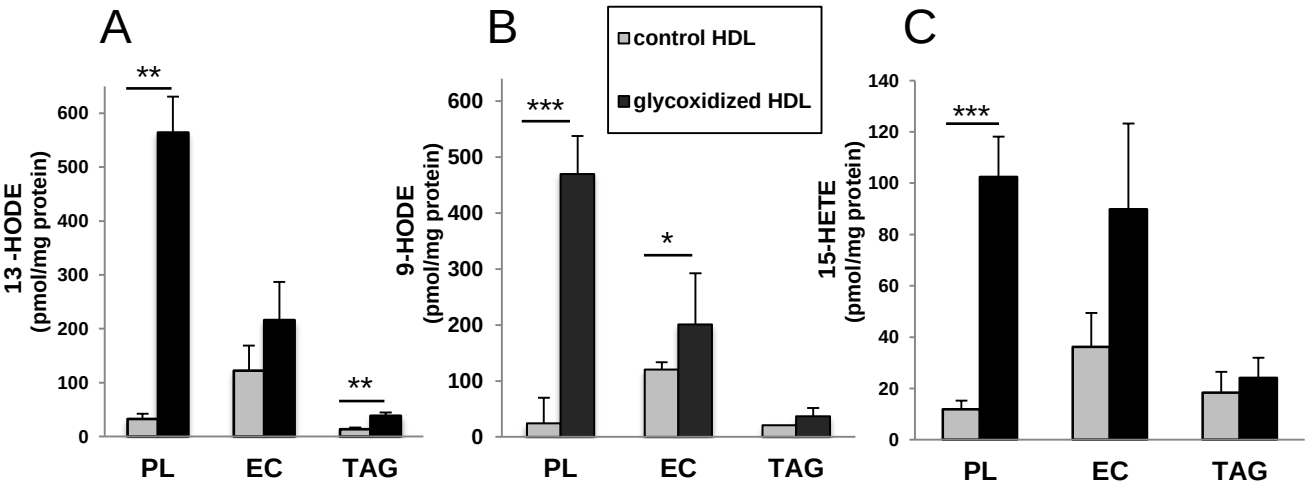
## FIGURE LEGENDS

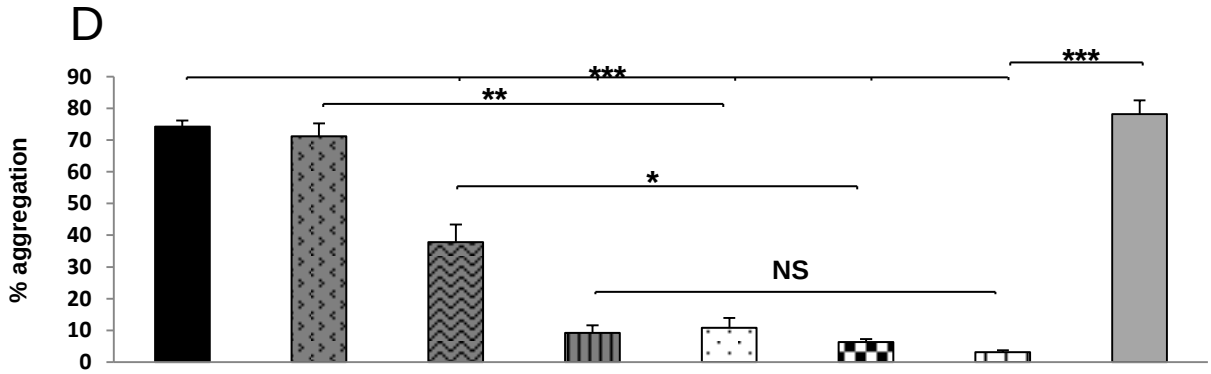
**Figure 1. Hydroxylated fatty acids in control and glycoxidized HDL. Effects of respective HDL on collagen-induced platelet aggregation.** Concentrations of 13-HODE (A), 9-HODE (B), 15-HETE (C) in control and glycoxidized HDL. Results are the means ± SEM of 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control HDL. (D) Platelet aggregation monitored in platelets isolated from healthy donors, following pre-incubation for 5 min at 37°C in the absence or presence of anti-SR-BI antibody, incubation with either control or glycoxidized HDL (25, 50 or 100 µg/mL) for 5 min at 37°C, and stimulation with collagen (2.5-5 µg/mL). Results are the means ± SEM of up to 10 experiments performed with 10 independent preparations of HDL and 10 independent platelet suspensions. Asterisks indicate significant differences (*, P<0.05; **, P <0.01; ***, P<0.001). NS, not significant.

**Figure 2. Effects of glycoxidized HDL on platelet p38 MAPK and $cPLA_2$ phosphorylation, and on collagen-induced increases of calcium concentrations in platelets.** Phosphorylation levels of p38 MAPK (A) and $cPLA_2$ (B) in platelets pre-incubated for 5 min at 37°C in the absence or presence of anti-SR-BI blocking antibody, then incubated with control or glycoxidized HDL (either 25 or 50 µg/mL) for 5 min at 37°C, and stimulated with collagen (2.5-5 µg/mL). Results, expressed as percentages of control, represent the means ± SEM of 5 independent experiments. Asterisks indicate significant differences (*, P<0.05; ***, P<0.001). NS, not significant. (C) Intracellular concentrations of calcium in Fura 2-AM loaded platelets pre-incubated in the absence or presence of 50 µg/mL control or glycoxidized HDL for 5 min at 37°C and stimulated with collagen (2.5-5 µg/mL). Tracings are representative of 4 independent experiments. a, $P < 0.001$ vs. (platelets + collagen) ; b, $P < 0.05$ vs. (platelets + control HDL + collagen).

**Figure 3. Effects of HDL enriched with oxidized phosphatidylcholine on collagen-induced platelet aggregation.** (A) Concentrations of HODE in HDL samples enriched or not with [PC(16:0/13-HODE)] or 2x [PC(16:0/13-HODE)]. **, P < 0.01 HDL enriched with PC(16:0/13-

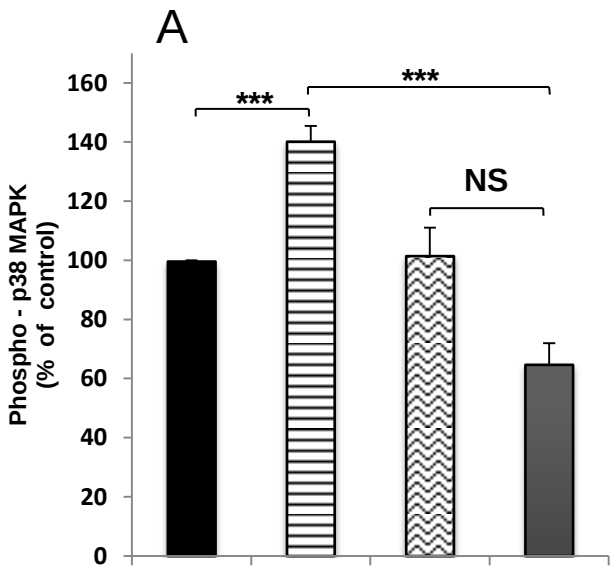
570 HODE) vs. control HDL. (B) Collagen-induced aggregation obtained in platelets pre-incubated for 5
571 min at 37°C in the absence (control HDL) or presence of HDL (50µg/mL) enriched with [PC(16:0/13-
572 HODE)] or 2x [PC(16:0/13-HODE)], and stimulated with collagen (2.5-5 µg/mL). Results are the
573 means ± SEM of 4 different preparations of various HDL and 4 different suspensions of platelets.
574 Asterisks indicate significant differences (*, $P$<0.05; **, $P$<0.01). NS, not significant.
575
576 **Figure 4. Hydroxylated fatty acids in HDL phospholipids from control healthy subjects and**
577 **from T2D patients. Effects of respective HDL on collagen-induced platelet aggregation.**
578 (A) Concentrations of 13-HODE, 9-HODE and 15-HETE in HDL PL from 5 control healthy subjects
579 and 5 patients with T2D. Results are the means ± SEM. **, $P$ < 0.01 vs. HDL from control subjects.
580 (B) Platelet aggregation monitored in platelets isolated from healthy donors, pre-incubated for 5 min at
581 37°C in the absence or presence of HDL (50 µg/mL) from control subjects and patients with T2D for 5
582 min at 37°C, and then stimulated with collagen (2.5-5 µg/mL). Results are the means ± SEM of 8
583 independent experiments performed with 8 different preparations of HDL and platelets. Asterisks
584 indicate significant differences (*, $P$<0.05; ***, $P$<0.001). (C) Concentrations of 13-HODE, 9-HODE
585 and 15-HETE in HDL samples enriched with PC(16:0/13-HODE) ("enriched HDL") or not (control
586 HDL), n=2. (D) Collagen-induced aggregation obtained in platelets pre-incubated for 5 min at 37°C
587 in the absence (control HDL) or presence of HDL enriched with [PC(16:0/13-HODE)] ("enriched
588 HDL") and stimulated with collagen. Results are the means ± SEM of 4 different preparations of
589 control and enriched HDL. Asterisks indicate significant differences (*, $P$<0.05).
590
591 **Figure 5. Summary diagram for the effects of glycoxidized HDL on platelet signaling pathways.**
592 Binding of glycoxidized HDL or HDL enriched with PC(16:0/13-HODE) to SR-BI receptor on
593 platelet membranes led to the inhibition of signaling pathways through decreased phosphorylated p38
594 MAPK and $cPLA_2$ levels and inhibited collagen-induced platelet aggregation.

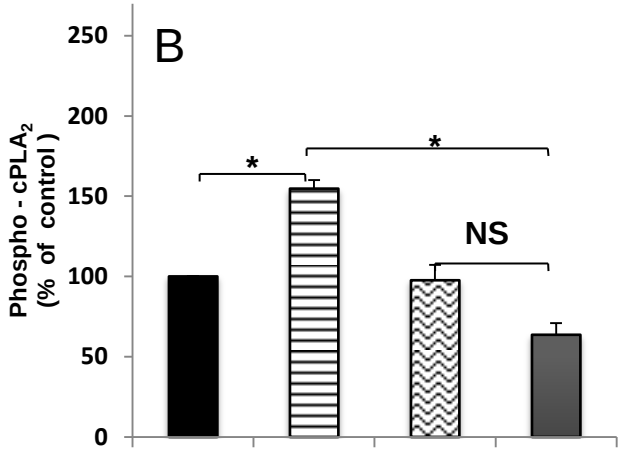


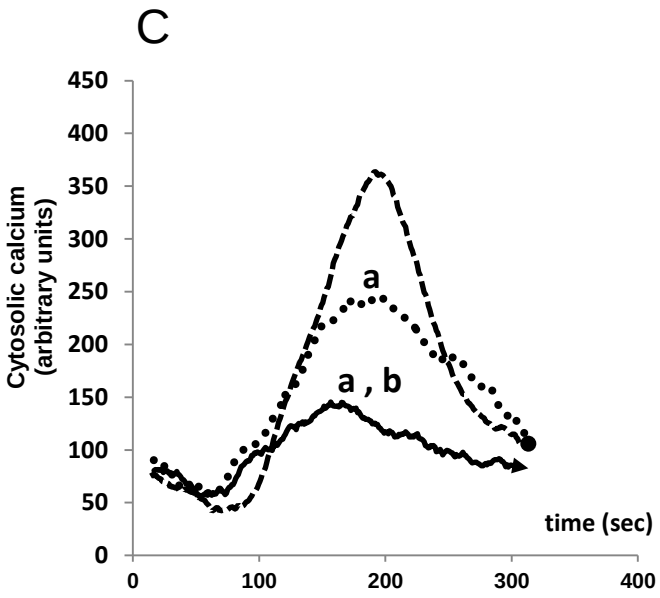



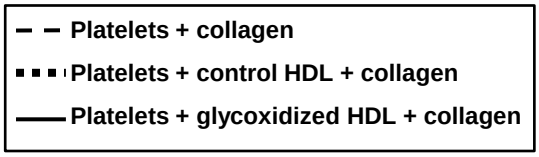

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Control HDL (µg/mL) | - | 25 | 50 | 100 | - | - | - | - |
| Glycoxidized HDL (µg/mL) | - | - | - | - | 25 | 50 | 100 | 100 |
| Collagen | + | + | + | + | + | + | + | + |
| SR-B1 antibody | - | - | - | - | - | - | - | + |

**Figure 1**




| | | | | |
|---|---|---|---|---|
| Control HDL | - | - | + | - |
| Glycoxidized HDL | - | - | - | + |
| Collagen | - | + | + | + |

**Figure 2**

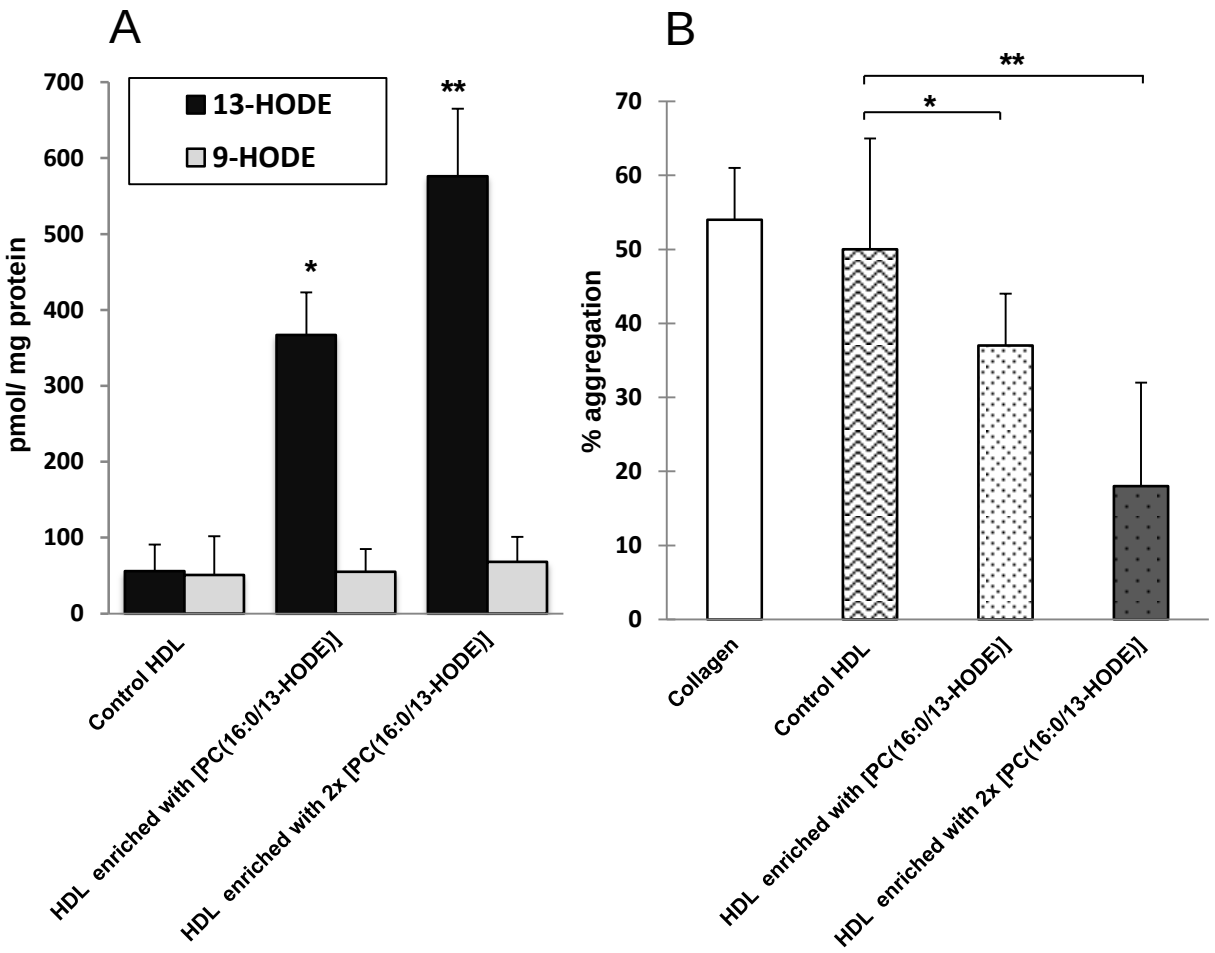

A
700
600
500
400
300
200
100
0
pmol/ mg protein
13-HODE
9-HODE
*
**
Control HDL
HDL enriched with [PC(16:0/13-HODE)]
HDL enriched with 2x [PC(16:0/13-HODE)]
B
70
60
50
40
30
20
10
0
% aggregation
**
*
Collagen
Control HDL
HDL enriched with [PC(16:0/13-HODE)]
HDL enriched with 2x [PC(16:0/13-HODE)]

**Figure 3**

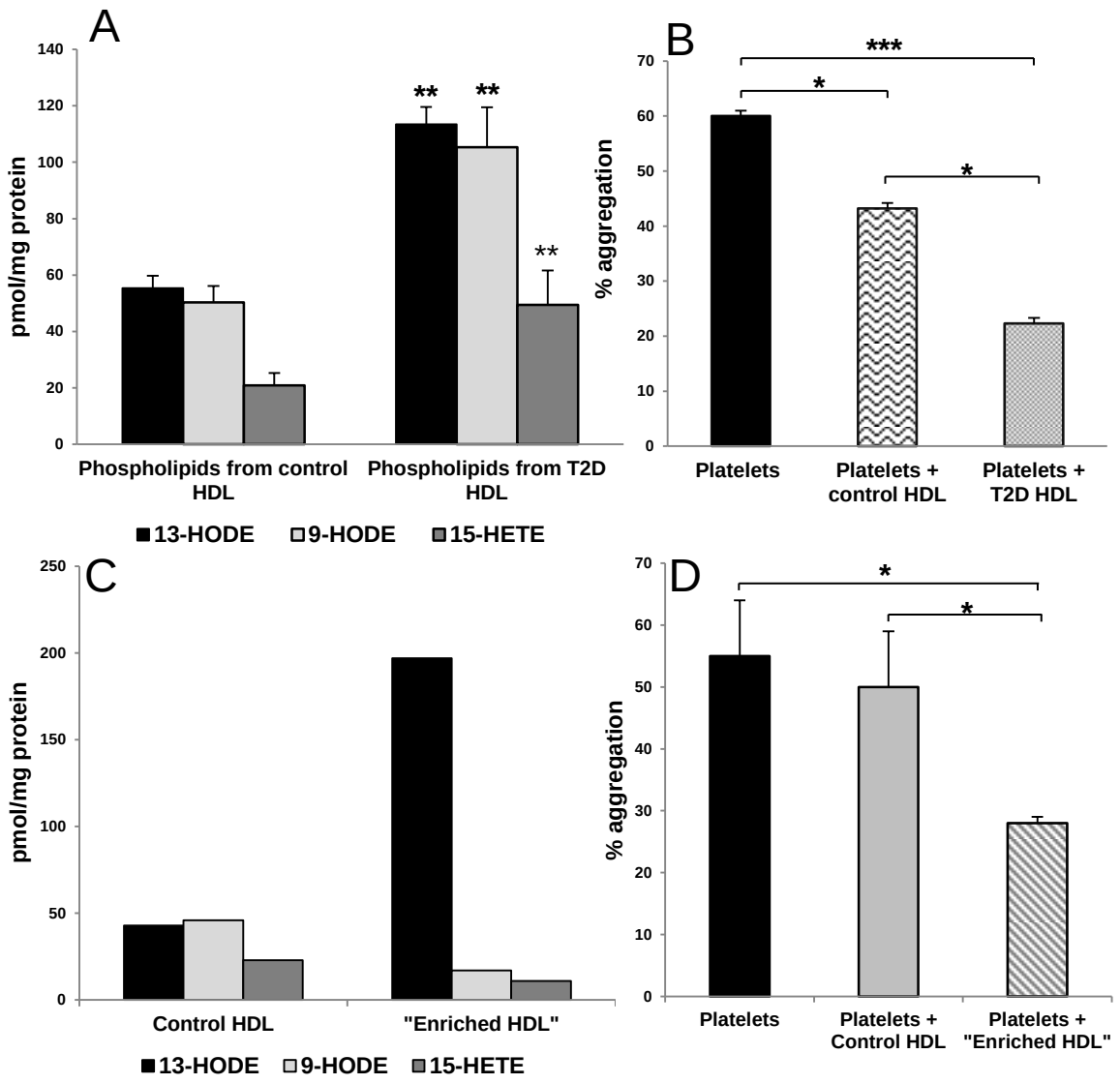

A
pmol/mg protein
140
120
100
80
60
40
20
0
**
**
**
Phospholipids from control HDL
Phospholipids from T2D HDL
13-HODE
9-HODE
15-HETE
B
% aggregation
70
60
50
40
30
20
10
0
***
*
*
Platelets
Platelets + control HDL
Platelets + T2D HDL
C
pmol/mg protein
250
200
150
100
50
0
Control HDL
"Enriched HDL"
13-HODE
9-HODE
15-HETE
D
% aggregation
70
60
50
40
30
20
10
0
*
*
Platelets
Platelets + Control HDL
Platelets + "Enriched HDL"

Figure 4

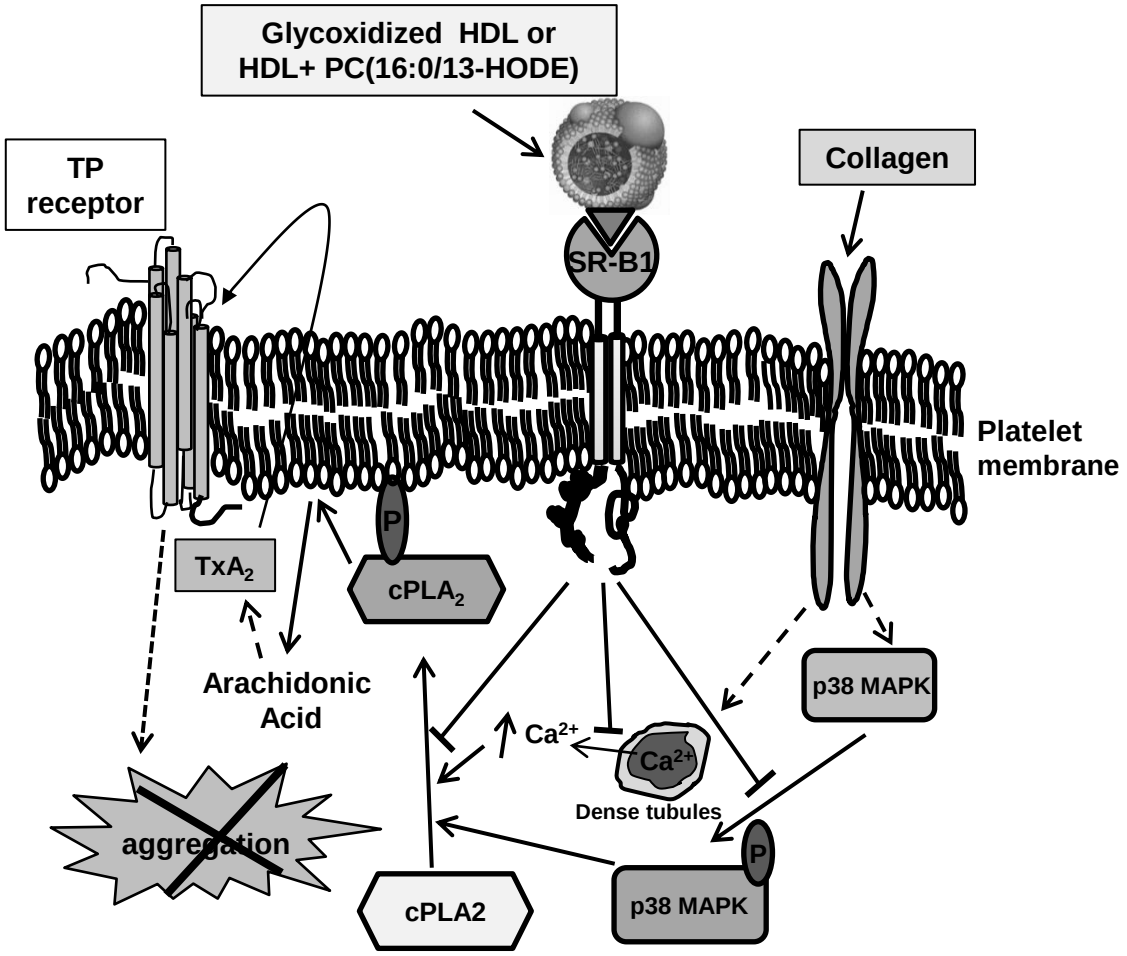

Glycoxidized HDL or
HDL+ PC(16:0/13-HODE)
TP
receptor
Collagen
SR-B1
Platelet
membrane
P
TxA2
cPLA2
Arachidonic
Acid
p38 MAPK
Ca2+
Ca2+
Dense tubules
aggregation
P
cPLA2
p38 MAPK

Figure 5

**Supplemental Methods**

**Fatty acid compositions of lipid classes**

Following the addition of appropriate internal standards (1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine, 1,2,3-triheptadecanoyl-sn-glycerol and heptadecanoyl cholesteryl ester) into HDL preparations and extraction with ethanol/chloroform (1:2, v/v) in the presence of BHT (50 μmol/L), lipid classes were separated by thin-layer chromatography (TLC) with hexane/diethylether/acetic acid (80:20:1, by vol.) to separate total PL, cholesteryl esters (CE) and triacylglycerols (TAG) (1). Corresponding silica zones were scraped off and treated with trifluoride boron/methanol (1.3 mol/L, 10%) 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 (0.25μm, 30m × 0.25mm, Supelco, Bellefonte, PA, USA) and a flame ionization detector.

**Quantification of monohydroxylated fatty acids**

Following lipid extraction in the presence of appropriate standards (PC(16:0/15-HEDE) and 15-HEDE) and separation of lipid classes by TLC as previously described, dried extracts were reduced by $NaBH_4$. Ester bonds were hydrolyzed with 0.5 mol/L potassium hydroxide for 30 min at 60°C followed by an acidification to pH 3 using acetic acid. Non esterified hydroxylated fatty acids and fatty acids were first extracted on an Oasis Sep-Pak cartridge column (Waters, Milford, MA) or were extracted with hexane. Then, non-esterified hydroxylated fatty acids were separated by TLC with hexane/diethylether/acetic acid (60:40:1, by vol.). The spots were scraped off and then extracted with methanol, separated by reverse-phase HPLC on X Bridge $C_{18}$ column (3.5 μm, 4.6 × 150 mm, Waters, Milford, MA) using a gradient solvent of acetonitrile and water (pH 3) and measured at 235 nm (2).

**Stereochemical analysis of hydroxylated fatty acids**

Hydroxylated fatty acids isomers (13(R,S)-HODE, 9(R,S)-HODE and 15(R,S)-HETE) were firstly separated by reverse-phase HPLC as previously described, collected, evaporated and transmethylated with diazomethane for 15 min at room temperature to obtain methyl esters of hydroxylated fatty acids. Each fraction was injected to chiral phase HPLC on a CHIRALCEL ®OD-H column (5 µm, 250 x 4.6 mm, Daicel), eluted isocratically with hexane/2-propanol/acetic acid (85:15:0.1 by vol ) at a flow rate of 1 mL/min at 25°C. Hydroxylated fatty acids were measured at 235 nm with a diode array detector.

**Malondialdehyde (MDA) determination**

HDL samples were mixed with thiobarbituric acid (TBA) (10 mmol/L), acetic acid and BHT (5 mmol/L) and the mixture was heated at 95°C for 60 min. The TBA-MDA adducts were extracted with ethyl acetate, separated onto a Nucleosil $C_{18}$ column (5µm, 4.6 × 250 mm, Macherey-Nagel, Hoerdt, France) by reverse-phase HPLC and measured fluorimetrically (excitation 515 nm, emission 553 nm) (3).

**Vitamin E determination**

HDL samples (1 vol.), containing tocol as an internal standard, were extracted twice with hexane (4 vol.) following the addition of ethanol (1 vol.). Tocopherol isomers were separated by reverse-phase HPLC onto a Nucleosil $C_{18}$ column (5µm, 4 × 150 mm) and measured fluorimetrically (excitation 295nm, emission 340nm) (4).

**Platelet p38 MAPK and cytosolic phospholipase $A_2$ activation**

Following platelet lysis, proteins were denatured, electrophoresed in 12% bis-Tris and transferred to nitrocellulose membranes. The membranes were incubated with either 1:2500 anti-p38 MAPK or anti-phospho-p38 MAPK, or anti-phospho-cytosolic phospholipase $A_2$ ($cPLA_2$) polyclonal antibodies, washed, and incubated with 1:5000 goat anti-rabbit horseradish peroxidase conjugate. P38 MAPK,

phospho-p38 MAPK and phospho-cPLA$_2$ were visualized by enhanced chemiluminescence, and bands were quantified by densitometry.

**Determination of intracellular $Ca^{2+}$ concentrations**

PRP was acidified to pH 6.4 with citric acid and incubated with 1 μmol/L Fura 2-AM for 45 min at 37°C in a water bath protected from light (5). Platelets were then isolated and suspended in Tyrode-HEPES buffer, and left at room temperature for at least 1 hour in the dark. Fura 2-AM loaded platelets were pre-incubated with 50 μg/ml control or glycoxidized HDL for 5 min then stimulated with collagen. The external $Ca^{2+}$ concentration in platelet suspensions was adjusted to 1 mmol/L using $CaCl_2$. Platelets were excited alternately at 340 and 380 nm and fluorescence emission was recorded at 510 nm. Intracellular $Ca^{2+}$ concentrations were calculated from the ratio of fluorescence emission to excitation.

**References**

1. Lagarde M, Drouot B, Guichardant M, Dechavanne M. In vitro incorporation and metabolism of some icosaenoic acids in platelets. Effect on arachidonic acid oxygenation. Biochim Biophys Acta 1985; 833:52-58.

2. Colas R, Sassolas A, Guichardant M, Cugnet-Anceau C, Moret M, Moulin P, Lagarde M, Calzada C. LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets. Diabetologia 2011; 54:2931-2940.

3. Therasse J, Lemonnier F. Determination of plasma lipoperoxides by high-performance liquid chromatography. J Chromatogr 1987; 413:237-241.

4. Calzada C, Coulon L, Halimi D, Le Coquil E, Pruneta-Deloche V, Moulin P, Ponsin G, Véricel E, Lagarde M. In vitro glycoxidized low-density lipoproteins and low-density lipoproteins isolated from

T2D patients activate platelets via p38 mitogen-activated protein kinase. J Clin Endocrinol Metab 2007; 92:1961-1964.

5. Nofer JR, Walter M, Kehrel B, Wierwille S, Tepel M, Seedorf U, Assmann G. HDL3-mediated inhibition of thrombin-induced platelet aggregation and fibrinogen binding occurs via decreased production of phosphoinositide-derived second messengers 1,2-diacylglycerol and inositol 1,4,5-tris-phosphate. Arterioscler Thromb Vasc Biol 1998; 18:861-869.