

1 **Glycoxidized HDL, HDL enriched with oxidized phospholipids and HDL from diabetic patients**  
2 **inhibit platelet function**

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14

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30

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32

33 **ABSTRACT**

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

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

40 **Results:** Compared to control HDL, *in vitro* glycoxidized HDL had decreased proportions of linoleic  
41 (LA) and arachidonic (AA) acids in phospholipids and cholesteryl esters, and increased concentrations  
42 of hydroxy-octadecadienoic acids (9-HODE and 13-HODE) and 15-hydroxy-eicosatetraenoic acid  
43 (15-HETE), derived from LA and AA respectively, especially hydroxy derivatives esterified in  
44 phospholipids. Glycoxidized HDL dose-dependently decreased collagen-induced platelet aggregation  
45 by binding to SR-BI. Glycoxidized HDL prevented collagen-induced increased phosphorylation of  
46 platelet p38 MAPK and cytosolic phospholipase A<sub>2</sub>, as well as intracellular calcium mobilization.  
47 HDL enriched with oxidized phospholipids, namely PC(16:0/13-HODE) dose-dependently inhibited  
48 platelet aggregation. Increased concentrations of 9-HODE, 13-HODE and 15-HETE in phospholipids  
49 (2.1, 2.1 and 2.4-fold increase respectively) were found in HDL from patients with T2D, and these  
50 HDL also inhibited platelet aggregation *via* SR-BI.

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

54

55 **Abbreviations:**

56 AA, arachidonic acid; BHT, butylated hydroxytoluene; CE, cholesteryl esters; cPLA<sub>2</sub>, cytosolic  
57 phospholipase A<sub>2</sub>; 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<sub>4</sub>, 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<sub>2</sub>, thromboxane A<sub>2</sub>,  
63 T2D, type 2 diabetes.

64

## 65 INTRODUCTION

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

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## 93 **SUBJECTS AND METHODS**

### 94 *Study subjects*

95 Eight T2D patients (5 men and 3 women, aged  $66 \pm 3.5$  years) and eight healthy subjects (6 men and 2  
96 women, aged  $25 \pm 2.9$  years) were included. The patients had poorly controlled diabetes (fasting  
97 glycemia :  $8.7 \pm 1.4$  mmol/L ; glycated hemoglobin HbA<sub>1C</sub> :  $8.3 \pm 0.6\%$ , 67 mmol/mol). They had  
98 mild hypertriglyceridemia (triglycerides:  $1.9 \pm 0.4$  mmol/L), normal LDL-cholesterol ( $2.4 \pm 0.2$   
99 mmol/L) and low HDL-cholesterol ( $1.1 \pm 0.1$  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 glycooxidation*

112 Glycooxidized 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  $\mu$ mol/L CuCl<sub>2</sub> for 1 day at 37°C. Control HDL  
114 were prepared by incubating native HDL in the presence of butylated hydroxytoluene (BHT)  
115 (5 $\mu$ mol/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<sub>2</sub>PO<sub>4</sub>, 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<sub>4</sub>) 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<sub>2</sub>O, 65:35:4, by vol.). Fractions containing the product were further purified  
143 by ion exchange chromatography using Amberlyst resin (chloroform/methanol/H<sub>2</sub>O, 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<sub>2</sub> activation***

187 Chemiluminescent Western blotting detection of p38 MAPK, phospho-p38 MAPK and phospho-  
188 cPLA<sub>2</sub> was performed as described in the Supplemental Methods.

189

190 ***Determination of intracellular Ca<sup>2+</sup> 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.  $2.04 \pm 0.28$  nmol/mg HDL protein in control HDL, n=5, P<0.001).  
215 Gamma-tocopherol concentrations decreased by 75% in glycoxidized HDL ( $0.08 \pm 0.01$  in  
216 glycoxidized HDL vs.  $0.32 \pm 0.02$  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 ( $1.93 \pm 0.32$  nmol/mg protein in glycoxidized HDL vs.  
221  $0.18 \pm 0.13$  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<sub>2</sub> 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<sub>2</sub>. Pre-incubation of collagen-stimulated platelets with  
255 glycoxidized HDL fully prevented the collagen-induced increased phosphorylation of both enzymes.

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

260 inhibited collagen-induced intracellular  $\text{Ca}^{2+}$  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<sub>2</sub>,  
263 and  $\text{Ca}^{2+}$  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  $\text{NaBH}_4$  (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- $\omega$ 6-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  $\mu$ g/mL patients HDL inhibited collagen-induced platelet  
299 aggregation by 63% whereas 50  $\mu$ g/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 glycooxidation 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* glycooxidized HDL prevented both the collagen-induced increased  
310 phosphorylation of p38 MAPK, the stress kinase responsible for the phosphorylation of cPLA<sub>2</sub> (24)  
311 and that of cPLA<sub>2</sub>, 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  $\mu$ g/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<sub>2</sub> 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

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385

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

Fatty acid (mol %)	Phospholipids		Cholesteryl esters	
	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.

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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  $\pm$  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  $\mu\text{g}/\text{mL}$ ) for 5  
550 min at 37°C, and stimulation with collagen (2.5-5  $\mu\text{g}/\text{mL}$ ). Results are the means  $\pm$  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<sub>2</sub> phosphorylation, and**  
556 **on collagen-induced increases of calcium concentrations in platelets.** Phosphorylation levels of p38  
557 MAPK (A) and cPLA<sub>2</sub> (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  $\mu\text{g}/\text{mL}$ ) for 5 min at 37°C, and stimulated with collagen (2.5-5  $\mu\text{g}/\text{mL}$ ). Results, expressed as  
560 percentages of control, represent the means  $\pm$  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  $\mu\text{g}/\text{mL}$   
563 control or glycoxidized HDL for 5 min at 37°C and stimulated with collagen (2.5-5  $\mu\text{g}/\text{mL}$ ). 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 HDODE) 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.**

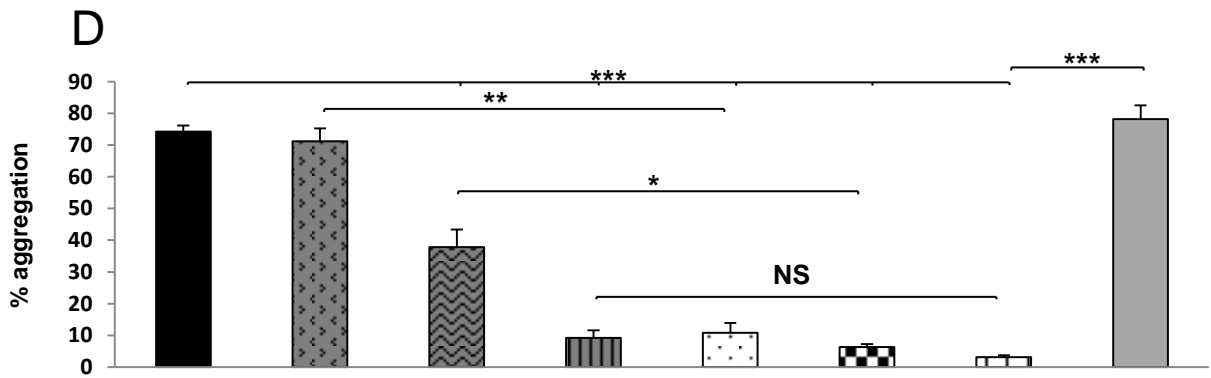
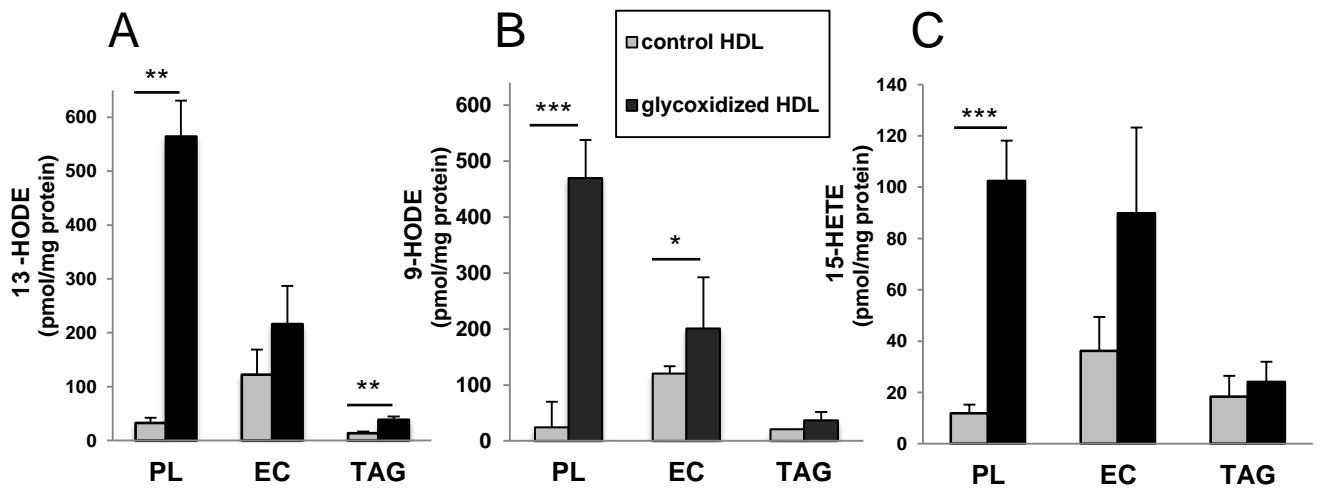
578 (A) Concentrations of 13-HODE, 9-HODE and 15-HETE in HDL PL from 5 control healthy subjects  
579 and 5 patients with T2D. Results are the means ± SEM. \*\*,  $P < 0.01$  vs. HDL from control subjects.

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.**

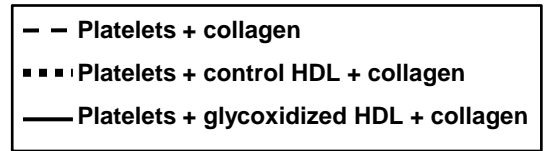
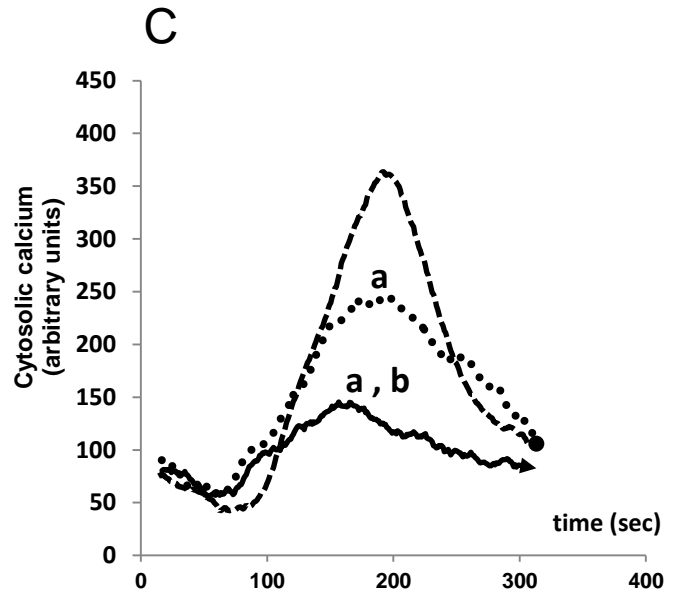
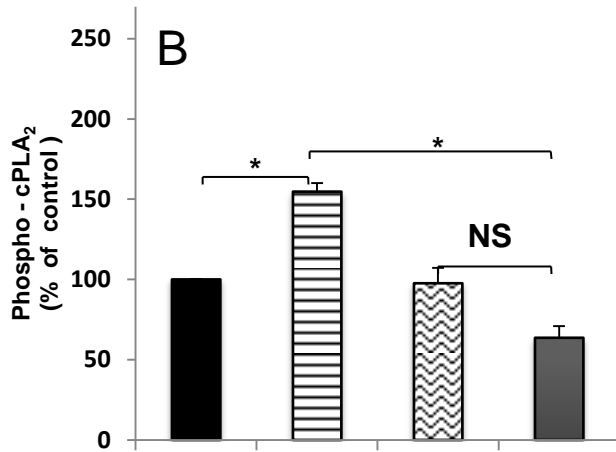
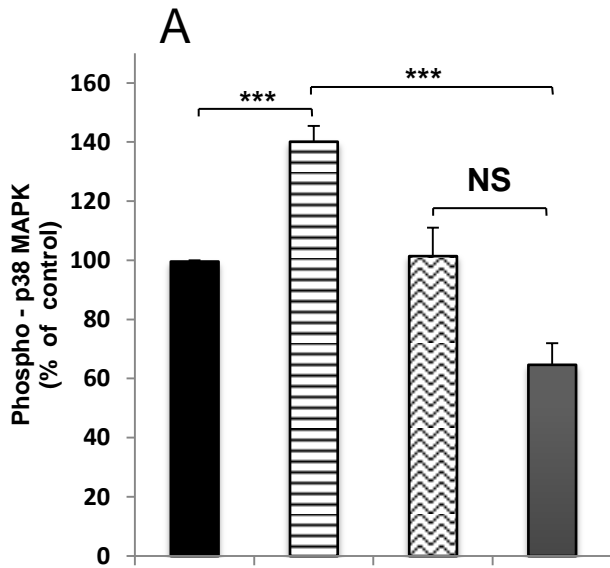
592 Binding of glycoxidized HDL or HDL enriched with PC(16:0/13-HODE) to SR-BI receptor on  
593 platelet membranes led to the inhibition of signaling pathways through decreased phosphorylated p38  
594 MAPK and cPLA<sub>2</sub> levels and inhibited collagen-induced platelet aggregation.



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

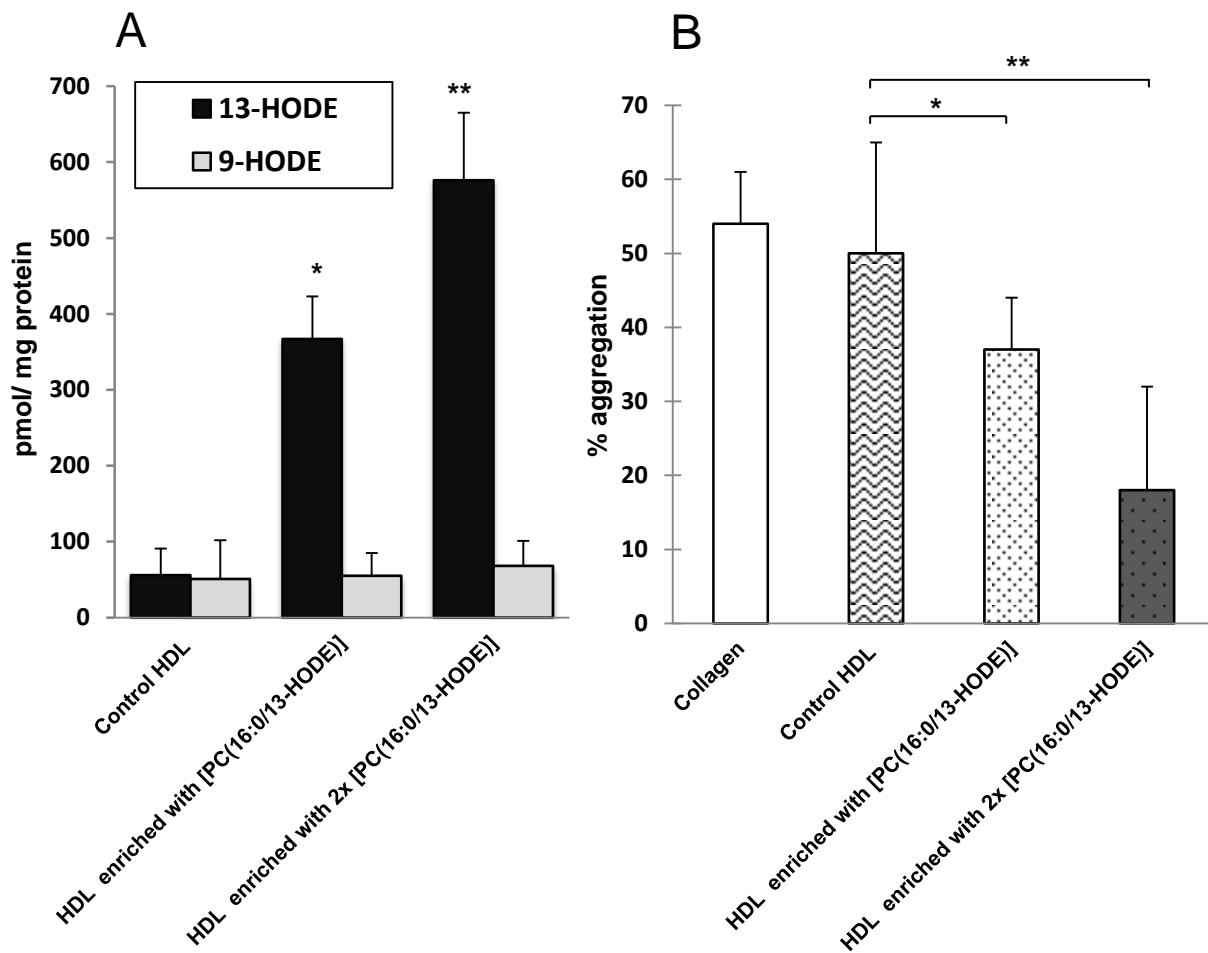
**Figure 1**





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

**Figure 2**



**Figure 3**

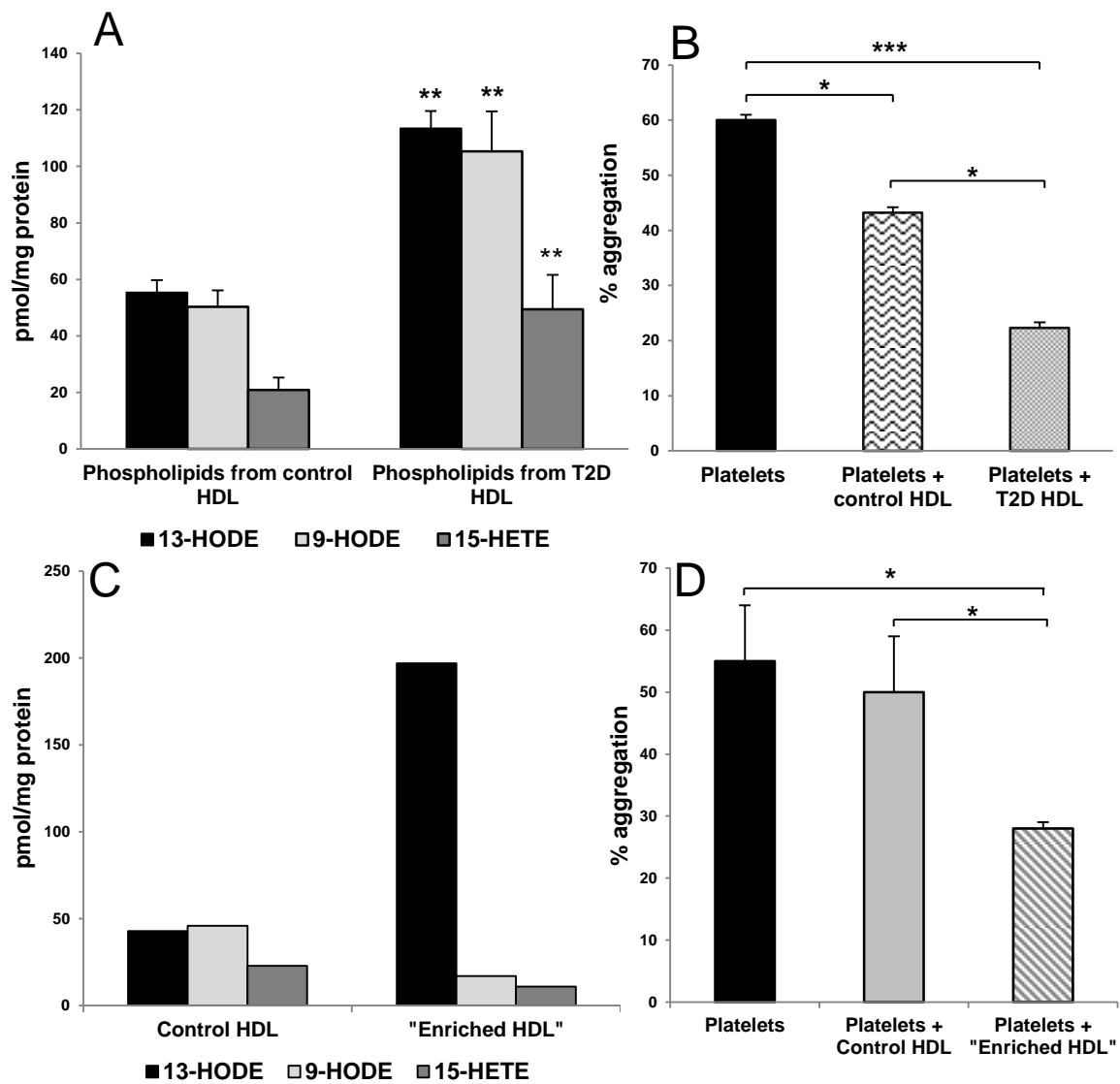


Figure 4

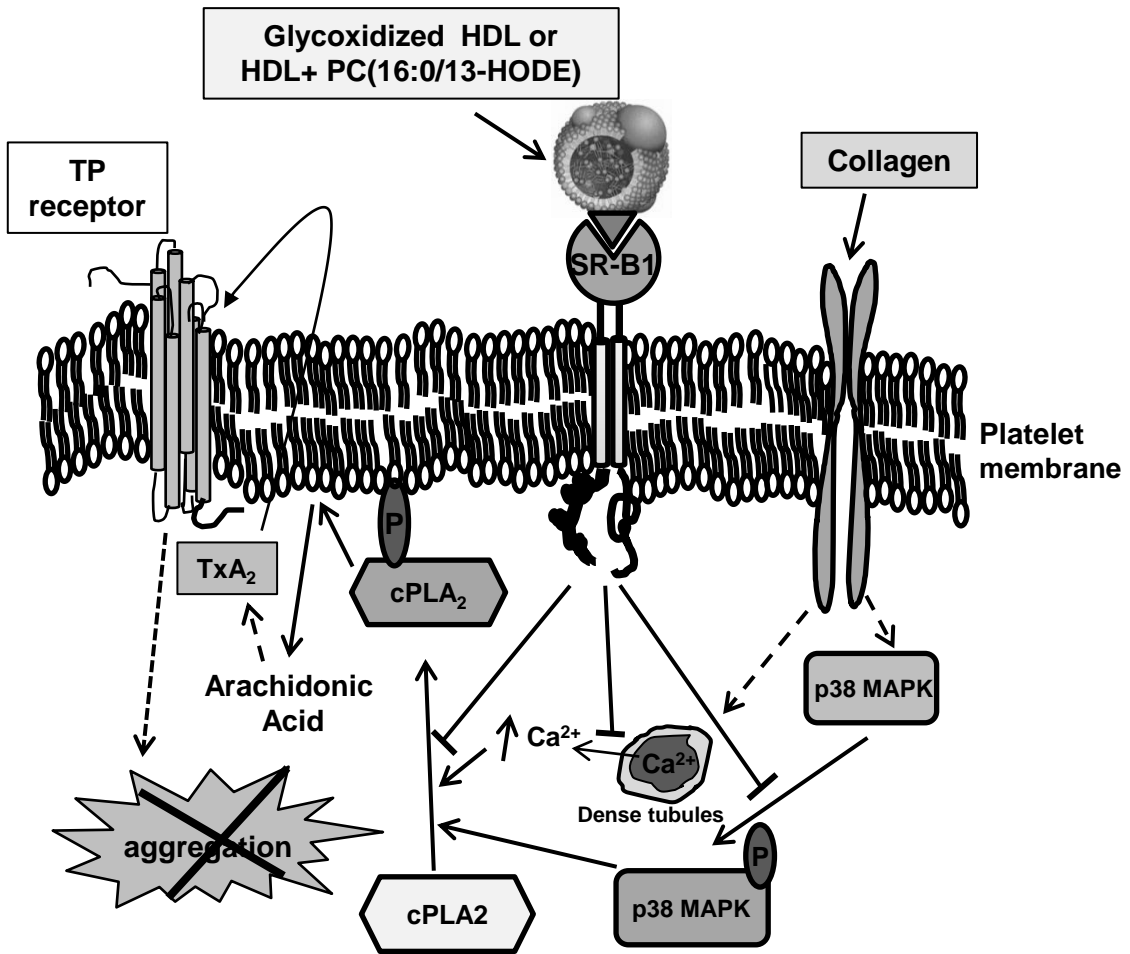


Figure 5

## **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  $\mu\text{mol/L}$ ), 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 (0.25 $\mu\text{m}$ , 30m  $\times$  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  $\text{NaBH}_4$ . 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  $\text{C}_{18}$  column (3.5  $\mu\text{m}$ , 4.6  $\times$  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<sub>18</sub> column (5µm, 4.6 × 250 mm, Macherey-Nagel, Hoerd, 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<sub>18</sub> column (5µm, 4 × 150 mm) and measured fluorimetrically (excitation 295nm, emission 340nm) (4).

### **Platelet p38 MAPK and cytosolic phospholipase A<sub>2</sub> 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<sub>2</sub> (cPLA<sub>2</sub>) polyclonal antibodies, washed, and incubated with 1:5000 goat anti-rabbit horseradish peroxidase conjugate. P38 MAPK,

phospho-p38 MAPK and phospho-cPLA<sub>2</sub> were visualized by enhanced chemiluminescence, and bands were quantified by densitometry.

### **Determination of intracellular Ca<sup>2+</sup> 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<sup>2+</sup> concentration in platelet suspensions was adjusted to 1 mmol/L using CaCl<sub>2</sub>. Platelets were excited alternately at 340 and 380 nm and fluorescence emission was recorded at 510 nm. Intracellular Ca<sup>2+</sup> concentrations were calculated from the ratio of fluorescence emission to excitation.

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