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.

55 **Abbreviations:**

56 AA, arachidonic acid; BHT, butylated hydroxytoluene; CE, cholesteryl esters; cPLA$_2$, cytosolic
57 phospholipase A$_2$; DHA, docosahexaenoic acid; DMA, dimethylacetal; EPA, eicosapentaenoic acid;
58 HDL, high-density lipoproteins; HEDE, hydroxy-eicosadienoic acid; HETE, hydroxy-eicosatetraenoic
59 acid; HODE, hydroxy-octadecadienoic acid; LA, linoleic acid; LDL, low-density lipoproteins; MDA,
60 malondialdehyde; $NaBH_4$, sodium borohydride; PC, phosphatidylcholine; PL, phospholipids; PRP,
61 platelet-rich plasma; PUFA, polyunsaturated fatty acids; SR-BI, scavenger receptor BI; TAG,
62 triacylglycerols; TBA, thiobarbituric acid; TLC, thin-layer chromatography; TxA$_2$, thromboxane A$_2$,
63 T2D, type 2 diabetes.

64

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.

93 **SUBJECTS AND METHODS**

94 ***Study subjects***

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

104

105 ***Isolation of HDL by ultracentrifugation***

106 Blood was collected on EDTA and HDL were immediately isolated from plasma by potassium
107 bromide stepwise ultracentrifugation (16). HDL were extensively dialyzed against PBS (pH 7.35) in
108 the presence of 1 mmol/L EDTA. The concentration of proteins was estimated using a Lowry assay
109 (17).

110

111 ***Preparation of HDL modified by glycoxidation***

112 Glycoxidized HDL consisted of HDL incubated with 50 mmol/L glucose for 5 days at 37°C, dialyzed
113 in PBS to remove excess glucose, and treated with 5 µmol/L $CuCl_2$ for 1 day at 37°C. Control HDL
114 were prepared by incubating native HDL in the presence of butylated hydroxytoluene (BHT)
115 (5µmol/L) and EDTA (1mmol/L) for 6 days at 37°C. All HDL were finally dialyzed against EDTA-
116 free PBS just before their interaction with platelets.

117

118 ***Platelet isolation***

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

121 had not ingested any aspirin or anti-inflammatory drugs in the previous ten days. Platelet-rich plasma
122 (PRP) was prepared by centrifugation of the blood at 200g for 15 min at 20°C. Platelets were isolated
123 as previously described (18).
124

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

126 1-palmitoyl,2-(13(S)-hydroperoxy-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/13(S)-
127 HpODE) was enzymatically synthesized from 1-palmitoyl,2-linoleoyl-sn-glycero-3-phosphocholine
128 (PLPC or PC(16:0/18:2), Cayman Chemicals, Ann Arbor, MI, USA) following incubation of
129 PC(16:0/18:2) with soybean 15-lipoxygenase (type V) in the presence of deoxycholate for 30 min
130 under a continuous flux of oxygen (19). Phosphatidylcholine hydroperoxide formed was reduced by
131 sodium borohydride ($NaBH_4$) into phosphatidylcholine hydroxide. 1-palmitoyl,2-(13(S)-hydroxy-
132 octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/13-HODE)) concentration was determined
133 spectrophotometrically by UV absorbance at 235 nm and its purity was checked by HPLC.
134

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

136 Firstly, 1-palmitoyl,2-eicosadienoyl-sn-glycero-3-phosphocholine PC(16:0/20:2n-6) was chemically
137 synthesized. To a solution of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (0.2 mmol), eicosa-
138 11,14-dienoic acid (20:2n-6) (0.08 mmol) was added in anhydrous chloroform (1 mL). A solution of
139 freshly recrystallized 4-Pyrrolidinopyridine (PPyr) (20) (0.09 mmol) and dicyclohexylcarbodiimide
140 (DCC) (0.09 mmol) in 1 mL of chloroform was then added drop wise (21). After 40 h, the reaction
141 mixture was concentrated and the product was then purified by flash chromatography on silica gel
142 (chloroform/methanol/H_2O, 65:35:4, by vol.). Fractions containing the product were further purified
143 by ion exchange chromatography using Amberlyst resin (chloroform/methanol/H_2O, 65:35:4, by vol.)
144 to give PC(16:0/20:2). 1-palmitoyl,2-(15(S)-hydroperoxy-eicosadienoyl)-sn-glycero-3-phosphocholine
145 (PC(16:0/15(S)-HpEDE) was then enzymatically synthesized from PC(16:0/20:2) as described above
146 and reduced into PC(16:0/15(S)-HEDE), and used as an internal standard in relevant analyses.
147
148

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

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

154

155 ***Characterization of HDL***

156 *Fatty acid compositions of lipid classes*

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

159

160 *Quantification of monohydroxylated fatty acids*

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

164

165 *Stereochemical analysis of hydroxylated fatty acids*

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

168

169 *Malondialdehyde (MDA) determination*

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

172

173 *Vitamin E determination*

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

176

177 ***Platelet aggregation***

178 Aggregation was measured in isolated platelets in a Chrono-log dual-channel aggregometer (Coulter,
179 Margency, France) according to the method of Born (22). Platelet suspensions were pre-incubated for
180 5 min at 37°C in the presence or absence of different preparations of HDL and then stimulated with
181 threshold concentrations of collagen (Nycomed, Linz, Austria) with continuous stirring at 1000 rpm.
182 The threshold concentration of collagen was defined as the concentration of collagen that induced
183 approximately a 60% increase in light transmission. The extent of platelet aggregation was expressed
184 in terms of percentage of change in light transmission 4 min after the addition of collagen.

185

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

187 Chemiluminescent Western blotting detection of p38 MAPK, phospho-p38 MAPK and phospho-
188 $cPLA_2$ was performed as described in the Supplemental Methods.

189

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

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

193

194 ***Statistical analysis***

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

197

198 **RESULTS**

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

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

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

204 eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids decreased by 54%, 91%,
205 56% and 91% respectively. The proportions of 16:0 dimethylacetals (DMA), 18:0 DMA and 18:1 n-9
206 DMA issued from the alkenyl residue of alkenyl,acyl-glycero-phosphoethanolamine (ethanolamine
207 plasmalogens) decreased in PL from glycoxidized HDL compared to those from control HDL. By
208 contrast, proportions of palmitic (16:0), stearic (18:0) and oleic (18:1n-9) acids increased in modified
209 HDL. In CE, glycoxidation of HDL also led to decreased proportions of LA, AA and EPA, by 53%,
210 92% and 86% respectively, and increased proportions of palmitic, stearic and oleic acids.
211
212 *Vitamin E concentration*
213 The concentrations of alpha-tocopherol strongly decreased in glycoxidized HDL compared to control
214 HDL (0 in glycoxidized HDL vs. 2.04 ± 0.28 nmol/mg HDL protein in control HDL, n=5, $P<0.001$).
215 Gamma-tocopherol concentrations decreased by 75% in glycoxidized HDL (0.08 ± 0.01 in
216 glycoxidized HDL vs. 0.32 ± 0.02 nmol/mg HDL protein in control HDL, n=5, $P<0.001$).
217
218 *MDA concentration*
219 The concentrations of MDA, a marker of overall lipid peroxidation, were more than 10-fold higher in
220 glycoxidized HDL compared to control HDL (1.93 ± 0.32 nmol/mg protein in glycoxidized HDL vs.
221 0.18 ± 0.13 nmol/mg protein in control HDL, n=4, $P<0.01$).
222
223 *Concentrations of hydroxylated fatty acids in lipid classes*
224 The concentrations of the stable primary products of PUFA peroxidation, 13-HODE and 9-HODE,
225 derived from LA, and 15-HETE, derived from AA, were assessed in HDL. In control HDL, most
226 hydroxylated fatty acids were present in CE, followed by PL and TAG, while most of them were
227 present in PL of glycoxidized HDL (Figure 1). HODE concentrations increased strongly in PL of
228 glycoxidized HDL compared to those of unmodified HDL (Figures 1A and 1B). 13-HODE
229 concentrations tended to increase (1.8-fold) and 9-HODE concentrations significantly increased in CE
230 from glycoxidized HDL. In TAG, 13-HODE concentrations increased in glycoxidized HDL (2.7-fold)
231 and 9-HODE concentrations tended to increase (1.8-fold) compared to control HDL. 15-HETE

232 concentration increased in PL from glycoxidized HDL (8.5-fold) while 15-HETE concentration tended
233 to increase in CE and TAG from glycoxidized HDL compared to control HDL (Figure 1C).
234

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

236 Compared to platelets incubated with collagen, pre-incubation of platelets with glycoxidized HDL for
237 5 min at 37°C resulted in a dose-dependent inhibition of collagen-induced platelet aggregation with
238 near complete inhibition at 100 µg/mL (Figure 1D). Control HDL had no effect at 25 µg/mL but
239 significantly inhibited collagen-induced platelet aggregation at concentrations greater than or equal to
240 50 µg/mL. Because scavenger receptor SR-BI binds modified HDL and is expressed in platelets (23),
241 its involvement in the mechanism of action of HDL on these cells was sought. Pre-incubation of
242 platelets with anti-SRBI blocking antibody for 5 min at 37°C fully prevented the inhibitory effects of
243 glycoxidized HDL on collagen-induced platelet aggregation (Figure 1D). Pre-incubation of platelets
244 with anti-SRBI also prevented the inhibition of collagen-induced platelet aggregation in presence of
245 control HDL (aggregation rate: 65 % in platelets incubated with anti-SRBI antibody and 100 µg/ml
246 control HDL vs. 12% in platelets incubated with 100 µg/ml control HDL). Anti-SRBI blocking
247 antibody had no effect on collagen-induced platelet aggregation, and pre-incubation with non-immune
248 isotype control antibody did not reduce the inhibitory effect of glycoxidized HDL on platelet
249 aggregation (data not shown).

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

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

260 inhibited collagen-induced intracellular Ca^{2+} increase in platelets by 47% while control HDL
261 decreased it by 32%. Pre-incubation of platelets with anti-SRBI blocking antibody for 5 min at 37°C
262 alleviated the inhibitory effects of glycoxidized HDL on phosphorylation of p38 MAPK and $cPLA_2$,
263 and Ca^{2+} mobilization (data not shown).
264
265 **Effects of HDL enriched with 1-palmitoyl,2-(13-hydroxy-octadecadienoyl)-sn-glycero-3-**
266 **phosphocholine on platelet aggregation**
267 To determine the role of oxidized LA-containing PL for anti-aggregating effects of HDL, HDL were
268 pre-loaded with two different concentrations of PC(16:0/13-HODE) prepared by 15-lipoxygenation of
269 LA esterified to phosphatidylcholine, followed by chemical reduction with $NaBH_4$ (Figure 3A). As
270 expected, higher concentrations of 13-HODE, close to those found in glycoxidized HDL, were present
271 in enriched HDL compared to control HDL, while concentrations of 9-HODE were similar in control
272 and enriched HDL, confirming the absence of non enzymatic lipid peroxidation during the preparation
273 of enriched HDL particles. As shown in Figure 3B, HDL enriched with PC(16:0/13-HODE) inhibited
274 collagen-induced platelet aggregation. The more HDL were enriched with PC(16:0/13-HODE), the
275 more platelet aggregation was inhibited. HDL enriched with native PC(16:0/18:2) had no significant
276 effect on collagen-induced platelet aggregation compared to platelets incubated with control HDL and
277 stimulated with collagen (data not shown).
278
279 **Effects of glycoxidized HDL obtained from patients with T2D on platelet aggregation**
280 The concentrations of hydroxylated fatty acids were assessed in HDL PL from poorly controlled T2D
281 patients and control subjects. 13-HODE and 9-HODE concentrations were two-fold higher in HDL PL
282 from T2D patients compared to those in control subjects. 15-HETE concentration was 2.4-fold higher
283 in patient HDL PL compared to its concentration in control HDL (Figure 4A). To determine whether
284 hydroxylated fatty acid products originated from enzymatic or non enzymatic lipid peroxidation, chiral
285 phase HPLC was carried out on each isomer. Concerning hydroxylated linoleic acid metabolites, 9(R)-
286 HODE and 9(S)-HODE enantiomers were equally present in HDL from T2D patients (51% 9(S)-
287 HODE and 49% 9(R)-HODE) suggesting that the great majority of 9-HODE originated from non-

288 enzymatic lipid peroxidation. 13-HODE consisted of 78% S isomer and 22% R isomer indicating the
289 involvement of enzymatic lipid peroxidation, presumably by 15-/ω6-lipoxygenase, together with non-
290 enzymatic lipid peroxidation in the formation of 13-HODE. 15-HETE comprised 84% S and 16% R
291 isomer suggesting again the involvement of 15-lipoxygenase in the oxidation of AA. By comparison,
292 unmodified HDL from control healthy subjects contained equal ratios of 9(R/S)-HODE, 60% S isomer
293 and 40% R isomer for 13-HODE and 15-HETE, suggesting that the lipoxygenation was a minor
294 process compared to what occurred in T2D.
295 The pre-incubation of platelets with HDL from T2D patients resulted in an inhibition of collagen-
296 induced platelet aggregation compared with control platelets incubated with collagen (Figure 4B). The
297 inhibitory effect of patients HDL on collagen-induced platelet aggregation was stronger than the one
298 induced by control HDL because 50 µg/mL patients HDL inhibited collagen-induced platelet
299 aggregation by 63% whereas 50 µg/mL control HDL inhibited it by 28%. To establish the role of
300 oxidized PL in the anti-aggregating effects of HDL, HDL were enriched with PC(16:0/13-HODE) to
301 obtain similar amounts of hydroxylated fatty acid as those found in T2D HDL (Figure 4C).
302 PC(16:0/13-HODE) enriched HDL inhibited collagen-induced platelet aggregation to a similar extent
303 as patient HDL (Figure 4D).
304
305 **DISCUSSION**
306 The present results indicate that HDL modified by glycoxidation inhibited platelet aggregation *via* SR-
307 BI in a dose-dependent manner and displayed higher anti-aggregatory potency than control HDL.
308 Regarding the platelet signaling cascade involved in the mechanism of action of HDL, we present new
309 data showing that *in vitro* glycoxidized HDL prevented both the collagen-induced increased
310 phosphorylation of p38 MAPK, the stress kinase responsible for the phosphorylation of $cPLA_2$ (24)
311 and that of $cPLA_2$, the key enzyme involved in the release of AA from membrane PL (25) which
312 constitutes a rate-limiting step in the biosynthesis of biologically active eicosanoids. Supporting our *in*
313 *vitro* results, we show for the first time that HDL from T2D patients also inhibited platelet aggregation
314 *via* SR-BI at concentrations as low as 50 µg/mL. Our results are in line with our previous results
315 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

343 We also establish the contribution of PL esterified with oxidized LA, especially PC(16:0/13-HODE),
344 in the anti-aggregatory properties of glycoxidized HDL. First, HODEs and 15-HETE were strongly
345 increased in PL from *in vitro* glycoxidized HDL. It was associated with decreased proportions of LA
346 and AA in this class of lipids, increased MDA levels and vitamin E consumption. Increased
347 concentrations of HODE and 15-HETE, originating from both enzymatic and non enzymatic lipid
348 peroxidation, were also observed in HDL PL from T2D patients compared to control HDL. It should
349 be underlined that the concentrations of HODEs and HETE in HDL PL were negatively associated
350 with collagen-induced platelet aggregation (r = -0.65, P=0.004). This confirms PL esterified with
351 oxidized LA as important biologically active components supporting protective effects of HDL against
352 platelet aggregation. Second, *in vitro* enrichment of HDL in phosphatidylcholine carrying out 13(S)-
353 HODE inhibited platelet aggregation whereas enrichment of HDL with non-oxidized
354 phosphatidylcholine had no significant effect on platelet aggregation. Similar increases of 13-HODE,
355 9-HODE and 15-HETE have been recently described in HDL total lipids from patients with diabetes
356 compared with those from patients without diabetes (37) but this is the first evidence of increased
357 content of hydroxylated fatty acids in the phospholipid class of HDL from T2D patients correlated
358 with atheroprotective activities of HDL. Moreover, the association of higher levels of oxidized PL in
359 HDL and lower response to aggregation are in line with the Cardiovascular Risk in Young Finns study
360 suggesting that an elevated cardiovascular risk profile was associated with lower oxidized HDL lipids
361 levels (38). Due to their localization at the surface of HDL particles, it is conceivable that PL are more
362 accessible targets of free radicals compared to CE and TAG localized in the core of HDL, and are
363 more likely to interfere with platelets following SR-BI interaction. In addition, the structure of
364 lipoproteins in the physiological conditions might increase the preferential oxidation of linoleate into
365 HODE over cholesterol as shown in human plasma (39). A limitation of our study is that lipidomic
366 analyses were limited to HODE and HETE species, including their stereo-isomers. The contribution of
367 other oxidized lipid species should be explored in order to decipher the molecules involved in the
368 protective role of glycoxidized HDL on platelet aggregation, although inhibition of platelet
369 aggregation by exogenous PC(16:0/13-HODE) was very similar to that observed by endogenous ones
370 in glycoxidized HDL as well as in HDL from DT2 patients.

371

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

379

380 **ACKNOWLEDGEMENTS**

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

385

386

387 **REFERENCES**

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

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

392

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

395

396 4. Bowry VW, Stanley KK, Stocker R. High density lipoprotein is the major carrier of lipid
397 hydroperoxides in human blood plasma from fasting donors. Proc Natl Acad Sci USA 1992;
398 89:10316-10320.
399
400 5. Proudfoot JM, Barden AE, Loke WM, Croft KD, Puddey IB, Mori TA. HDL is the major
401 lipoprotein carrier of plasma F2-isoprostanes. J Lipid Res 2009; 50:716-722.
402
403 6. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian
404 A, Fonarow GC, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation
405 of mildly oxidized low density lipoprotein: Steps 2 and 3. J Lipid Res 2000; 41:1495-1508.
406
407 7. Nakano T, Nagata A. Immunochemical detection of circulating oxidized high-density lipoprotein
408 with antioxidized apolipoprotein A-I monoclonal antibody. J Lab Clin Med 2003; 141:378-384.
409
410 8. Nakajima T, Origuchi N, Matsunaga T, Nakajima T, Origuchi N, Matsunaga T, Kawai S, Hokari S,
411 Nakamura H, Inoue I, Katayama S, Nagata A, Komoda T. Localization of oxidized HDL in
412 atheromatous plaques and oxidized HDL binding sites on human aortic endothelial cells. Ann Clin
413 Biochem 2000; 37:179-186.
414
415 9. Ueda M, Hayase Y, Mashiba S. Establishment and evaluation of 2 monoclonal antibodies against
416 oxidized apolipoprotein A-I (apoA-I) and its application to determine blood oxidized apoA-I levels.
417 Clin Chim Acta 2007; 78:105-111.
418
419 10. Nobécourt E, Jacqueminet S, Hansel B, Hansel B, Chantepie S, Grimaldi A, Chapman MJ,
420 Kontush A. Defective antioxidative activity of small dense HDL3 particles in T2D: relationship to
421 elevated oxidative stress and hyperglycaemia. Diabetologia 2005; 48:529-538.
422

423 11. Sorrentino SA, Besler C, Rohrer L, Meyer M, Heinrich K, Bahlmann FH, Mueller M, Horváth T,
424 Doerries C, Heinemann M, Flemmer S, Markowski A, Manes C, Bahr MJ, Haller H, von Eckardstein
425 A, Drexler H, Landmesser U. Endothelial-vasoprotective effects of high-density lipoprotein are
426 impaired in patients with T2D mellitus but are improved after extended-release niacin therapy.
427 Circulation 2010; 121:110-122.
428
429 12. Low H, Hoang A, Forbes J, Thomas M, Lyons JG, Nestel P, Bach LA, Sviridov D. Advanced
430 glycation end-products (AGEs) and functionality of reverse cholesterol transport in patients with T2D
431 and in mouse models. Diabetologia 2012; 55:2513-2521.
432
433 13. de Vries R, Groen AK, Perton FG, Dallinga-Thie GM, van Wijland MJ, Dikkeschei LD,
434 Wolffenbuttel BH, van Tol A, Dullaart RP. Increased cholesterol efflux from cultured fibroblasts to
435 plasma from hypertriglyceridemic T2D patients: roles of pre [beta]-HDL, phospholipid transfer
436 protein and cholesterol esterification. Atherosclerosis 2008; 196:733-741.
437
438 14. Assinger A, Schmid W, Eder S, Eder S, Schmid D, Koller E, Volf I. Oxidation by hypochlorite
439 converts protective HDL into a potent platelet agonist. FEBS Lett 2008; 582:778–784.
440
441 15. Valiyaveettil M, Kar N, Ashraf, M.Z, Byzova, TV, Febbraio, M, Podrez, EA. Oxidized high-
442 density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI. Blood 2008;
443 111:1962-1971.
444
445 16. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally
446 separated lipoproteins in human serum. J Clin Invest 1955; 34:1345-1353.
447
448 17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol
449 reagent. J Biol Chem 1951; 193:265-275.
450

451 18. Lagarde M, Bryon PA, Guichardant M, Dechavanne M. A simple and efficient method for platelet
452 isolation from their plasma. Thromb Res 1980; 17:581-588.
453
454 19. Januel C, El Hentati FZ, Carreras M, Arthur JR, Calzada C, Lagarde M, Véricel E. Phospholipid-
455 hydroperoxide glutathione peroxidase (GPx-4) localization in resting platelets, and compartmental
456 change during platelet activation. Biochim Biophys Acta 2006; 1761:1228-1234.
457
458 20. Schmidpeter A, Luber J. Spirophosphoranes from Acylhydrazines and
459 Phosphorus(III)Compounds. Angew Chem Int Ed Engl 1972; 11:306-307.
460
461 21. Duclos RI. Synthesis of 1-palmitoyl-2-hexadecyl-sn-glycero-3-phosphocholine (PHPC). Chem
462 Phys Lipids 1993; 66:161-170.
463
464 22. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962;
465 194:927-929.
466
467 23. Imachi H, Murao K, Cao W, Tada S, Taminato T, Wong NC, Takahara J, Ishida T. Expression of
468 human scavenger receptor B1 on and in human platelets. Arterioscler Thromb Vasc Biol 2003;
469 23:898-904.
470
471 24. Börsch-Haubold AG, Kramer RM, Watson SP. Phosphorylation and activation of cytosolic
472 phospholipase A2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets.
473 Eur J Biochem 1997; 245:751-759.
474
475 25. Kramer RM, Sharp JD. Structure, function and regulation of Ca2+-sensitive cytosolic
476 phospholipase A2. FEBS Lett 1997; 410:49-53.
477

478 26. Calzada C, Véricel E, Colas R, Guillot N, El Khoury G, Drai J, Sassolas A, Peretti N, Ponsin G,
479 Lagarde M, Moulin P. Inhibitory effects of in vivo oxidized high-density lipoproteins on platelet
480 aggregation: evidence from patients with abetalipoproteinemia. FASEB J 2013; 27:2855-2861.
481
482 27. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic
483 oxidative stress in type 2 diabetes. Diabetes 2004; 53:119-124.
484
485 28. Ferroni P, Basili S, Falco A, Davì G. Platelet activation in type 2 diabetes mellitus. J Thromb
486 Haemost. 2004; 2:1282-1291.
487
488 29. Véricel E, Januel C, Carreras M, Moulin P, Lagarde M. Diabetic patients without vascular
489 complications display enhanced basal platelet activation and decreased antioxidant status. Diabetes
490 2004; 53:1046-1051.
491
492 30. Colas R, Sassolas A, Guichardant M, Cugnet-Anceau C, Moret M, Moulin P, Lagarde M, Calzada
493 C. LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and
494 activate platelets. Diabetologia 2011; 54:2931-2940.
495
496 31. Assinger A, Koller F, Schmid W, Zellner M, Babeluk R, Koller E, Volf I. Specific binding of
497 hypochlorite-oxidized HDL to platelet CD36 triggers proinflammatory and procoagulant effects.
498 Atherosclerosis 2010; 212:153-160.
499
500 32. Nagano Y, Arai H, Kita T. High density lipoprotein loses its effect to stimulate efflux of
501 cholesterol from foam cells after oxidative modification. Proc Natl Acad Sci USA 1991; 88:6457-
502 6461.
503
504 33. Cavallero E, Brites F, Delfly B et al Abnormal reverse cholesterol transport in controlled type II
505 diabetic patients. Studies on fasting and postprandial LpA-I particles. Arterioscler Thromb Vasc Biol
506 1995; 15:2130-2135.

507 34. Morgantini C, Natali A, Boldrini B, Imaizumi S, Navab M, Fogelman AM, Ferrannini E, Reddy
508 ST. Anti-inflammatory and antioxidant properties of HDLs are impaired in type 2 diabetes. Diabetes
509 2011; 60:2617-2623.
510
511 35. Francis GA, Mendez AJ, Bierman EL, Heinecke JW. Oxidative tyrosylation of high density
512 lipoprotein by peroxidase enhances cholesterol removal from cultured fibroblasts and macrophage
513 foam cells. Proc Natl Acad Sci USA 1993; 90:6631-6635.
514
515 36. Van Der Stoep M, Korporaal SJ, Van Eck M. High-density lipoprotein as a modulator of platelet
516 and coagulation responses. Cardiovasc Res 2014; 103:362-371.
517
518 37. Morgantini C, Meriwether D, Baldi S, Baldi S, Venturi E, Pinnola S, Wagner AC, Fogelman AM,
519 Ferrannini E, Natali A, Reddy ST. HDL lipid composition is profoundly altered in patients with type 2
520 diabetes and atherosclerotic vascular disease. Nutr Metab Cardiovasc 2014; 24:594-599.
521
522 38. Kresanov P, Ahotupa M, Vasankari T, Kaikkonen J, Kähönen M, Lehtimäki T, Viikari J, Raitakari
523 OT. The associations of oxidized high-density lipoprotein lipids with risk factors for atherosclerosis:
524 the Cardiovascular Risk in Young Finns Study. Free Radic Biol Med 2013; 65:1284-1290.
525
526 39. Yoshida Y, Niki E. Relative susceptibilities of linoleates and cholesterol to oxidation assessed by
527 total hydroxyoctadecadienoic acid and 7-hydroxycholesterol. J Oleo Sci 2008; 57:407-414.

528 **Table. Fatty acid composition of phospholipids and cholesteryl esters in control and glycoxidized**
529 **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 ***		

530
531 Results, expressed as mol % of main fatty acids, are means ± SEM of 5 independent experiments. *, *P*
532 < 0.05; **, *P* < 0.01; ***, *P* < 0.001 vs. control HDL. Dimethylacetal (DMA) sum corresponds to the
533 sum of 16:0 DMA, 18:0 DMA and 18:1 n-9 DMA.
534
535
536
537
538
539
540
541

542 **FIGURE LEGENDS**

543

544 **Figure 1. Hydroxylated fatty acids in control and glycoxidized HDL. Effects of respective HDL**
545 **on collagen-induced platelet aggregation.** Concentrations of 13-HODE (A), 9-HODE (B), 15-HETE
546 (C) in control and glycoxidized HDL. Results are the means ± SEM of 5 independent experiments. *,
547 $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control HDL. (D) Platelet aggregation monitored in platelets
548 isolated from healthy donors, following pre-incubation for 5 min at 37°C in the absence or presence of
549 anti-SR-BI antibody, incubation with either control or glycoxidized HDL (25, 50 or 100 µg/mL) for 5
550 min at 37°C, and stimulation with collagen (2.5-5 µg/mL). Results are the means ± SEM of up to 10
551 experiments performed with 10 independent preparations of HDL and 10 independent platelet
552 suspensions. Asterisks indicate significant differences (*, P<0.05; **, P <0.01; ***, P<0.001). NS, not
553 significant.

554

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

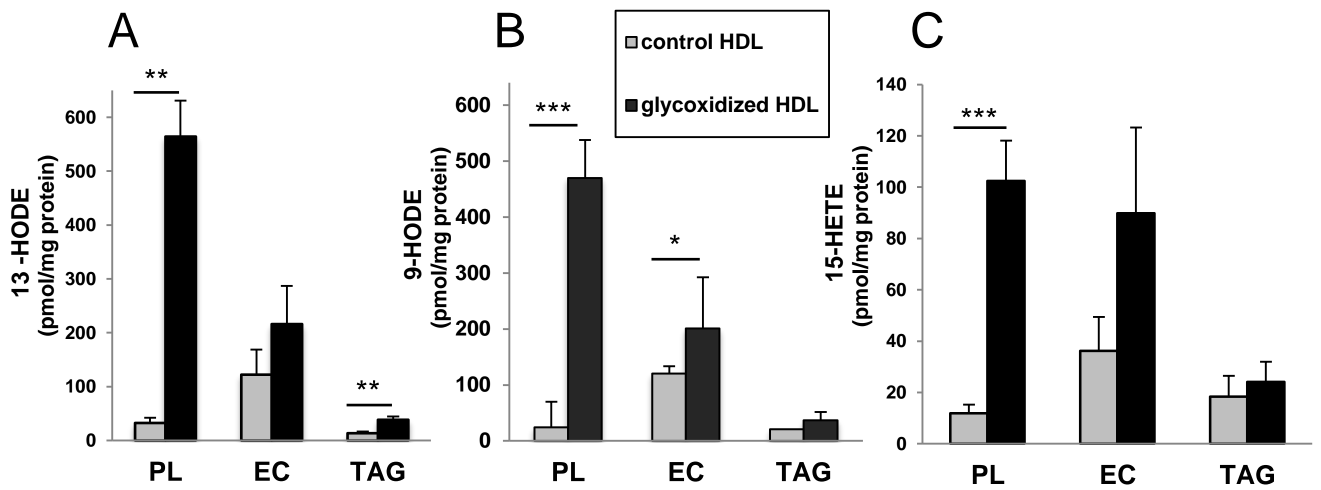
566

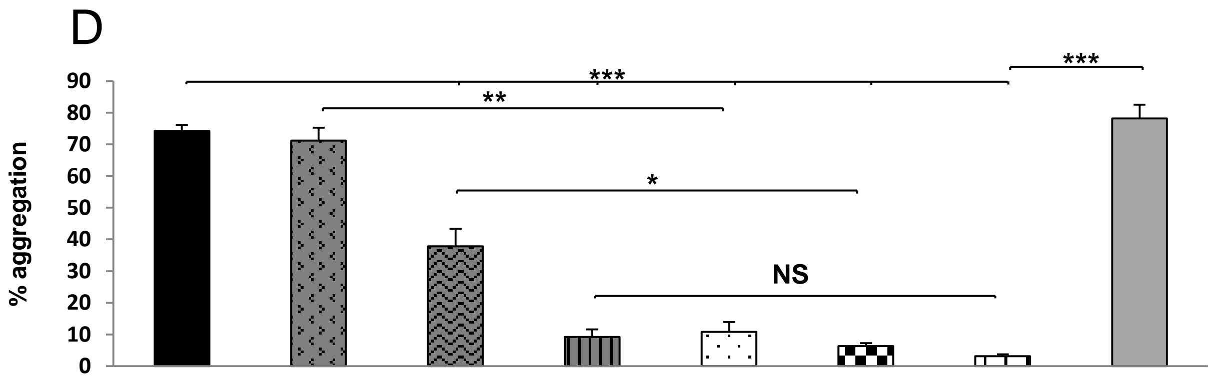
567 **Figure 3. Effects of HDL enriched with oxidized phosphatidylcholine on collagen-induced**
568 **platelet aggregation.** (A) Concentrations of HODE in HDL samples enriched or not with
569 [PC(16:0/13-HODE)] or 2x [PC(16:0/13-HODE)]. **, P < 0.01 HDL enriched with PC(16:0/13-

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

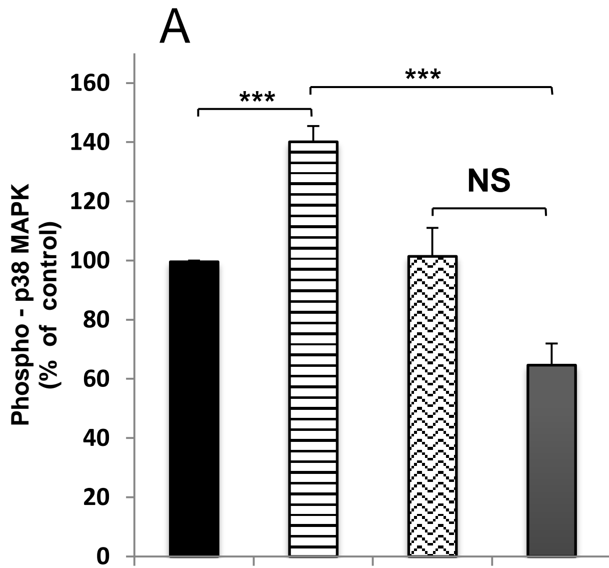
591 **Figure 5. Summary diagram for the effects of glycoxidized HDL on platelet signaling pathways.**
592 Binding of glycoxidized HDL or HDL enriched with PC(16:0/13-HODE) to SR-BI receptor on
593 platelet membranes led to the inhibition of signaling pathways through decreased phosphorylated p38
594 MAPK and $cPLA_2$ levels and inhibited collagen-induced platelet aggregation.

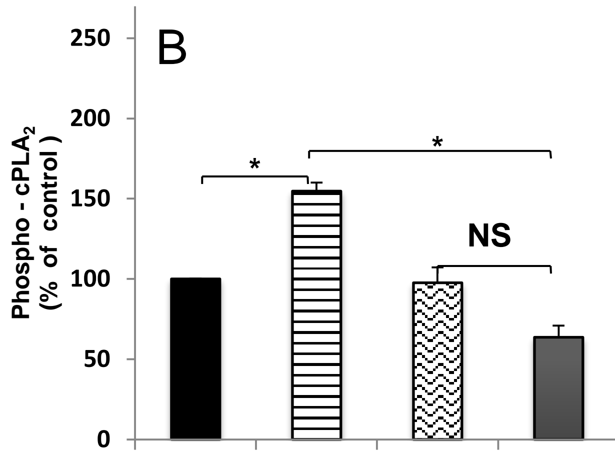


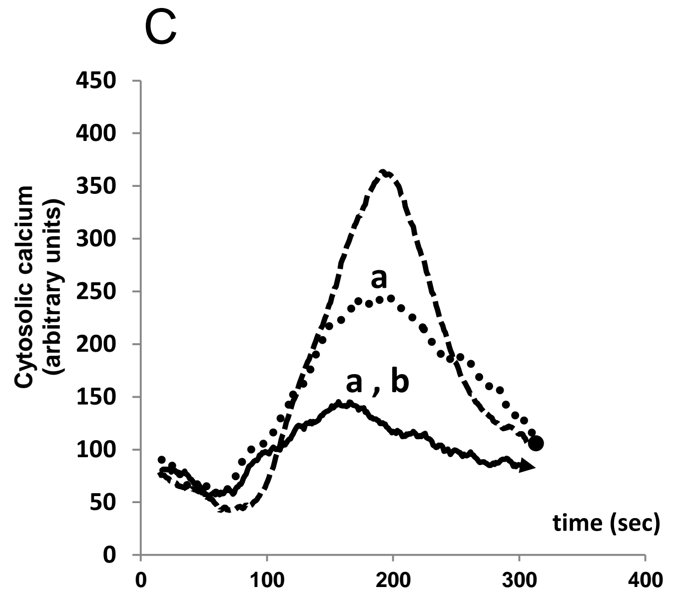


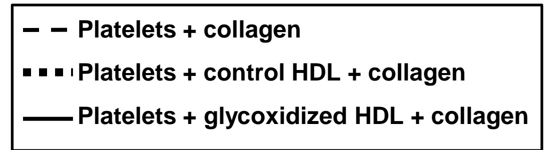
Control HDL (µg/mL)	-	25	50	100	-	-	-	-
Glycoxidized HDL (µg/mL)	-	-	-	-	25	50	100	100
Collagen	+	+	+	+	+	+	+	+
SR-B1 antibody	-	-	-	-	-	-	-	+

Figure 1









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

Figure 2

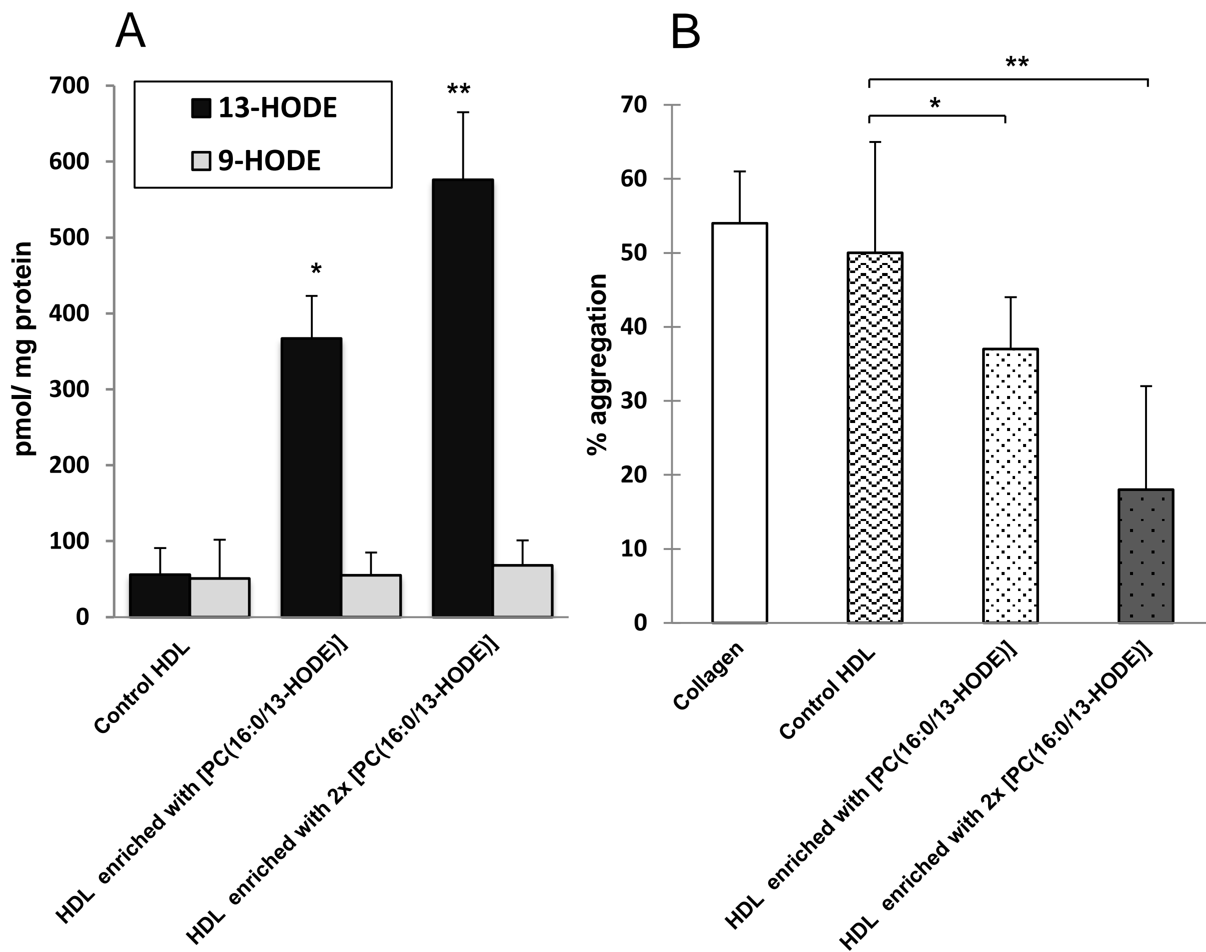


Figure 3

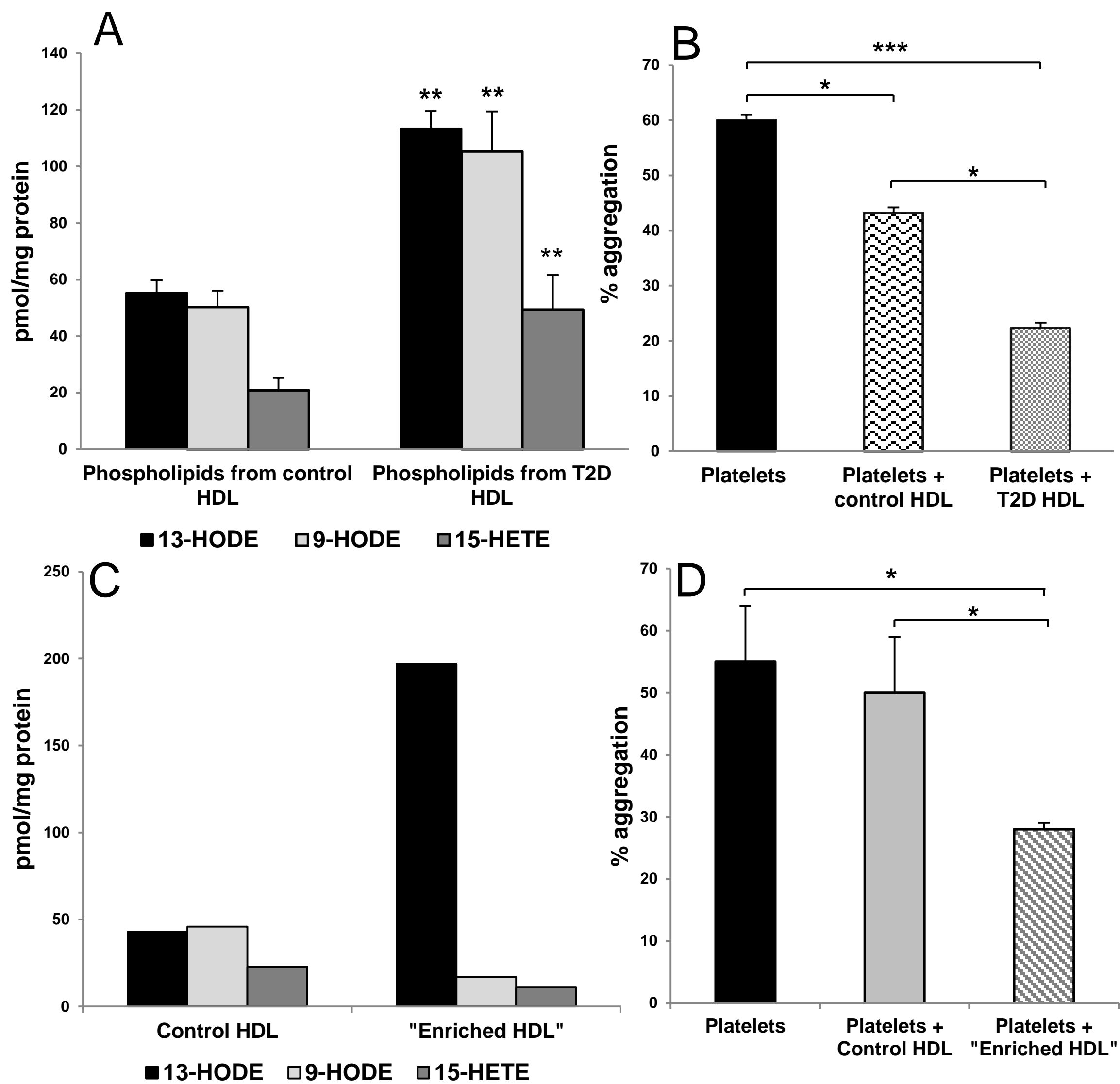


Figure 4

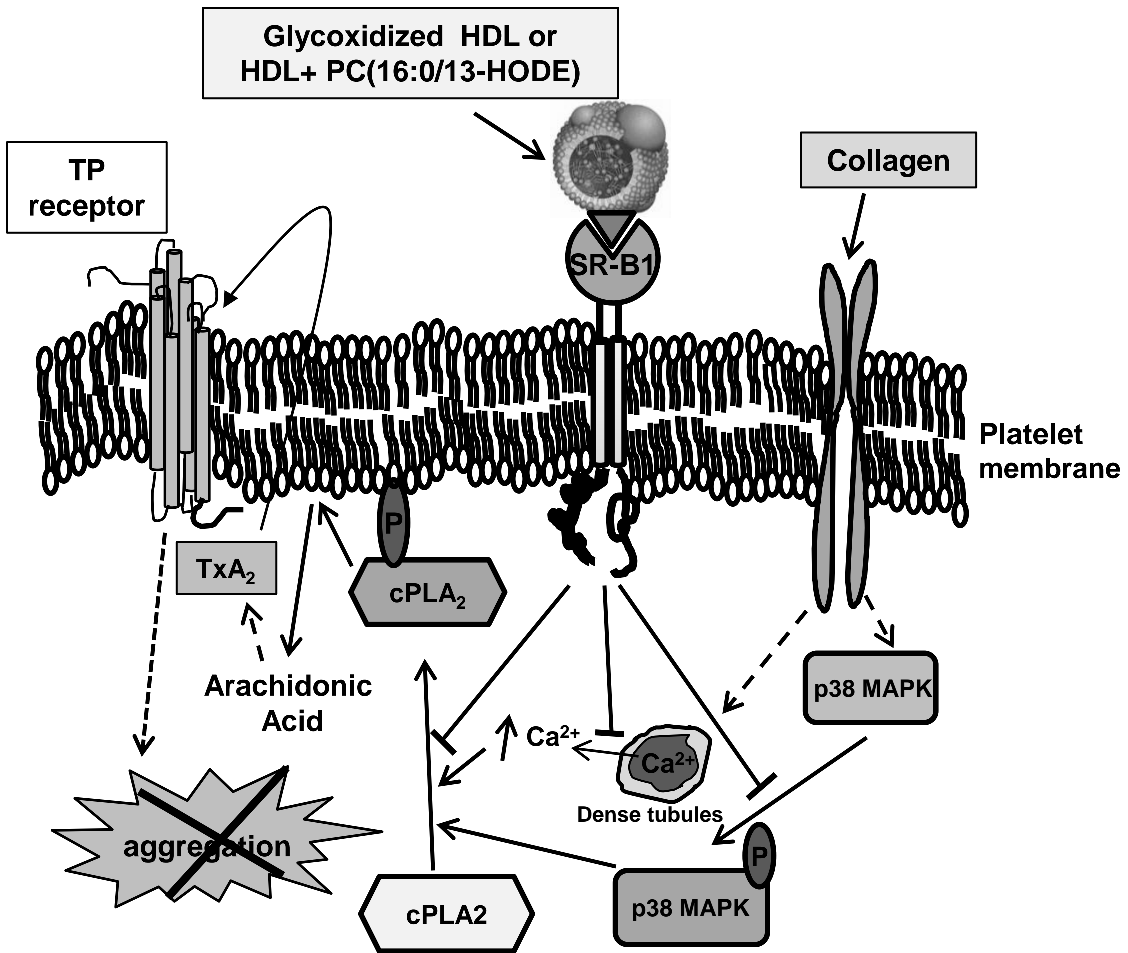


Figure 5

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.